SERUM-MEDIATED ISOENZYME CONVERSION IN HUMAN PLACENTAL ALKALINE PHOSPHATASE

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by

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DECLARATION

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree. The work is my own except where specifically acknowledged.

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SUMMARY

1) Work in this laboratory has established that there exist at least three distinct forms of human placental alkaline phosphatase, including two classically described soluble forms (A- and B-PLAP), and a membrane form specific to the syncytiotrophoblastic microvillous membrane, designated M-PLAP.

2) I have extended this work by purifying the three isoenzymes of PLAP from placental tissue by developing a purification system that exploits their differential hydrophobicity.

3) Non-reducing SDS-PAGE was used to resolve the dimers of PLAP, which were then visualised either by Coomassie Blue or by direct enzyme staining. By this means, I was able to confirm the findings of Abu-Hasan and Sutcliffe (1985), namely that M-PLAP runs as a double banded zone of activity with a mobility corresponding to a molecular size of around 100,000 daltons, while A-PLAP runs with a mobility corresponding to a molecular size of around 115,000 daltons. Dimers of B-PLAP were resolved for the first time as a single band with a mobility corresponding to a molecular size of around 110,000 daltons.

4) Analysis of the subunits on SDS-PAGE has shown that a single Coomassie Blue-staining component present in A-PLAP is also common to B-PLAP and M-PLAP, suggesting that

all three are composed, at least in part, of monomers of similar molecular weights. However, a band of lower molecular weight was consistently observed in M-PLAP and B-PLAP even when special care was taken to work at 4°C and to minimise artifactual proteolytic cleavage. This has not been previously reported in PLAP purified from the placenta. Independent evidence for the presence of another PLAP polypeptide has come from the work of Nickson, Livingstone and Sutcliffe (1986), who showed that two PLAP polypeptides (56kD and 58kD) could be specifically immunoprecipitated from the in vitro translation of term placental villous mRNA.

5) Unpublished observations by Abu-Hasan and Sutcliffe showed that M-PLAP could not be detected in maternal serum either by electrophoresis or gel filtration. This was intriguing because it was already established (Abu-Hasan and Sutcliffe, 1985) that about 95% of the PLAP molecule, including its active site, protrudes from the surface of the microvillus, and is bathed in maternal blood in the intervillus spaces. They suspected that serum contained a factor capable of degrading M-PLAP to the A- and B-PLAP isoenzymes. The present work has confirmed and explained these findings, as follows.

6) Serum can convert M-PLAP to isoenzymes indistinguishable from A- and B-PLAP on starch gel electrophoresis. These processed PLAP products also have

dimer mobilities identical to A- and B-PLAP on non-reducing SDS gel electrophoresis. It was proposed that a factor in normal human serum mediated the conversion of M-PLAP to the A- and B-PLAP isoenzymes.

7) This conversion of M-PLAP to the A and B isoenzymes can proceed in the presence of protease inhibitors and is not accompanied by any change in the subunit size of the polypeptide within the resolving limits of reducing SDS PAGE. It was therefore concluded that the conversion was not proteolytic in nature.

8) I now transferred my efforts to the analysis of the conversion factor in serum. We formed a working hypothesis that removal of lipid moieties may be involved in the conversion of the hydrophobic M-PLAP to the less hydrophobic A- and B-PLAP. Since size fractionation of serum indicated that the factor had a size of about 60 kilodaltons, the serum enzymes lipoprotein lipase and lecithin-cholesterol acyltransferase were tested for conversion activity, but were found to be ineffective on M-PLAP substrate.

9) Attempts to analyse the kinetics of the conversion activity were hampered by the lack of a quantitative measure of the extent of conversion. Such an assay was developed in a model system, in which known ratios of A-, B- and M-PLAP could be assayed accurately and reproducibly. However, for reasons not determined, the

assay system could not be used to examine the conversion status of converted M-PLAP.

10) A purification system was developed for the purification of the conversion factor from serum, using conventional and FPLC column chromatography. When the resultant material was radiolabelled with ^{125}I and examined on SDS-PAGE, major components were identified with molecular weights of 27,000 and 17,000 daltons.

11) The purified, radiolabelled conversion activity was also analysed by 2-dimensional antibody antigen crossed electrophoresis (AACE). Precipitin arcs containing labelled protein were identified by their relative mobilities. These included an arc corresponding to the mobility of alpha-lipoprotein, which is comprised of two major polypeptide species of 27 and 17kD (apolipoproteins A-I and A-II respectively).

12) Rabbit antiserum raised against human apolipoproteins A-I and A-II was conjugated onto Sepharose and used in the antibody affinity chromatography of normal human serum. Depletion of the alpha-lipoprotein polypeptides coincided with depletion of conversion activity, while the bound and eluted fraction was found to contain conversion activity.

13) These results lead us to propose that serum apolipoproteins A-I and A-II are associated with the <u>in vitro</u> processing of M-PLAP to A- and B-PLAP.

ABBREVIATIONS

AACE - Antibody-antigen crossed electrophoresis

ADP - Adenosine diphosphate

AP - Alkaline phosphatase

ATP - Adenosine triphosphate

A-PLAP - A-form alkaline phosphatase

BSA - Bovine serum albumin

B-PLAP - B-form alkaline phosphatase

^{o}C - Degrees Centigrade

$CaCl_2$ - Calcium chloride

cDNA - Complementary DNA

CL - Cross-linked

CnBr - Cyanogen bromide

ConA - Concanavalin A

CPS - Counts per second

DEAE - Diethylaminoethane

DNA - Deoxyribonucleic acid

EC - European Commission

EDTA - Ethylenediaminetetra-acetic acid

ELISA - Enzyme-linked immunoadsorbant assay

FPLC - Fast protein liquid chromatography

HCl - Hydrochloric acid

HDL - High density lipoproteins

HLA - Human lymphocyte antigens

HPL - Human placental lactogen

HS - Human serum

IAP - Intestinal alkaline phosphatase

kD - Kilodalton

LCAT - Lecithin-cholesterol acyl transferase

LDL - Low density lipoproteins

M - Molar

mA - Milliamps

mfVSG - Membrane-form variant surface glycoprotein

mg - Milligramme

$MgCl_2$ - Magnesium chloride

ml - Millilitre

M-PLAP - M-form placental alkaline phosphatase

mRNA - Messenger RNA

mw - Molecular weight

NaBr - Sodium bromide

NaCl - Sodium chloride

NaOH - Sodium hydroxide

ND - Not determined

NM - Not measured

NP - Not performed

NR - Non-reduced

PAGE - Polyacrylamide gel electrophoresis

PAPP - Pregnancy-associated plasma protein

pH - Acidity

PLAP - Placental alkaline phosphatase

PMSF - Phenylmethylsulphonylfluoride

PSCA	-	Phenyl Superose-derived conversion activity
RNA	-	Ribonucleic acid
RPM	-	Revolutions per minute
SDS	-	Sodium dodecylsulphate
sVSG	-	Soluble-form variant surface glycoprotein
TBS	-	Tris-buffered saline
TLX	-	Trophoblast cross-reactive antigen
Tris	-	Tris(hydroxymethyl) aminoethane
ug	-	Microgramme
ul	-	Microlitre
w/v	-	Weight/volume

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

This chapter presents a review of the current state of knowledge of mammalian alkaline phosphatases, with special emphasis on the human placental form of the enzyme. Alkaline phosphatases comprise one of the most studied groups of all enzymes (see McComb et al, 1979), with different molecular forms having characteristic patterns of expression in different species, between members of a single species, between tissues of a single organism, and within a particular tissue. These differences have been identified by a variety of physical, structural and functional properties (Fernley, 1971; Fishman, 1974). These include molecular and subunit properties, electrophoretic, immunochemical, thermal stability properties and differential response to substrates and effectors of catalytic activity. Much of the mechanisms by which such diversity arises is known, and will be reviewed below.

The theme of the current researches is human placental alkaline phosphatase, which is located on the placental syncytiotrophoblast membrane. In order to understand the location and possible functional significance of this enzyme, it is necessary to quickly survey the development, structure and function of the human placenta.

1.1 The Human Placenta

The placenta is an essential, multifunctional, component of the mammalian reproductive system, whose role is to maintain the fetal environment throughout gestation. One major function is to transport nutrients, water, and gases to the developing fetus and to excrete fetal waste products into the maternal circulation. Further, by means of the secretion of hormones, the placenta is able to modify the maternal metabolism at various stages of pregnancy. This organ may also be involved in the maintenance of the pregnancy as an allograft, and in the inhibition of the intrinsic clotting system, which normally is activated when blood comes into contact with non-endothelial surfaces.

1.1.1 Human Placental Development

Following implantation of the developing blastocyst in the uterine wall, the trophoblast infiltrates the endometrial endothelium until, by the 11-12th day, the blastocyst is completely embedded. At this stage, the trophoblast cells differentiate into cytotrophoblast, which becomes less common as the placenta matures, surrounded by primitive syncytiotrophoblast. Up to the 25th day of pregnancy, there is a period of intense growth and differentiation as the "fingers" of chorionic

villi become established. As pregnancy proceeds, the villi proliferate and become thinner. Secondary villi develop from these primary villi which contain various cell types and specialised areas. Finally, tertiary villi are produced which contain the fetal capillaries.

1.1.2 Placental Microvilli

Mature villi are composed of an outer layer of syncytiotrophoblast, a layer of cytotrophoblast and an inner layer of connective tissue which contains collagen fibres, fibroblasts, Hofbauer cells and placental capillaries. The syncytiotrophoblast is uniform in thickness and has a free surface covered by microvilli whose cytoplasm is continuous with that of the syncytiotrophoblast. These syncytiotrophoblastic microvilli are suffused on their outer, or maternal, side with extra-epithelial blood. Thus, the syncytiotrophoblast forms the fetal-maternal interface across which all exchange takes place. As the fetus grows, and its requirements for exchange increase, so the syncytium becomes thinner, the microvilli increase in number, and the placental capillaries move closer to the surface. It has been estimated that, at term, the total surface area available across which exchange could take place exceeds $10m^2$ (Aherne and Dunnill, 1966).

1.1.3 Morphology of the Cytotro- and Syncytiotrophoblast

There are considerable morphological differences between the syncytiotrophoblastic and cytotrophoblastic layers. The cytotrophoblast cells show considerable variation in shape, dependent upon location and gestation period. They have little detectable intracellular structure, having a scarcity of endoplasmic reticulum and organelles (Boyd and Hamilton, 1970). In contrast, the syncytium has a high concentration of mitochondria, lysosomes, vesicles, vacuoles, microtubules, microfilaments and endoplasmic reticulum (Wynn, 1975). This structural evidence has led to the hypothesis that the syncytiotrophoblast is the major endocrinologically active tissue of the placenta.

1.1.4 Placental Proteins

Studies of placental brush border protein components has been stimulated primarily by the discovery of Smith, Brush and Luckett (1974) that placental preparations enriched in microvillous membranes could be obtained by differential centrifugation of a saline extract of whole placental tissue. Using SDS-PAGE electrophoresis, workers have in general found between 16 and 20 polypeptide species (Carlson *et al*, 1976; Smith *et al*, 1979; Kelley *et al*, 1979). Albumin, transferrin and alkaline phosphatase are major components in all preparations.

Albumin is present as a contaminant and is derived from contaminating fetal and maternal blood. Transferrin is present in relatively high concentration due to binding to the large number of transferrin receptors on the membrane (Loh et al, 1980). Placental alkaline phosphatase is the major external structural protein component of the microvilli (Jones and Fox, 1976; Doellgast and Benirschke, 1979).

Several groups have tried to identify further placental brush border protein components either by analysis of proteins present in microvillous membrane preparations, or by cytochemistry. These species can be classified as binding sites, enzymes and other proteins (for review, see Truman and Ford, 1984). Those membrane proteins defined as binding sites include specificities for immunoglobulin G (Van der Meulen et al, 1980; Johnson and Brown, 1981), transferrin (Loh et al, 1980), low density lipoproteins (Alsat et al, 1982), insulin (Whitsett and Lessard, 1978) and epidermal growth factor (Hock et al, 1979; Ogbimi et al, 1979). Those enzymes found in microvillous membrane preparations include 5'-nucleotidase (Truman et al, 1981) and various ATPases. Other proteins thought to be specifically synthesised during pregnancy, though not exclusively by the trophoblast tissues, include the pregnancy associated proteins SP1, PP5, and PAPP-A (Sutcliffe et al, 1980;

Klopper, 1980). A recent review of placental specific proteins has been written by major contributors to the field, edited by Rosen (1986).

1.1.5 Placental Immunology

A major area of investigation is the mechanisms by which the placenta is maintained as an allograft. On an *a priori* basis, the fetal/placental unit is a 'good candidate for eliciting a maternal immune response, since trophoblast cells are in direct physical contact with both the maternal blood supply and the maternal endometrium. Indeed, several studies have shown that the syncytiotrophoblastic microvilli are sufficiently immunogenic when injected into mice to elicit an immune response (Beer *et al*, 1972a,b). Maternal lymphocyte recognition of paternal determinants on the trophoblast surface is an area of contention at present. Whereas Davies *et al* (1982a, 1982b) provided evidence of such a phenomenon, their results remain to be confirmed by other workers. Nickson and Sutcliffe (1986), using a passive haemaglutination assay, failed to detect anti-trophoblast antibodies in maternal serum. Similarly, recent work by Hole *et al* (1987), using an ELISA assay with solubilised placental plasma membrane proteins, detected no significant difference in titre between pregnancy sera and normal male sera. Nevertheless, it is known that the

syncytiotrophoblastic membranes do not express histocompatibility antigens, on the basis of a negative reaction on immunohistochemistry (Goodfellow et al, 1976; McIntyre and Faulk, 1979).

The mechanisms by which the placental allograft is maintained is not known, although three possible hypotheses have been formulated, none of which are mutually exclusive. It is possible that the fibrinoid layer at the utero-placental interface may act as a physical barrier to maternal lymphocytes or other cellular elements (Sutcliffe et al, 1982). Maternal antibodies raised against paternal antigens on the fetal-maternal interface may bind and act "block" the sensitised maternal lymphocytes and cellular components from reaching their targets. Other blocking factors have been described by Rocklin et al (1976). A third hypothesis was proposed by Wynn (1975) in which trophoblastic exfoliation acts to divert the maternal immune system from the conceptus.

It has been proposed that a degree of immune recognition is necessary for the maintenance of pregnancy. Couples in which the woman has had habitual recurrent spontaneous abortions are thought to express a greater degree of sharing of HLA determinants than would be expected with reference to couples with no such history (Beer et al, 1981). Faulk (1981) proposed that

recognition of the paternally derived TLX specificities on the placental surface is necessary for maintenance, although these specificities have never been identified biochemically. Evidence supporting the hypothesis of immune recognition was gleaned by Mowbray et al (1985) who found that immunisation of recurrent aborters with their partner's lymphocytes, and hence, hypothetically, the generation of antibodies against paternal determinants, appeared to result in an increase in successful pregnancy.

In short, therefore, the syncytiotrophoblastic microvilli are important in the transport of nutrients, gases and waste material between the maternal and fetal blood supplies. The brush border, or its components, may have a role to play in making the fetal/placental unit an immunologically privileged site. The membranes are also instrumental in steroid/hormone signaling between the mother and the developing embryo, as well as synthesising a number of enzymes and other proteins of unknown function. One example of such an enzyme, and a major protein of the term syncytiotrophoblastic microvilli, is placental alkaline phosphatase.

1.2 Mammalian Alkaline Phosphatases

Alkaline phosphatases (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) are a

group of nonspecific phosphomonoesterases located primarily in the plasma membrane of the cells in which they occur. In vitro studies of the enzyme has revealed that they also exhibit phosphotransferase (Herraez et al, 1980) and protein phosphatase activities (Swarup et al, 1981; Lau et al, 1977). The lack of substrate specificity and the broad spectrum of activities exhibited by alkaline phosphatases have rendered the elucidation of the physiological function of the enzyme difficult.

Alkaline phosphatases are among the most widely studied groups of enzymes, and have been found in bacteria, plants, and animals (see McComb et al, 1979). In man, they exist as highly glycosylated homodimers, and as such are found in essentially all tissues, although they have been best studied in those tissues containing the greatest activities, namely liver, bone, kidney, intestine and placenta. Table 1 presents a number of the properties of particular alkaline phosphatase species.

1.2.1 Physiological Function of Alkaline Phosphatases

The functional significance of mammalian alkaline phosphatases have yet to be established. Many lines of evidence have indicated that there is a correlation between alkaline phosphatase and fat absorption and/or phosphatidate hydrolysis (Fishman, 1974; Ehle et al, 1985; Sumikawa et al, 1987). Alkaline phosphatases are

Isoenzyme Type	Tissue Unspecific L/B/K AP	Tissue Specific IAP	Tissue Specific PLAP	Further Loci Early IAP	Further Loci PLAP-like
MW (kD)	136-170	140-170	116-125	N.D.	subunit 65
Allelic diversity	-	-	+++	-	-
Cloned /Sequenced	+	+	+	-	-
pH optima	10.1	10.1	10.7	10.1	10.6
Heat Stability:					
56°C 15min	+/++	++	+++	++	+++
65°C 5min	-	-	+++	-	+++
Amino acid sensitivity (5mM)					
l-phenylalanine	-	+++	+++	+++	+++
l-homoarginine	+++	-/+	-/+	-/+	-/+
l-leucine	+	++	+	++	+++
Electrophoretic mobility	fast	slow	intermediate	fast	intermediate
Sialic acid	+	-	+	+	+
Lectin Binding	+	-	+++	+	+
Immunoreactivity to antisera to:					
Liver AP	+++	-	-	-	-
Bone AP	+++	-	-	-	-
Kidney AP	+++	-	-	-	-
Intestinal AP	-	+++	+	++	+
Placental AP	-	+	+++	+	+++

TABLE 1.1. Multiple Forms of Human Alkaline Phosphatase; adapted from Stigbrand (1984).

also thought to be intimately related to mineralisation of bone and ossifying growth-plate cartilage (Burch et al, 1985). Alternatively, other workers have proposed that alkaline phosphatase contributes to the regulation of cell growth by selective tyrosyl dephosphorylation (Risk and Johnson, 1985), and could hence be important in controlling the interaction of cell surface receptors with epidermal growth factor and insulin (Avruch et al, 1982; Hunter, 1982). Again, initial researches in this laboratory, and by Hutton et al (1980), have suggested that placental alkaline phosphatase is important in the inhibition of blood clotting, via the degradation of ADP.

1.2.2 Genetic Diversity in Alkaline Phosphatases

Analysis of the immunological, catalytic and electrophoretic properties of the alkaline phosphatases extracted from mammalian tissues indicates that there are several classes of mammalian alkaline phosphatases, and that they are encoded by at least three structural genes (Seargeant and Stinson, 1979; McKenna et al, 1979; Goldstein et al, 1980a). For a review of multilocus enzyme systems as illustrated by mammalian alkaline phosphatase see Harris (1982).

1.2.2.1 Tissue unspecific Alkaline Phosphatase (L/B/K AP)

One form of alkaline phosphatase is found predominantly in osteoblasts, fibroblasts, leukocytes, and in cells from a variety of different tissues, including liver, kidney, breast and brain. This is known as the tissue-unspecific, or liver/bone/kidney form of alkaline phosphatase (L/B/K AP). The isoenzymes extracted from these cells and tissues are known to be immunochemically identical (Boyer, 1963), and are similarly sensitive to inhibition by l-homoarginine (Fishman and Sie, 1970).

1.2.2.2 Intestinal-type Alkaline Phosphatase (IAP)

Intestinal alkaline phosphatase (IAP) is one example of a tissue-specific AP, and as such is largely confined to the brush border membranes of the small intestine. It can be distinguished from other APs by specific antisera, though some cross-reaction with antisera raised against placental alkaline phosphatase can be seen. It shares several properties with placental alkaline phosphatase in that it is partially heat stable, resistant to catalytic inhibition with l-homoarginine, and sensitive to inhibition with l-phenylalanine (Ghosh and Fishman, 1966). IAP is a glycoprotein, but it does not contain sialic acid residues, neither does it bind to concanavalin nor lentil lectin (Lehmann, 1980).

Studies using monoclonal antibodies, tryptic peptide and cyanogen bromide peptide mapping indicates that there exists a form of intestinal alkaline phosphatase expressed in the fetus up to to 30 weeks gestation, which is similar, but not identical to the adult isoenzyme (Vockley et al, 1984a; Vockley et al, 1984b). It is proposed that this is expressed from a further alkaline phosphatase gene locus, although some developmentally-related difference in gene-rearrangement or messenger-processing cannot be excluded.

1.2.2.3 Placental-type Alkaline Phosphatase (PLAP)

Placental alkaline phosphatase (PLAP) is the other major tissue-specific alkaline phosphatase, and is mainly confined to the placenta of higher primates including orangutan, chimpanzee and man (Doellgast, 1984). Although catalytically similar to the adult intestinal isoenzyme, it can be distinguished with some monoclonal antibodies and by its marked heat-stability (Neale et al, 1965; Beratis and Hirschorn, 1972). PLAP is also slightly more sensitive to inhibition with l-leu-gly-gly and l-phe-gly-gly (Doellgast and Meis, 1979).

Alkaline phosphatase activity has been detected in the placenta as early as the first 6-10 weeks of pregnancy. This form of alkaline phosphatase was found to consist of two heat sensitive and l-homoarginine

inhibitable species, one of which shared antigenic determinants with L/B/K AP, the other being antigenically unique (Fishman et al, 1976; Sakiyama et al, 1979). Between the eleventh and thirteenth weeks of pregnancy, the expression of AP is in a state of flux, during which the early-type, or developmental phase-specific alkaline phosphatases are replaced by the classically described PLAP. From there, placental alkaline phosphatase serum concentrations rise exponentially to a peak level at delivery of around 250ng/ml, and clearing from the blood within six days of delivery (Holmgren et al, 1978).

Heat-stable, l-homoarginine resistant and l-phenylalanine resistant alkaline phosphatases have been detected in trace amounts in the normal human testis (Chang et al, 1980), in lung, cervix, and thymus (Nozawa et al, 1980; Goldstein et al, 1980b; Goldstein et al, 1982a), and in intestine and liver (Garattini, et al, 1985). However, these PLAP-like enzymes are also sensitive to inhibition with l-leucine and EDTA, and some question remains as to whether they represent a further AP locus, or whether they are differently processed transcripts or polypeptides. This picture is confused by the existence of a rare allelic variant of PLAP, namely the D- or 18-variant, which is indistinguishable from the PLAP-like alkaline phosphatase described above (Doellgast and Fishman, 1976).

1.2.3 Predicted Amino Acid Sequences of Mammalian APs

The cDNA sequence for one form of intestinal AP and one form of L/B/K AP has been cloned and sequenced (Weiss *et al*, 1986; Henthorn *et al*, 1987). In addition, Henthorn *et al* (1986) have cloned and sequenced the products of two common alleles encoded by the locus for human placental alkaline phosphatase, namely type 1 and type 3. The amino acid sequence predicted from the cDNA sequence of all four is presented in Table 2.

The analysis of Henthorn *et al* (1986) identified ten single nucleotide substitutions between the two alleles of placental alkaline phosphatase, seven of which result in a difference in the amino acid sequence presented in Table 2. This amount of variation between the two alleles is unusual, but may be related to the high degree of polymorphism at this locus.

Millan (1986) was able to isolate and sequence an almost full length clone for type 1 PLAP. However, this differed from the type 1 sequence published by Henthorn *et al* (1987) at two nucleotide positions within the protein coding region. One of these differences results in the substitution of a proline for an arginine at amino acid position 209. If these differences exist, then this may represent microheterogeneity within the type 1 phenotype, a hypothesis supported by monoclonal antibody

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-17                                                  M--LLLLLL  LGLRLQLSLG  PLAP
-19                                                  MQGPWVLLL  LGLRLQLSLG   IAP
-17                                                    MISPFLV  LAIGTCLTNS  L/B/K
                                                       +++++      +++  ++

+1      L                                                          V        PLAP3
+1    IIPVEEENPD  FWNREAAEAL  GAAKKLQPAQ  TA-AKNLIIFL  GDGMGVSTVT  PLAP1
+1    VIPAEEENPA  FWNRQAAEAL  DAAKKLQPIQ  KV-AKNLILFL  GDGLGVPTVT   IAP
+1    LVPEKEKDPK  YWRDQAQETL  KYALELQKLN  TNVAKNVIMFL  GDGMGVSTVT  L/B/K
       +++ ++ +    +++ + +    ++ ++  ++  ++                +

+51   AARILKGQKK  DKLGPEIPLA  MDRFPYVALS  KTYNVDKHVP  DSGATALAYL   PLAP
+51   ATRILKGQKN  GKLGPETPLA  MDRFPYLALS  KTYNVDRQVP  DSAATATAYL   IAP
+52   AARILKGQLH  HNPGEETRLE  MDKFPFVALS  KTYNTNAQVP  DSAGTATAYL  L/B/K
       +     ++  +++ + ++ +                  ++++    ***   +

+101  CGVKGNFQTI  GLSAAARFNQ  CNTTRGNEVI  SVMNRAKKAG  KSVGVVTTTR   PLAP
+101  CGVKANFQTI  GLSAAARFNQ  CNTTRGNEVI  SVMNRAKQAG  KSVGVVTTTR   IAP
+102  CGVKANEGTV  GVSAATERSR  CNTTQGNEVT  SILRWAKDAG  KSVGIVTTTR  L/B/K
         ++          +++++      +   +      ++  +

+151  VQHASPAGTY  AHTVNRNWYS  DADVPASARQ  EGCQDIATQL  ISNM-DIDVIL   PLAP
+151  VQHASPAGTY  AHTVNRNWYS  DADMPASARQ  EGCQDIATQL  ISNM-DIDVIL   IAP
+152  VNHATPSAAY  AHSADRDWYS  DNEMPPEALS  QGCKDIAYQL  MHNIRDIDVIM  L/B/K
         + +         ++*+      +    + ++  +   +    +  +

+201                                                      H        PLAP3
+201  GGGRKYMFRM  GTPDPEYPDD  YSQGGTRLDG  KNLVQEWL---GE  RQGARYVWNR  PLAP1
+201  GGGRKYMFPM  GTPDPEYPAD  ASQNGIRLDG  KNLVQEWL---AK  HQGAWYVWNR   IAP
+203  GGGRKYMYPK  NKTDVGYESD  EKARGTRLDG  LDLVDTWKSFKPR  YKHSHFIWNR  L/B/K
               +  +++ ++ ++   ++++ +      ++  ++ +   ++  +++++

+251      R          A                                              PLAP3
+251  TELMQASLDP  S-VTHLMGLFE  PGDMKYEIHR  DSTLDPSLME  MTEAALRLLS  PLAP1
+251  TELMQASLDQ  S-VTHLMGLFE  PGDTKYEIHR  DPTLDPSLME  MTEAALRLLS   IAP
+256  TELM--TLDP  HNVDYLLGLFE  PGDMQYELNR  NNVTDPSLSE  MVVVAIQILR  L/B/K
       +     +  +  ++          ++   +     ++++   +    +++  +  +

+301  RHPRGFFLFV  EGGRIDHGHH  ESRAYRALTE  TIMFDDAIER  AGQLTSEEDT   PLAP
+301  RNPRGFYLFV  EGGRIDHGHH  EGVAYQALTE  AVMFDDAIER  AGQLTSEEDT   IAP
+305  KNPKGFFLLV  EGGRIDHGHH  EGKAKQALHE  AVEMDRAIGQ  AGSLTSSEDT  L/B/K
       +     +     ++ ++  +   + ++ +  ++    +   +

+351                    C       G                                    PLAP3
+351  LSLVTADHSH  VFSFGGYPLR  GSSFIGLAAP  K-ARDRKAYTV  LLYGNGPGYP  PLAP1
+351  LTLVTADHSH  VFSFGGYTLR  GSSIFGLAPS  K-AQDSKAYTS  TLYGNGPGYP   IAP
+355  LTVVTADHSH  VFTFGGYTPR  GNSIFGLAPM  LSDTDKKPFTA  ILYGNGPGYK  L/B/K
                     +++      + ++    +   + ++ +    +  +         +

+401  LKDGARPDVT  ESESGSPEYR  QQSAVPLDEE  THAGEDVAVF  ARGPQAHLVH   PLAP
+401  FNSGVRPDVN  ESESGSPDYQ  QQAAVPLSSE  THGGEDVAVF  ARGPQAHLVH   IAP
+406  VVGGERENVS  MVDYAHNNYQ  AQSAVPLRHE  THGGEDVAVF  SKGPMAHLLH  L/B/K
       +++ + ++ +  ++ + +++ +  + +    ++              +   +

+451  GVQEQTFIAH  VMAFAACLEP  YTACDLAPPA  CTTDAAHPGR  SVVPALLPLL   PLAP
+451  GVQEQSFVAH  VMAFAACLEP  YTACDLAPPA  CTTDAAHP--  --VAASLPLL   IAP
+456  GVHEQNYVPH  VMAYAACIGA  NLGHCAPASS  AGSLAAGPLL  VALALYPLSV  L/B/K
       +   +                +  ++ +++  ++  ++ +   + ++  ++  +++++

+501  AGTLLLLETA  TAP                                              PLAP
+497  AGTLLLLGAS  AAP                                               IAP
+506  LF                                                          L/B/K
       ++     +++
```

Table 1.2. Comparisons of the predicted amino acid sequences for intestinal alkaline phosphatase (IAP), term placental type 1 AP (PLAP1) and known substitutions in the type 3 allele (PLAP3), and liver/bone/kidney AP (L/B/K). Gaps that have been introduced to maximise pairing of homologous amino acids are indicated (-), with non-conservative substitution of amino acids shown (+). Residues that interact with the phosphate substrate (*) are also indicated. Amino acid +1 corresponds to the first residue in each of the mature proteins. The sequences are shown in single-letter code, which corresponds to the amino acids as follows: A, Alanine; C, Cysteine; D, Aspartic acid; E, Glutamic acid; F, Phenylalanine; G, Glycine; H, Histidine; I, Isoleucine; K, Lysine; L, Leucine; M, Methionine; N, Asparagine; P, Proline; Q, Glutamine; R, Arginine; S, Serine; T, Threonine; V, Valine; W, Tryptophan; Y, Tyrosine.

Adapted from Weiss et al (1986), Henthorn et al (1986) and Berger et al (1987a).

studies (Slaughter et al, 1983).

Kam et al (1985) also published a sequence for human placental alkaline phosphatase. However, no attempt was made to identify the PLAP morph cloned. On comparison with the sequences of Henthorn et al (1986), the sequence of Kam et al (1985) differs from the type 1 polypeptide by 7 amino acids, and from the type 3 polypeptide at 12 amino acid positions. In addition, it was unique at two silent site nucleotide positions, and at one 3'-untranslated position. This morph was argued by Henthorn et al (1986) as representing one of the many rare alleles of PLAP.

1.2.4 Allelic Diversity in Alkaline Phosphatases

Placental alkaline phosphatase is the only isoenzyme of alkaline phosphatase with a well documented polymorphism in the true sense of the term. It has long been known that placental alkaline phosphatase is peculiar in that it is extremely polymorphic (Boyer, 1961). Three common alleles give rise to six common phenotypes, with types 1, 2 and 3 representing homozygotes and types 2-1, 3-1 and 3-2 representing heterozygotes (Robson and Harris, 1965; Beckman et al, 1966). In addition, at least 18 rare alleles occur, producing rare variant electrophoretic phenotypes, most of which comprise a rare allele in combination with one

of the common alleles (Robson and Harris, 1967; Harris et al, 1974). Although individually uncommon, such rare variants occur in 3-5% of placentas (Donald and Robson, 1974). In comparison, the average heterozygosity for rare alleles at 42 different loci has been estimated as occurring in about 0.11% of cases (Harris et al, 1974). The reasons for this high degree of allelic diversity at the PLAP locus is not known, and may involve an unusual degree of intragenic crossing-over, gene conversion, single base mutations, or other phenomena. There are no differences between these enzyme variants with respect to pH optima, Km values or inhibition with l-phenylalanine. However, variations in thermostability are detected (Holmgren and Stigbrand, 1976). Variants can also be discriminated by allozyme-absorbed antisera (Wei and Doellgast, 1980). The structural basis for such polymorphism has not been fully elucidated, except in the cases of the two common alleles described above.

1.2.5 Evolution of Mammalian Alkaline Phosphatases

The predicted amino acid sequence comparison of the alkaline phosphatases shown in Table 2 indicates that there exist several areas of conserved amino acid sequence, particularly in those regions comprising the active site of the enzyme, as identified by binding to substrate phosphate. PLAP shows a greater overall amino acid homology to IAP (86.5%) than to L/B/K AP (52%) with

IAP and PLAP sequences showing an approximately equal divergence from the tissue-unspecific AP. This is consistent with the biochemical and immunological characterisation of the AP proteins (Lehmann, 1975; Doellgast, 1984). Comparisons of protein and DNA suggest that PLAP is the result of a recent duplication of the gene for IAP, followed by divergence of both sequences.

PLAP and IAP genes both contain a relatively long 3'-untranslated region, which can be used to estimate the time in evolution when the gene duplication giving rise to placental and intestinal APs occurred. Assuming that this region is not under selection, the percentage of divergence (26.1%) can be compared to the divergence between presumably unselected DNA sequences in other pairs of species. Using this method, Henthorn et al (1987) estimated that gene duplication took place before the divergence of New World monkeys and the lineage that gave rise to both the Old World monkeys and the hominoids, perhaps as early as the time of the divergence of prosimians (lemurs, tree shrews, lorises and tarsiers) from anthropoids (monkeys and apes). This estimate is consistent with the finding of PLAP in the placentas of the great apes but not in the placentas of many other primate species (Goldstein et al, 1982b).

1.2.6 Post-Translational Modification of Mammalian APs

Differences observed between alkaline phosphatase species cannot wholly be attributed to expression of alleles from different genetic loci, and much evidence has been gathered which shows that differential post-transcriptional modification of the mRNA transcript and/or of the nascent polypeptide chain takes place.

1.2.6.1 Glycosylation of Alkaline Phosphatases

As mentioned above, the isoenzymes of tissue unspecific alkaline phosphatase are immunochemically identical, and are similarly inhibited by l-homoarginine. However, different electrophoretic patterns are observed for these isoenzymes following electrophoresis in agar gels (Smith et al, 1973), and are inactivated at widely different rates by urea and temperature (Moss and Whitby, 1975). Since treatment of these isoenzymes with neuraminidase abolishes these differences, they are considered to be largely the result of differences in carbohydrate moieties (Stinson and Seargeant, 1981; Moss, 1984).

1.2.6.2 Membrane Binding of Alkaline Phosphatases

Recent results bearing on the mechanisms by which alkaline phosphatases may be bound to the cell membrane, as well as bearing on the results herein reported, will be presented in the general discussion. In short, most

evidence currently available suggests that at least some of the mammalian alkaline phosphatase protein family should be regarded as members of a new class of membrane proteins. These proteins are bound to the plasma membrane through a carboxy-terminal amino acid linkage to phosphatidylinositol. The first such protein to be discovered and fully characterised was the variant surface glycoprotein (VSG) of the parasite Trypanosoma brucei. This glycoprotein is synthesised with a hydrophobic carboxy-terminal domain that is very rapidly (within 1 minute) cleaved and replaced by a glycophospholipid, which then anchors the VSG to the membrane. An endogenous enzyme with phosphatidylinositol-specific phospholipase C activity is then able to specifically cleave the surface coat. Whether this mechanism is also employed in alkaline phosphatases is presently not known, particularly in the case of the placental isoenzyme, but the hypothesis should be borne in mind that post-translational modification may be important in generating membrane-bound and soluble forms of alkaline phosphatase.

1.2.7 Further Variants of Mammalian APs

As well as the well characterised alkaline phosphatases from defined genetic loci and their allelic variants, there are a number of forms of alkaline phosphatase whose origins are less well understood.

1.2.7.1 Tumour Alkaline Phosphatases

In 1968, Fishman et al (a,b) detected an alkaline phosphatase isoenzyme in the serum and tumour tissue of a patient with bronchogenic carcinoma. On examination, this AP had properties identical to those of PLAP. This was called the Regan isoenzyme after the patient, and was shown to be indistinguishable from PLAP with regard to heat stability, inhibition with l-phenylalanine, optimum pH, electrophoretic migration, action of neuraminidase and specific reaction with rabbit antisera to PLAP. Cleeve and Tua (1983) have since proposed that the Regan and PLAP isoenzymes are products of the same structural genes.

Another tumour-associated variant of heat-stable alkaline phosphatase, detected initially by Nakayama et al (1970), is referred to as the Nagao isoenzyme. This has all the properties of the Regan isoenzyme, but is additionally sensitive to l-leucine and EDTA. The existence of a PLAP-like isoenzyme in tumours was confirmed by Inglis et al (1973) who, on examination of 39 cancer patient sera containing alkaline phosphatase activity similar to PLAP, found that approximately half of the APs were inhibitable by l-leucine.

Increased levels or inappropriate expression of PLAP or PLAP-like isoenzymes have been associated with a number of different benign and malignant tumours of

trophoblastic and non-trophoblastic tissues and organs. These include malignancies of the pancreas, lung, breast, colon, lymph nodes, kidney, stomach, and bladder (see Higashino et al, 1972; Fishman and Stolbach, 1979; Stigbrand and Engvall, 1982), although higher levels are consistently found in seminomas (Nathanson and Fishman, 1971; Wahren et al, 1979; Jeppsson et al, 1984), and in ovarian and uterine cancers (Benham et al, 1978; Nouwen et al, 1985).

It remains to be established whether or not there is a relationship between the rare 18-, or D-, variant of PLAP, the Nagao isoenzyme, and the l-leucine sensitive isoenzyme expressed in trace amounts in normal tissues. However, it seems likely that, at least in some cases, the transformation of a cell line from normal to malignant is associated with the over-expression of the PLAP-like enzyme from the same locus (Millan et al, 1982; Millan and Stigbrand, 1983). It is also unknown what significance can be placed on the finding of a rare allele so commonly expressed in tumours. Does this imply that there is a predisposition for cancer among individuals of this phenotype; does the variant normally arise through aberrant post-transcriptional modification, a pathway which is forced in the case of malignant tissues; or does tumour expression represent re-expression from a different gene or pseudogene, whose transcript is fortuitously similar to that of a PLAP

allele?

PLAP or PLAP-like alkaline phosphatases are not the only APs found associated with transformed tissues. A tumour enzyme identified in a patient with a hepatoma was found to be temperature-sensitive, l-phenylalanine and l-leucine sensitive, but insensitive to inhibition with l-homoarginine. Since it also reacted with anti-intestinal AP antibodies, this Kasahara variant was seen as a IAP-like isoenzyme (Warnock and Reisman, 1969; Higashino et al, 1975). This variant has also been described in renal cell carcinomas (Hada et al, 1978).

1.2.8 Alkaline Phosphatase Components

Starch gel electrophoresis of tissue extracts has demonstrated that all APs contain at least two groups of components. The most anodal form represents the dimeric form of the enzyme and is usually the most abundant. The other form moves more slowly and is thought to contain higher molecular weight species. In PLAP, these forms are designated A-PLAP and B-PLAP respectively (Ghosh and Fishman, 1968; Beratis et al, 1970). Both forms show the same polymorphisms, and possess similar heat stability, suggesting that both are comprised, at least in part, of the same enzymatic polypeptide. However, further studies showed that the forms had different isoelectric points, mobilities on acrylamide gels and elution points on ion-exchange chromatography, and that B-PLAP had a higher

native molecular weight of around 200,000 daltons. Doellgast et al (1977) found that B-PLAP had a lower specific activity than A-PLAP, suggesting that B-PLAP was associated with material that did not contribute to the enzymatic activity. These results led workers to the hypothesis that B-PLAP was associated with hydrophobic and basic proteinaceous material, although the exact basis for the difference between A- and B-PLAP remains unclear. B-PLAP was thought by Doellgast et al (1977) to represent the membrane-bound form of the enzyme.

1.2.9 Microvillous Human Placental Alkaline Phosphatase

Work by the Sutcliffe group demonstrated that, as extracted from syncytiotrophoblastic microvilli, there existed a third, highly abundant form of AP, named M-PLAP (Abu-Hasan, Davies and Sutcliffe, 1984; Abu-Hasan and Sutcliffe 1984, 1985). M-PLAP was found to share the same genetic polymorphism, sensitivity to specific inhibitors, and similar kinetic behaviour to A-PLAP, suggesting that the same enzymatic polypeptide was involved. Furthermore, examination of the properties of M-PLAP by antigenic cross-reactivity, heat-stability, CNBr and protease cleavage maps, amino terminal amino acid sequencing, and subunit molecular weight strongly argued against the involvement of two distinct genes for the expression of the A and M forms of PLAP.

Despite the structural similarities between A- and

M-PLAP, the M form had rather different properties. M-PLAP required the presence of detergent in order to enter and resolve in starch or polyacrylamide gels. This was explained by the two findings of the relative hydrophobicity of M-PLAP, in as much as it remained bound to an alkyl Sepharose column under conditions in which A- and B-PLAP were eluted, and of the high molecular weight of 725,000 daltons for M-PLAP on size exclusion chromatography. Such behaviour argued in favour of M-PLAP being a membrane form of the enzyme. Further analysis (Abu-Hasan and Sutcliffe, 1985) found that M-PLAP could be cleaved from intact microvilli with the protease subtilisin, resulting in the liberation of an active polypeptide composed of subunits reduced in molecular weight by around 2,000 daltons. This corresponded to the protease susceptible site found by other workers using bromelain (Jemmerson et al, 1984; Kottel and Hanford, 1980). Amino acid sequencing by Abu-Hasan and Sutcliffe (1985), revealed that cleavage was taking place towards the carboxy-terminus, thus indicating that M-PLAP was bound to the membrane at its carboxy-terminus. The deduction of Abu-Hasan and Sutcliffe (1985) that there existed a membrane attachment site at the carboxy-terminus of PLAP was supported by the recent analysis of PLAP cDNA by Kam et al (1985). However, it remained to be determined whether the predicted hydrophobic amino acid sequence is present in the mature polypeptide.

M-PLAP remained distinct from other forms of PLAP under all extraction and storage procedures used, including solubilisation with butanol, chloroform-methanol, nonidet, Triton X-100, and sodium deoxycholate, indicating not only that M-PLAP was not an artifact of one particular extraction procedure, but also that A- and M-PLAP probably differed by the presence or absence of a hydrophobic moiety bound tightly to the molecule.

1.3 Strategy of My Investigations

The aims of my researches have essentially focussed on three distinct questions, all of which bear on the structural relationships between the A, B and M forms of human placental alkaline phosphatase.

Several unpublished observations from the Sutcliffe group had established that a human serum factor had the property of "converting" M-PLAP to forms with the electrophoretic mobility of A- and B-PLAP. It was therefore important to establish how similar the products were to the A- and B-PLAP as extracted from whole placental tissue. This would be attempted by purification of the isoenzymes by ion-exchange chromatography, and analysis of the monomer and dimer sizes of A-, B-, M- and converted M-PLAP on starch and acrylamide gels.

A second approach was to characterise the serum factor. The initial study of the conversion factor focussed on the question as to whether or not the

conversion was an enzymatic activity. An attempt at generating a quantitative assay for conversion would be made in order to accurately determine the effects of titration of conversion with time, concentration and temperature. A number of putative enzymes would also be tested for conversion activity.

The final part of the work was an attempt to purify and identify the serum factor. Purification would be done largely by conventional column chromatography and Fast Protein Liquid Chromatography (FPLC). Identification of the factor would involve immunological techniques including Western blotting, antibody-antigen crossed electrophoresis (AACE) and antibody affinity chromatography.

CHAPTER 2

THE PURIFICATION OF THE ISOENZYMES OF PLAP FROM THE PLACENTA AND AN ANALYSIS OF THE PRODUCTS OF CONVERSION OF M-PLAP

CHAPTER 2

The Purification of the Isoenzymes of PLAP from the Placenta and an Analysis of the Products of Conversion of M-PLAP

2.1 Introduction

This chapter presents the methods used to purify and characterise the three isoenzymes of human placental alkaline phosphatase, and an initial study of the conversion activity of serum. Aims and approaches were as follows:

1) To purify the A-, B- and M-PLAP isoenzymes from the placenta, to confirm the properties of M and A and to investigate the characteristics of B-PLAP.

2) To convert M-PLAP and to compare the products of conversion with A- and B-PLAP purified from the placenta.

3) To use the purified M-PLAP as a substrate for preliminary investigations into the possible identity of the conversion factor.

2.2 PURIFICATION PROTOCOL AND RESULTS

2.2.1 Purification of PLAP isoenzymes

The fractionation of the isoenzymes of PLAP on DEAE-Sepharose is shown in Figure 2.1. The ion-exchange was carried out in equilibration buffer and hence in the presence of 0.5 per cent (v/v) Triton X-100. As the concentration of NaCl increased, M-PLAP eluted before A-PLAP. B-PLAP eluted as a rather broad peak at an intermediate mean concentration of NaCl. The fractions (numbers 18 to 29) containing M-PLAP and B-PLAP were pooled and subjected to a further round of ion-exchange under similar conditions. This resulted in a substantial separation of M-PLAP and B-PLAP. Fractions numbered 36 to 45 (Figure 2.1) were combined to give a pool of A-PLAP and B-PLAP. After dialysis against Tris-succinate buffer, the pool was divided in two. Half was re-chromatographed by ion-exchange in the presence of Triton X-100 as before. As shown in Table 2.1, B-PLAP eluted at around 0.08 M NaCl as expected, and A-PLAP eluted between 0.06 M and 0.11 M NaCl. The other half-pool was applied to a DEAE-Sepharose CL-6B column equilibrated with Tris-succinate buffer and eluted with an increasing salt gradient in the absence of Triton X-100. Under these conditions, B-PLAP was found to elute over a range of NaCl strength similar to that of A-PLAP, between 0.08 M

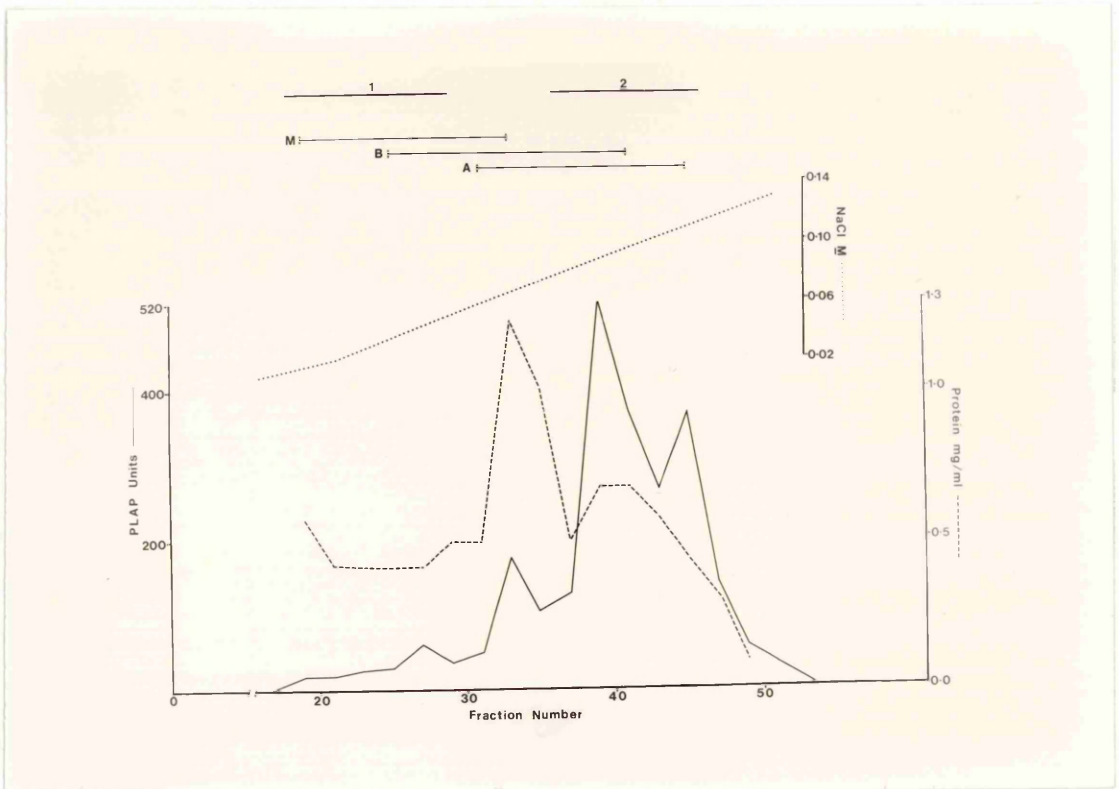


Figure 2.1. Ion-exchange chromatography of microvillous membrane alkaline phosphatase in the presence of 0.5% Triton X-100. DEAE-Sepharose CL-6B chromatography of alkaline phosphatase extracted with butanol from placental microvilli. Enzyme activity (in nmoles/min/ml), protein concentration and the concentrations of NaCl are plotted against fraction number. Aliquots of the fractions positive for enzyme activity were taken for analysis by starch gel electrophoresis. Horizontal bars show where the three isoenzymes eluted as well as which fractions were pooled for further purification.

and 0.11 M.

Table 2.1: Ion-exchange characteristics of A-, B- and M-PLAP: the range of NaCl concentrations required to elute PLAP isoenzymes from DEAE-Sepharose at 4°C in the presence and absence of Triton X-100. In brackets is shown the concentration of NaCl in the fraction containing the maximum activity of the respective isoenzyme.

Isoenzyme	Triton X-100 (%)	NaCl* (mM)
A-PLAP	0.5	70-110 (100)
	0.0	70-110 (100)
B-PLAP	0.5	60-80 (70)
	0.0	80-110 (90)
M-PLAP	0.5	30-50 (40)
	0.0	170-190 (180)

* In a buffer of 25mM succinic acid, 4mM Tris (pH 7.0).

Fractions from the second rounds of ion-exchange were selected to provide stocks of A-PLAP, B-PLAP, and M-PLAP with essentially no cross-contamination by the other PLAP isoenzymes. Typical recoveries from each ion-exchange were around 60 per cent for both protein and enzyme activity. Material isolated in the final pool of A-PLAP constituted 2.5 per cent activity and 0.05 per cent protein of the initial butanol extracts of microvilli. M- and B-PLAP preparations contained 0.6 per cent of the starting activity and 0.01 per cent of the

starting protein. The major loss in recovery of enzyme activity was into fractions containing more than one isoenzyme. Purity with respect to isoenzymic activity was determined by starch gel electrophoresis. Figure 2.2 shows the purified pools of A-, B- and M-PLAP together with the butanol-extracted microvillous membrane starting material as observed on detergent starch gels.

2.2.2 Subunits of PLAP isoenzymes

Figure 2.3 shows the purified pools of A-,B- and M-PLAP compared under reducing conditions. The single band of about 65,000 mol. wt present in A-PLAP (tracks 1 and 2) is also common to B-PLAP (tracks 3 and 4) and M-PLAP (tracks 5 and 6) preparations, suggesting that all three are composed, at least in part, of monomers of similar molecular weights. The lower band of mol. wt of approximately 63,000 was consistently found in B- and M-PLAP preparations from different placentae (tracks 3, 4, 5 and 6). This 63kD band corresponds in mobility to the band generated from PLAP by subtilisin cleavage (compare Figure 2.4, tracks 1 and 2, and tracks 4 and 5), as reported by Abu-Hasan and Sutcliffe (1985). To minimise artifactual cleavage of PLAP during the preparation, syncytiotrophoblast membranes were isolated in the presence of 2 mM PMSF and 1.0 mg/ml TAME, and special care was taken to keep the material on ice and as cold as

Figure 2.2. Starch gel analysis of ion-exchange fractions. The isoenzymic purity of A-, B- and M-PLAP was examined on detergent starch gels following purification by ion-exchange from butanol-extracted placental microvilli (St). The gel was stained for enzyme activity.

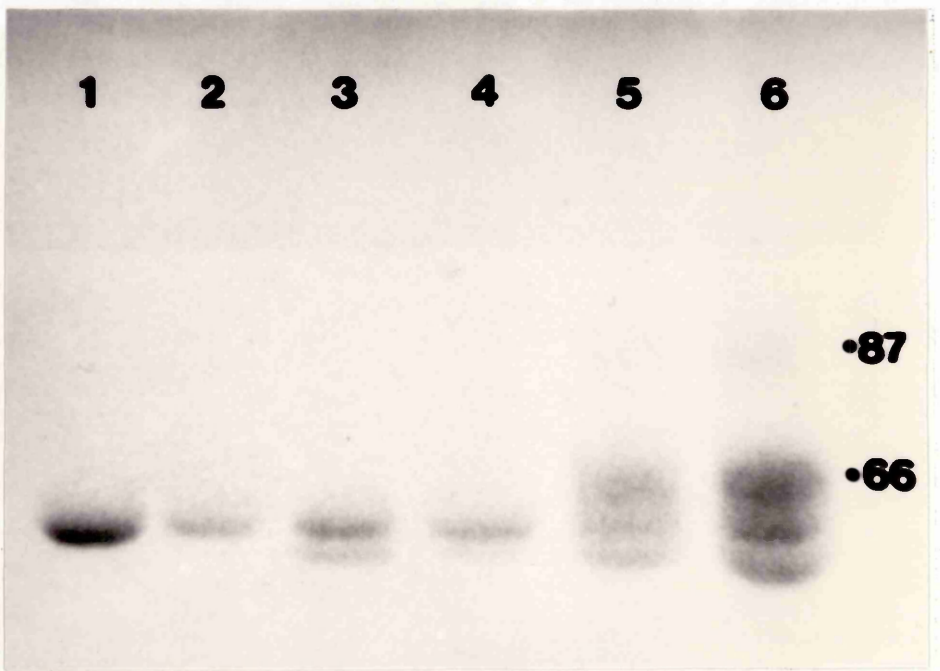


Figure 2.3. SDS-PAGE analysis of ion-exchange fractions. The subunit composition of A-, B- and M-PLAP was visualized by protein staining following SDS-PAGE gel electrophoresis. Tracks 1 and 2 contain A-PLAP (1.0 ug and 0.5 ug respectively); tracks 3 and 4 contain B-PLAP (1.0 ug and 0.5 ug respectively); tracks 5 and 6 contain M-PLAP (1.5 ug and 3.00 ug respectively). Molecular weight calibration is provided from the standard proteins transferrin (87kD) and albumin (66kD) run in the same gel.

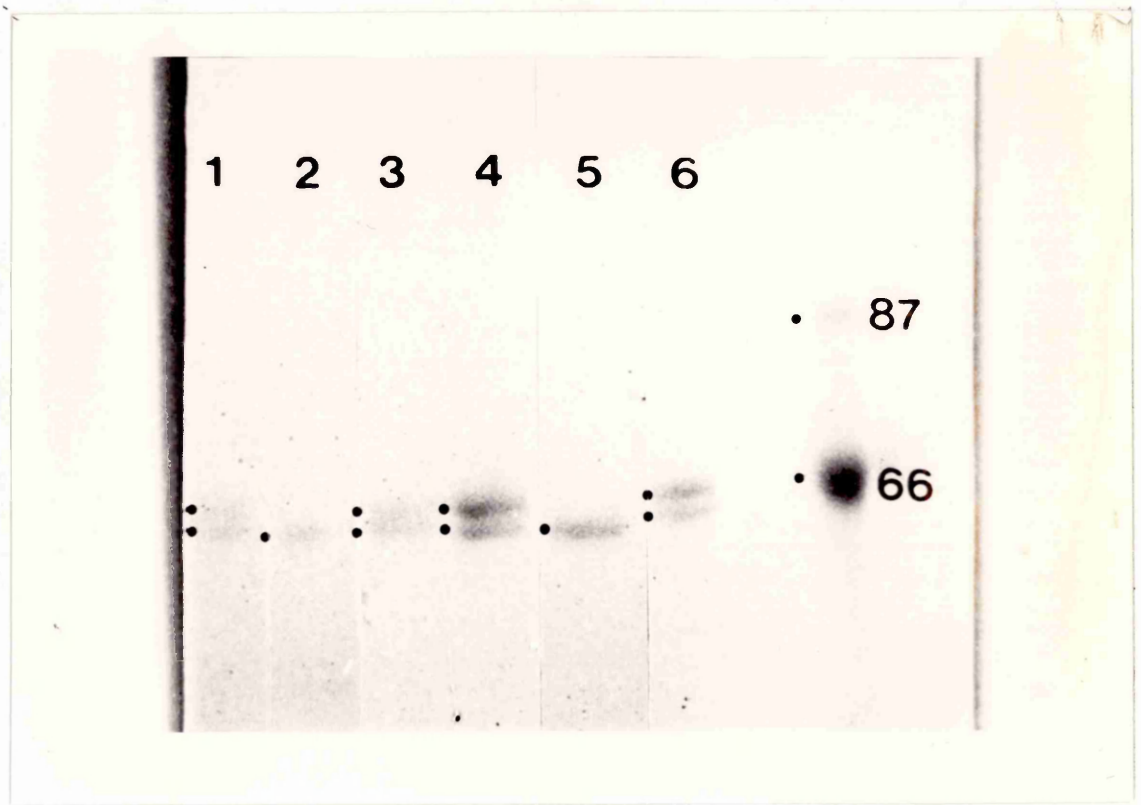


Figure 2.4. Subtilisin treatment of M- and B-PLAP. Subtilisin-treated B-PLAP (track 2) and M-PLAP (track 5) were run under reducing conditions in SDS-PAGE, and the gel subsequently stained for protein. Tracks 1 and 3 contain control B-PLAP, and tracks 4 and 6 contain control M-PLAP. The end track contains marker proteins with the molecular weights indicated (transferrin (86kD) and albumin (66kD)). Spots mark the positions of polypeptide bands in the neighbouring tracks.

possible. However, B-PLAP and M-PLAP still showed a double-banded pattern on reducing gels and the same pattern of enzymatically active bands as shown in Figure 2.5. On no occasion did the A-PLAP preparation show the double bands. The band at 67,000 mol. wt in M-PLAP (tracks 5 and 6) was identified as serum albumin, with which it was found to co-migrate.

Figure 2.5 shows A-, B- and M-PLAP run on SDS-PAGE under non-reducing conditions, stained for alkaline phosphatase activity, and subsequently counterstained for protein with Coomassie blue. A major band of A-PLAP activity (tracks 1 and 2) was found to migrate with a mobility corresponding to a mol. wt of around 115,000, together with minor zones of activity of faster mobilities which were detected when the sample is heavily loaded (track 2). B-PLAP showed a corresponding band of mobility together with one of slightly faster mobility, corresponding to a mol. wt of around 110,000 (tracks 3 and 4). M-PLAP had a double-banded zone of mobility considerably faster than either A- or B-PLAP corresponding to a mol. wt of around 100,000 (tracks 5 and 6), together with a protein band of faster mobility which co-migrates with the serum albumin marker in the adjacent track.

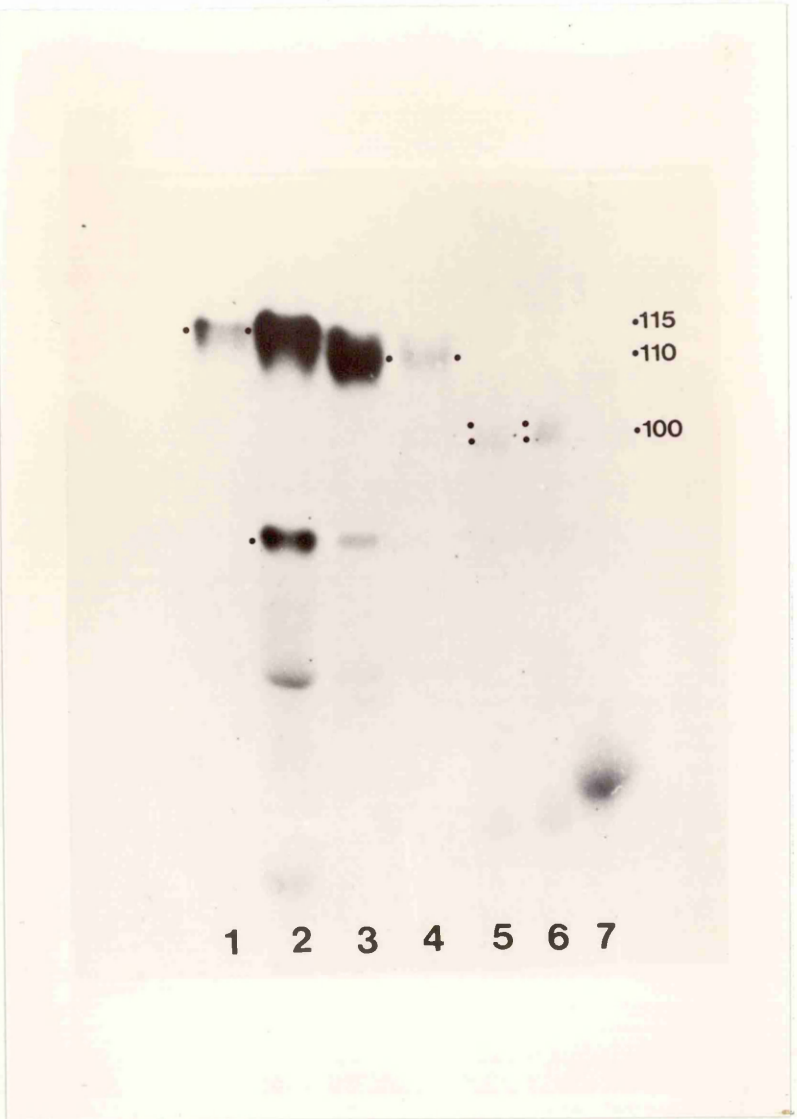


Figure 2.5. Non-reduced acrylamide gel analysis of ion-exchange fractions following PAGE under non-reducing conditions. Enzyme activity of A-,B- and M-PLAP was visualized by enzyme staining, and those bands which stained are indicated by spots. The gel was subsequently counterstained for protein. Tracks 1 and 2 contain A-PLAP (1.0 ug and 2.0 ug respectively); tracks 3 and 4 contain B-PLAP (1.5 ug and 0.3 ug respectively); tracks 5 and 6 contain M-PLAP (0.5 ug and 1.0 ug respectively). Track 7 contains 1.0 ug of serum albumin. Corresponding molecular weights were calculated from standard proteins run in the same gel under reducing conditions.

2.2.3 Conversion of M-PLAP to A- and B-PLAP

Figure 2.6 shows the effect on the starch gel mobilities of the isoenzymes following incubation of PLAP with normal human serum. Tracks 1, 3 and 5, where PLAP was incubated in the absence of serum, were compared with tracks 2, 4 and 6, where PLAP was treated with serum. A- and B-PLAP showed no change on incubation with serum, but M-PLAP showed generation of activities corresponding to the mobilities of A- and B-PLAP.

The effect on conversion activity of increasing the concentration of serum, increasing the duration of incubation (0 to 3 hours) and increasing temperature of incubation (4°C to 37°C) was studied in detergent starch gels by comparing aliquots of M-PLAP incubated under a variety of conditions. The results are shown in Figure 2.7. Conversion is greater during incubation with undiluted serum than is observed during incubation with a tenfold dilution of serum (compare tracks 4, 5 and 6 with tracks 10, 11 and 12). Secondly, during incubation at 4°C with 1/10 diluted serum, conversion is more extensive after 3 hours than after 1 hour or 10 minutes (tracks 1, 2 and 3). Thirdly, more conversion activity is detected at 37°C than at 4°C (comparing tracks 7, 8 and 9 with tracks 10, 11 and 12).

The products of conversion of M-PLAP to A-PLAP and B-PLAP were analysed on SDS-PAGE. Figure 2.8 (panel 1)

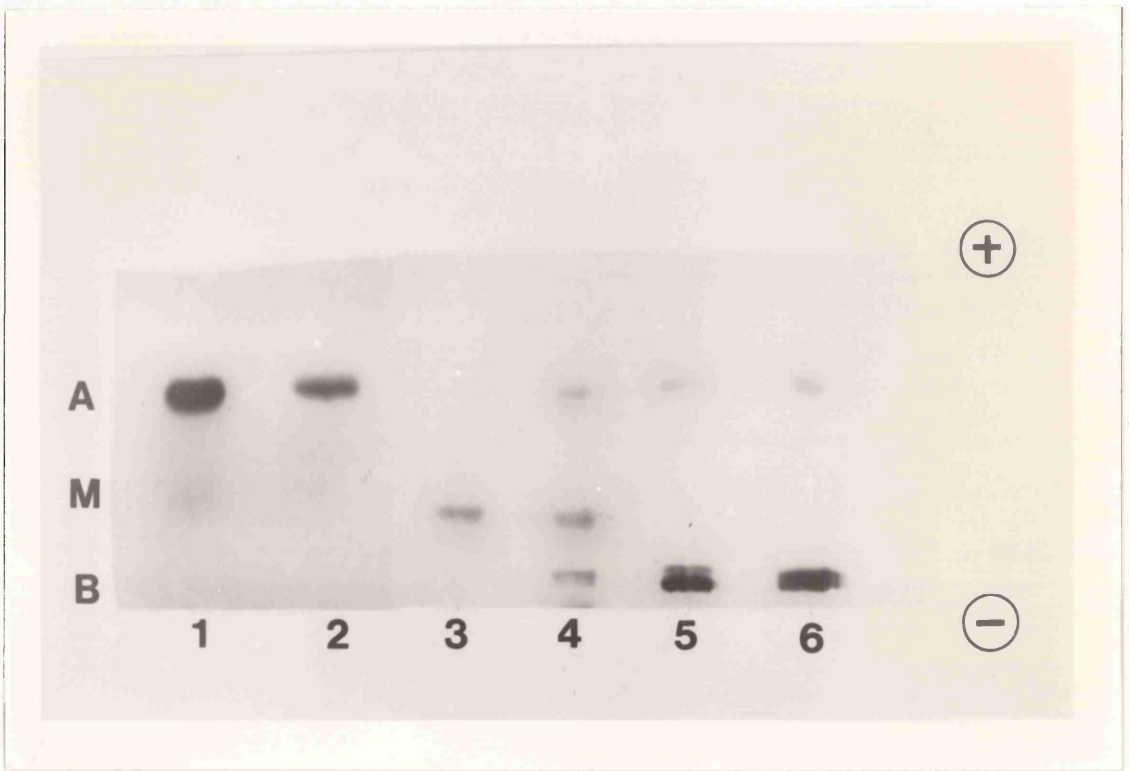
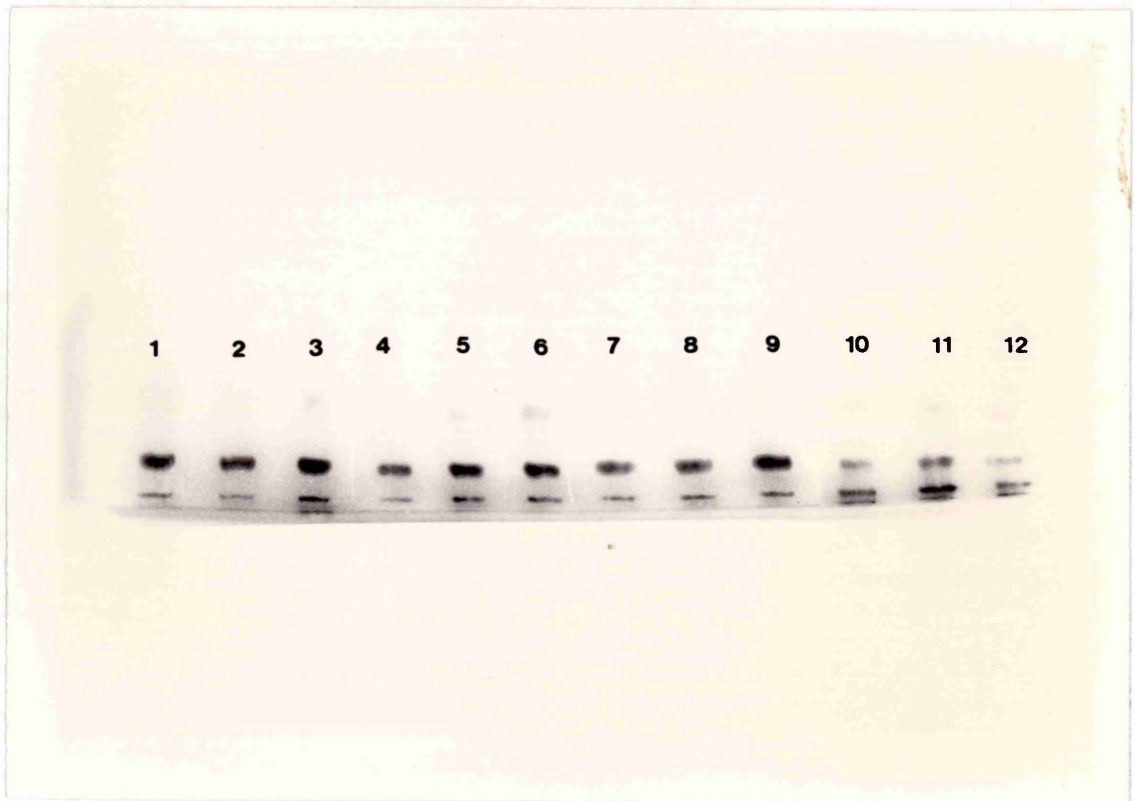


Figure 2.6. Starch gel electrophoresis of PLAP incubated with or without serum. Aliquots of A-, B- and M-PLAP were incubated either in the presence (even-numbered tracks) or absence (odd-numbered tracks) of normal human serum for 4 hours at 37°C in the presence of protease inhibitors, then run on starch gels. Tracks 1 and 2 contain A-PLAP, tracks 3 and 4 contain M-PLAP and tracks 5 and 6 contain B-PLAP. The gel was stained for enzyme activity.



Ti	10	60	180	10	60	180	10	60	180	10	60	180
Tm	4	4	4	37	37	37	4	4	4	37	37	37
Dn	D	D	D	D	D	D	U	U	U	U	U	U

Figure 2.7. Conversion of M-PLAP titrated against time, concentration and temperature as visualized by enzyme staining on detergent starch gels. Time (Ti). Incubations were performed for ten minutes, or for 60 minutes, or for 180 minutes as indicated below the tracks 4°C or at 37°C as indicated below the tracks. Serum concentration (Dn). Incubations were performed with either serum diluted 1/10 (D) or with undiluted serum (U) as indicated below the tracks.

shows an SDS gel in the absence of reduction in which control tracks of A-, M- and B-PLAP (tracks 1, 3 and 5, respectively), incubated without serum, are compared with similar material (tracks 2, 4 and 6) incubated in the presence of serum and protease inhibitors. A-PLAP shows a single major band of activity, B-PLAP has a band of similar mobility and a band of faster mobility. Neither A-PLAP nor B-PLAP are affected by serum. M-PLAP shows two major zones of activity of considerably faster mobility than that of A- or B-PLAP. However, when treated with serum, M-PLAP generated two new zones of activity which co-migrated with the zones for A-PLAP and B-PLAP.

Figure 2.8 (panel 2) shows the same incubations, together with molecular weight markers and tracks containing semi-purified convertase alone run under reducing conditions. There is no observable reduction of the monomeric molecular weight of A-, B- or M-PLAP. The patterns remain as observed in Figure 2.3.

2.2.4 Further investigation of the serum factor

Partial characterization and purification of the conversion factor was achieved by means of chromatographic and physiological criteria. On ion-exchange on DEAE-Sepharose CL-6B, the factor eluted as a unimodal peak of activity at a salt strength of 0.09 M NaCl. A similar elution point has been reported for

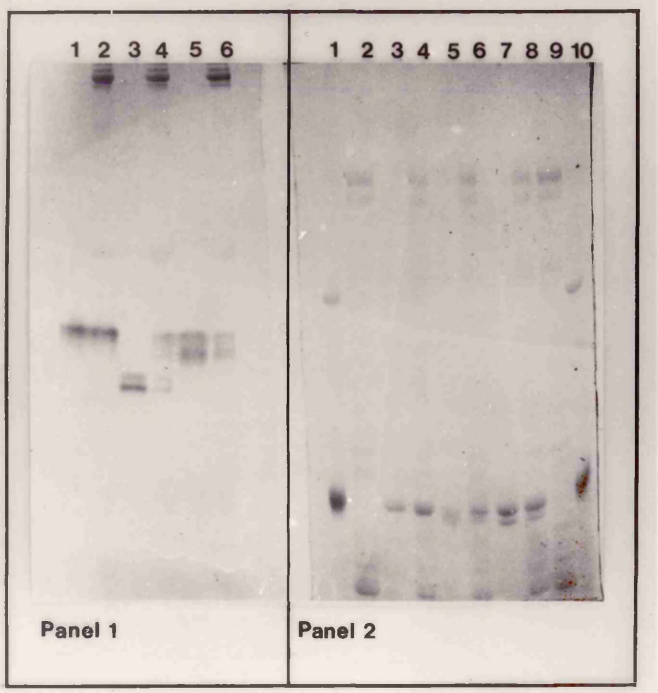


Figure 2.8. Serum-converted products on SDS-PAGE. Panel 1. Control A-,M- and B-PLAP (tracks 1, 3 and 5) and A-, M- and B-PLAP following incubation with normal serum in the presence of protease inhibitors (tracks 2, 4 and 6) were visualized by protein staining on polyacrylamide gels in the absence of reduction. Panel 2. The same material was also run under reducing conditions, and stained for protein. Tracks 3 and 4 contain A-PLAP, tracks 5 and 6 contain M-PLAP and tracks 7 and 8 contain B-PLAP. Tracks 2 and 9 contain semi-purified convertase alone. Tracks 1 and 10 contain serum albumin (66000) and transferrin (87000).

lecithin cholesterol acyltransferase (LCAT) by Chong *et al* (1983), but purified LCAT fractions did not contain active converting factor. The factor appears to be a glycoprotein since it binds to a ConA-Sepharose affinity column. Treatment of a volunteer (RGS) with 5,000 units of intravenous heparin did not result in an increase in activity of serum conversion when tested against M-PLAP, as might have been expected had lipoprotein lipase been the conversion agent (Hahn, 1943; Korn, 1959). In three independent preparations the conversion activity was found in the post-lipoprotein fraction of serum; it was therefore not found to be associated with the lipoprotein fractions of serum.

The heat stability of the converting agent(s) was investigated by preincubating serum at a range of temperatures prior to incubation with M-PLAP for 8h at 37°C. The proportion of M-PLAP converted to A- and B-PLAP was then estimated on detergent starch gels; the results are presented in Figure 2.9. Conversion activity is present in serum preincubated for 1 hour at 45°C (track 7), but is much reduced in serum preincubated for 30 min at 53°C (track 4).

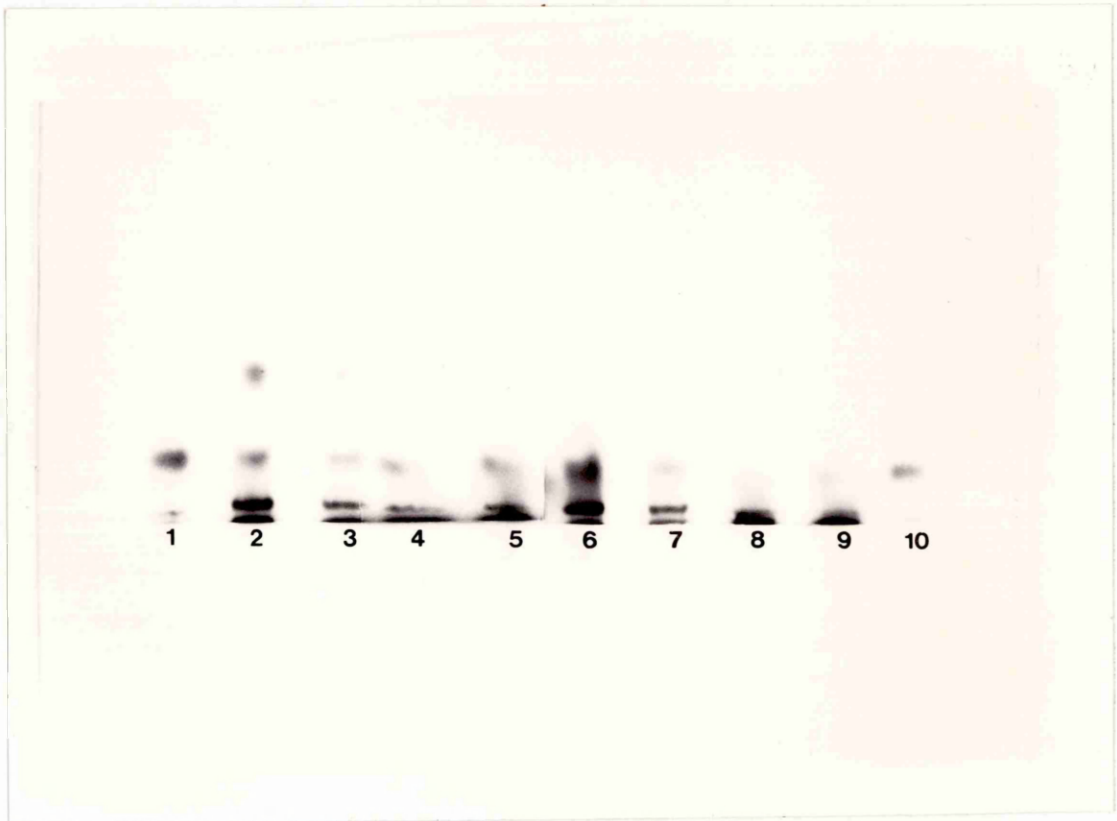


Figure 2.9. Heat inactivation of the serum factor. Serum was preincubated under the following conditions and the conversion activity was visualised by enzyme staining on detergent starch gels. Serum in tracks 2 to 5 was incubated for 1 hour, while serum in tracks 6 to 9 was incubated for 30 minutes. Incubation was performed at 37°C (tracks 2 and 6), at 45°C (tracks 3 and 7), at 53°C (tracks 4 and 8), and at 60°C (tracks 5 and 9). Tracks 1 and 10 contain control M-PLAP.

2.3 DISCUSSION

2.3.1 Structural analysis

Ion-exchange chromatography has been used to fractionate three isoenzymes of human placental alkaline phosphatase (PLAP). The method took advantage of the differential effect of non-ionic detergent on the elution of the B and M isoenzymes from diethylaminoethyl-(DEAE)-Sepharose CL-6B. Abu-Hasan and Sutcliffe (1984) proposed that this chromatographic effect was due to the binding of DEAE-Sepharose CL-6B to a hydrophobic component in M-PLAP which is reduced in the presence of Triton X-100. The present data show that B-PLAP also has hydrophobic properties, a suggestion previously advanced by Doellgast *et al* (1977). However, the shift in the elution point caused by the detergent is less for B-PLAP than for M-PLAP (Table 1), suggesting that the order of hydrophobicity is M-PLAP > B-PLAP > A-PLAP. This order is supported by the higher affinity of M-PLAP for alkyl agarose (Abu-Hasan and Sutcliffe, 1984) and the finding that M-PLAP can only be resolved in starch gels in the presence of detergent. Detergent causes B-PLAP to have a slightly increased mobility in detergent gels but has no effect on the mobility of A-PLAP.

Although there was some contaminating albumin in the preparation of M-PLAP (Figure 2.3, tracks 5 and 6), the

results showed that a major polypeptide of mol. wt 65,000 is common to all three isoenzymes, as has previously been reported (Doellgast et al, 1977; Abu-Hasan, Davies and Sutcliffe, 1984; Abu-Hasan and Sutcliffe, 1985). The molecular weight estimation of 65,000 is in close correspondence with that described by Badger and Sussman (1976) and by Holmgren and Stigbrand (1976); the value of 69,000 previously reported by the Glasgow group has now been revised downwards, although some gel preparations still yield a PLAP polypeptide of mobility equal to 69,000 mol. wt. The basis of this variation is still unknown, but relates to variation between preparations of polyacrylamide gel.

Subtilisin cleavage of PLAP generates a band of very similar mobility to the 63,000 mol. wt band reported for M- and B-PLAP (Figure 2.4). The characteristics of subtilisin-cleaved M-PLAP are a PLAP molecule with a native mol. wt of around 130,000 and a mobility on detergent starch gels of slightly faster than A-PLAP. However, we believe that this 63,000 mol. wt band is not due to proteolytic activity since the 63,000 mol. wt band reported for M- and B-PLAP in this paper is present without there being these concomitant changes in the size or mobility of M-PLAP. Further, the conditions of purification at low temperature in the presence of phenylmethylsulphonyl fluoride (PMSF) and Na-p-tosyl-l-

arginine ester (TAME) were designed to counter possible protease activity. Also, in our hands, proteases are equally efficacious on M- and A-PLAP (Abu-Hasan and Sutcliffe, 1985), whereas the 63,000 mol. wt band is not found in A-PLAP. Since subtilisin activity does not visibly modify the 63,000 mol. wt band (Figure 2.4), the subtilisin site must either be absent, masked by conformation or carbohydrate, or very close to the carboxy terminus of the 63,000 mol. wt polypeptide.

Independent evidence for the presence of another PLAP polypeptide has recently come from the work of Nickson, Livingstone and Sutcliffe (1986), who found that two PLAP polypeptides of mol. wt 56,000 and 58,000 could be specifically immunoprecipitated from the *in vitro* translation of term placental villus messenger ribonucleic acid (mRNA). Similar results have been reported by Ovitt *et al* (1986) who also found that only one polypeptide was translated in JEG-3, JAR and BeWo choriocarcinoma cell lines, confirming the report of Ito and Chou (1983). The differences in mol. wt between the polypeptides translated *in vitro* and *in vivo* argue that this heterogeneity is not due to glycosylation or any other post-translational process. Instead it indicates a heterogeneity of PLAP mRNA which specifies two related PLAP polypeptides in the syncytiotrophoblast, but not in choriocarcinoma cells.

2.3.2 Isoenzyme Dimer Comparisons

Non-reducing sodium dodecyl sulphate (SDS) polyacrylamide gels were used to resolve the dimers of PLAP (Figure 2.5). This is particularly convenient as the dimers can be detected by enzymatic staining. M-PLAP (tracks 5 and 6) runs as a double-banded zone of activity of a mobility corresponding to a mol. wt of around 100,000. These properties had previously been observed for M-PLAP (Abu-Hasan and Sutcliffe, 1984). The two stained species of mol. wt 100,000 would appear to be components of M-PLAP, since the samples analysed were essentially free of B- and A-PLAP (Figure 2.2). The dimers of B-PLAP are now shown for the first time as a single band of mol. wt around 110,000 (tracks 3 and 4), whereas A-PLAP runs with a mobility corresponding to a mol. wt of around 115,000.

2.3.3 Isoenzyme Conversion

Despite repeated attempts, M-PLAP has never been detected in maternal serum (N.S. Abu-Hasan, 1985, unpublished). This fact can now be explained by the finding of a factor in serum which can convert M-PLAP. It also establishes M-PLAP as the membrane form of the enzyme, with the previously described A and B forms being created by conversion from M-PLAP. The products of

conversion are indistinguishable from A-PLAP and B-PLAP on starch gel electrophoresis (Figure 2.6, tracks 1, 4 and 5). Further, the processed products have dimer mobilities identical to A- and B-PLAP (Figure 2.8, panel 1, tracks 1, 4 and 5). This modification does not appear to involve a proteolytic step since there is no change in subunit molecular weight (Figure 2.8, panel 2, tracks 5 and 6), and can proceed in the presence of protease inhibitors. Within the limits of sensitivity of the starch gel system, the conversion activity appears to titrate with time, temperature and concentration (Figure 2.7), and so may be an enzymatic activity. The factor is subject to heat inactivation between 45°C and 53°C (Figure 2.9).

We pursued the hypothesis that the removal of lipid moieties might be involved in the conversion of M-PLAP to the less hydrophobic A and B forms. We used the knowledge that heparin displaces lipoprotein lipase (LPL) from vascular epithelial surfaces (Hahn, 1943; Korn, 1959), to increase the serum concentration of LPL in a volunteer (RGS). Since the subsequent serum was not found to contain increased conversion activity, the factor is unlikely to be LPL. Nor is it likely to be lecithin cholesterol acyltransferase, since purified LCAT preparations did not contain conversion activity. These conclusions must be treated with caution, since these

materials were tested under conditions which were optimal for conversion of M-PLAP by serum.

2.3.4 Molecular Heterogeneities

The original reports that M-PLAP is the major component of microvillous PLAP proposed that post-translational modification or mRNA heterogeneity could account for the heterogeneity of PLAP isoenzymes (Abu-Hasan and Sutcliffe, 1985). On the basis of the present work, and that of Nickson, Livingstone and Sutcliffe (1986) and Ovitt *et al* (1986) both hypotheses seem to be required. A-PLAP and B-PLAP have been shown in this chapter to be derived from M-PLAP. This does not occur spontaneously in solutions of M-PLAP, but requires a protein factor found in serum. This conversion is not accompanied by polypeptide modifications which are detectable on reducing SDS-PAGE, though it is detected on starch and non-reducing SDS gels. This conversion is directional; in our hands the A and B isoenzymes do not convert to M-PLAP. The existence of A-and B-PLAP as the two conversion products of M-PLAP suggested that M-PLAP might not be a simple multimer of homodimers (Abu-Hasan, Davies and Sutcliffe, 1984; Abu-Hasan and Sutcliffe, 1985), but that some assymmetry or heterogeneity exists in M-PLAP to generate both A- and B-PLAP. The evidence for the doublet 63,000 and 65,000 mol. wt bands in M- and

B-PLAP have been discussed above. Whether or not they represent post-transcriptional (mRNA) heterogeneity, the presence of the doublets in M- and B-PLAP but not A-PLAP indicates that M-PLAP is structurally heterogeneous and that when converted to the A- and B-PLAP isoenzymes, the 63,000 mol. wt band is present only in B-PLAP. Heterogeneity of M-PLAP in non-denaturing gels has previously been reported (Abu-Hasan and Sutcliffe, 1984). It is proposed that the polypeptide component represented by the 63,000 mol. wt band is important in the formation of M- and B-PLAP and may confer hydrophobicity upon them. How a polypeptide could confer this property is a matter for speculation, but it may force the protein to adopt a different tertiary structure in which more hydrophobic residue are exposed.

The work presented in this chapter was initially reported in modified form by Livingstone et al (1987).

CHAPTER 3

AN APPROACH TO A QUANTITATIVE ASSAY FOR CONVERSION

Chapter 3

An Approach to a Quantitative Assay for Conversion

3.1Introduction

The previous chapter included some experiments on the heat-stability of the conversion factor and the effects of time, concentration and temperature on the extent of M-PLAP converted. The precision of these studies would have been much improved had there been available a quantitative assay for the conversion of M-PLAP. The results presented in this chapter describe one approach to the development of such an assay.

It was a well established observation in Glasgow that M-PLAP did not migrate into starch gels during electrophoresis in the absence of detergent, but rather remained on the filter paper insert at the origin. Under the same conditions A- and B-PLAP are not retarded. My hypothesis was that these properties could be used to separate M-PLAP from the A and B isoenzymes produced during the conversion reaction. If this were so, then the alkaline phosphatase activity retained on a filter paper insert following electrophoresis would be directly related to the proportion of M-PLAP present. Performing this assay before and after incubation with serum or

serum proteins, by subtraction it would be possible to determine the level of conversion that had taken place during incubation.

3.1.1 Basic Procedure

M-PLAP stock was incubated with an equal volume of serum proteins and 10ul aliquots were spotted onto filter paper inserts and electrophoresed at 20V/cm for one hour at 4°C in a standard detergent-free starch gel. After this time, the filter was removed and placed in 200ul of an appropriate detergent solution and incubated at 37°C for one hour with frequent vortexing. Aliquots of 20ul were removed and assayed in the standard alkaline phosphatase assay for one hour at room temperature. This was compared with a control of the same sample spotted, washed and assayed in the absence of electrophoresis.

3.2 Results

3.2.1 Detergent Effects

The initial experiments were aimed at finding suitable detergent solutions in which alkaline phosphatase activity could be assayed. To this end, aliquots of M-PLAP were incubated at 37°C for up to one hour in tris-succinate buffer (pH 7.4) alone, or in buffer containing one of the following detergents0.5% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.5% (v/v) Nonidet NP40, 0.25% (w/v) sodium dodecyl sulphate, or 0.5% (v/v) Tween 20. The mean of triplicate experiments was calculated and is shown in Table 3.1 presented as a percentage of the control AP activity in buffer alone.

Table 3.1: The effect of detergent on M-PLAP activity in solution following incubation for 30 minutes or for 60 minutes.

Incubation Conditions	30 minutes	60 minutes
Buffer alone	100%	100%
Triton X-100 (0.5%)	129%	149%
Na Deoxycholate (0.5%)	211%	271%
Nonidet NP40 (0.5%)	172%	220%
SDS (0.25%)	28%	23%
Tween 20 (0.5%)	168%	225%

The above results show that incubation with four of the five detergents results in an increase in alkaline phosphatase activity. No alkaline phosphatase activity was detected in these detergent buffer solutions before addition of M-PLAP. Neither did the presence of detergent have an effect on the extinction coefficient of p-nitrophenol at 400 nm. The reason for this "detergent effect" was not determined but may be related to the relative hydrophobicity of the M-PLAP molecule. Detergent may allow a more efficient interaction with the aqueous phase and hence with substrate molecules. Having observed this effect we were able to allow for it in subsequent experiments.

3.2.2 Recovery of Activity from Filter Paper Inserts

To quantify the alkaline phosphatase activity retained on a filter paper insert following electrophoresis, it was important that the retained activity be efficiently recovered from the insert into the detergent wash. To examine the efficiency of a range of detergent washes, at the concentrations used in Figure 3.1, aliquots of M-PLAP were applied to filter paper inserts which were then dipped in the appropriate solution. This was compared with the addition of M-PLAP directly to the appropriate detergent wash (as a control). The detergent wash was performed for one hour at 37°C with frequent vortexing. Triplicate results are presented in Table 3.2.

Table 3.2: The efficiency of detergent solutions in washing M-PLAP from filter paper inserts.

Incubation Conditions		Measured Activities			Mean Activity	Detergent Effect	Recovered Activity
Buffer Control	-	0.264	0.348	0.296	0.303	100%	
	+	0.054	0.050	0.036	0.047		15%
NP40	-	0.418	0.476	0.424	0.439	145%	
	+	0.324	0.339	0.318	0.327		74%
Deoxy	-	0.567	0.575	0.617	0.586	194%	
	+	0.486	0.548	0.495	0.510		87%
Tx100	-	0.434	0.458	0.411	0.435	143%	
	+	0.345	0.296	0.352	0.331		76%
SDS	-	0.055	0.068	0.030	0.051	17%	
	+	0.043	0.024	0.032	0.033		65%
Tween	-	0.412	0.433	0.439	0.428	141%	
	+	0.289	0.310	0.269	0.290		68%

- Alkaline phosphatase solution added directly to detergent wash ie. no filter paper inserts used. The data under the heading "Detergent Effect" was calculated by:

$$\text{Percentage} = \frac{\text{Mean activity in test solution}}{\text{Mean activity in buffer control}} \times \frac{100}{1}$$

+ Filter paper inserts impregnated with alkaline phosphatase solution then washed in detergent. The data under the heading "Recovered Activity" was calculated by:

$$\text{Percentage} = \frac{\text{Mean activity washed into solution}}{\text{Mean activity added directly}} \times \frac{100}{1}$$

The results of this experiment show that 87% of the applied activity is washed from the filter in sodium deoxycholate buffer after one hour, compared with 15% for the buffer control. It was decided to use this detergent routinely in the convertase assay.

3.2.3 Concentration of Na Deoxycholate

Up to this stage in the investigation, detergents had been used at arbitrarily assigned concentrations. It was therefore decided to examine more closely the optimal concentration of Na Deoxycholate to use in the assay. Table 3.3 shows the results of washing the filter papers in one of five different concentrations. Controls were the aliquot of M-PLAP added directly into the detergent solutions, and the filters washed in buffer alone. Triplicate experiments were incubated at 37°C for one hour with frequent vortexing.

Table3.3: The efficiency of a range of concentrations of Na Deoxycholate in washing M-PLAP from filter paper inserts.

Incubation Conditions		Measured Activities			Mean Activity	Detergent Effect	Recovered Activity
Buffer Control	-	0.199	0.221	0.192	0.204	100%	
	+	0.036	0.024	0.013	0.024		12%
0.05%	-	0.291	0.308	0.305	0.301	148%	
	+	0.059	0.068	0.046	0.058		19%
0.16%	-	0.345	0.364	0.367	0.359	176%	
	+	0.093	0.095	0.071	0.086		25%
0.50%	-	0.394	0.404	0.422	0.407	200%	
	+	0.301	0.325	0.388	0.338		83%
1.10%	-	0.347	0.399	0.400	0.382	187%	
	+	0.377	0.332	0.338	0.349		91%
2.50%	-	0.323	0.344	0.316	0.328	161%	
	+	0.319	0.332	0.308	0.320		98%

- Alkaline phosphatase solution added directly to detergent wash ie. no filter paper inserts. For detergent effect:

$$\text{Percentage} = \frac{\text{Mean activity in test solution}}{\text{Mean activity in buffer control}} \times \frac{100}{1}$$

+ Filter paper inserts impregnated with alkaline phosphatase solution then washed in detergent. For recovered activity:

$$\text{Percentage} = \frac{\text{Mean activity washed into solution}}{\text{Mean activity added directly}} \times \frac{100}{1}$$

The results of this experiment demonstrate that the earlier fortuitous choice of 0.5% sodium deoxycholate was a good one, since lower concentrations are not as efficient in leaching the activity into the wash solution. At higher concentrations, the detergent effect is not as marked, possibly indicating denaturation of the alkaline phosphatase.

3.2.4 Conversion Assay Sensitivity to A-, B- and M-PLAP

The next important step in the testing of this assay system was to investigate whether there were any differences in the recoverable activities following electrophoresis for A-, B- and M-PLAP. In short, whether the assay can discriminate between M-PLAP and A- or B-PLAP. For this and subsequent experiments the AP was taken from the purified isoenzyme stocks shown in the previous chapter (Figure 2.2). Each stock contained a separate PLAP isoenzyme, but they contained differing amounts of contaminating protein (Figure 2.3). Thus, in order to load filter paper inserts with an equivalent alkaline phosphatase activity, it would be necessary to load differing amounts of protein. It was necessary to investigate the effects of different protein loadings simultaneously to loadings of A-, B- and M-PLAP. In short, this experiment involved loading an equivalent activity of A-, B- and M-PLAP in either buffer alone, or buffer and dialysed bovine serum albumin (4mg/ml) (dialysed to exclude possible salt effects). No alkaline phosphatase activity was detected in this BSA solution. Results are presented in Table 3.4, with triplicate filter paper inserts subjected to electrophoresis at 20V/cm for one hour at 4°C, then incubated at 37°C in 0.5% Na Deoxycholate for one hour with frequent vortexing. These are compared to washed filter papers

that have not been subjected to electrophoresis.

Table 3.4: The sensitivity of the conversion assay to A-, B- and M-PLAP in the presence and absence of 4mg/ml BSA.

Incubation Conditions		Measured Activities			Mean Activity	Recovered Activity
A-PLAP	-	0.150	0.153	0.168	0.158	
	+	0.033	0.039	0.030	0.035	22%
B-PLAP	-	0.274	0.286	0.289	0.284	
	+	0.101	0.087	0.106	0.099	35%
M-PLAP	-	0.434	0.355	0.368	0.386	
	+	0.253	0.204	0.239	0.233	60%
A+BSA	-	0.407	0.405	0.366	0.393	
	+	0.011	0.002	0.003	0.006	2%
B+BSA	-	0.708	0.706	0.683	0.700	
	+	0.032	0.043	0.024	0.034	5%
M+BSA	-	0.507	0.570	0.533	0.537	
	+	0.240	0.227	0.248	0.239	46%

\- No electrophoresis performed.

\+ Electrophoresis performed.

$$\text{Percentage} = \frac{\text{Mean Activity After Electrophoresis}}{\text{Mean Activity Before Electrophoresis}} \times \frac{100}{1}$$

The results show that considerably more AP activity remains on the filter paper following electrophoresis when applied in the form of M-PLAP than either A- or B-PLAP. I interpret this to be because the A and B isoenzymes are electrophoresed into the gel. Thus the assay is substantially specific for M-PLAP compared to A- or B-PLAP. The recovery for B-PLAP is higher than for A-PLAP. This may be because B-PLAP has a slower mobility than A-PLAP. Thus, more B-PLAP may have left the filter paper had the electrophoresis been prolonged. However,

since 95% of the applied B-PLAP left the paper in one hour, this possibility was not further investigated.

Wash solutions contained more AP activity following incubation in the presence of BSA compared to incubations in the absence of added protein (A-PLAP activity increases from 0.158 to 0.393; B-PLAP rises from 0.284 to 0.700; M-PLAP is increased from 0.386 to 0.537). The reason for this effect is not known, but may be due to alkaline phosphatase binding non-specifically and irreversibly to the filter papers, an effect saturated out by the added BSA. The results are further complicated by the observation that the mean A- and B-PLAP activity following electrophoresis was greater in the absence than in the presence of BSA (A-PLAP activity dropped from 0.035 to 0.006, while B-PLAP activity fell from 0.099 to 0.034). This may indicate the existence of further protein-binding sites on the filters which retain protein during electrophoresis but release it during the detergent wash. Thus, in the absence of BSA, AP binds to these sites and subsequently give an increase in the overall recovered activity. It is of interest that this effect is not observed for M-PLAP.

In short, these effects indicated that this assay would be potentially limited to comparing samples at the same protein concentration. We termed this limitation as being due to the "protein effect".

3.2.5 Reconstruction Experiment

A final test of the assay was performed using mixtures of M-, A- and B-PLAP to simulate the end products of a range of levels of conversion. No conversion (0%) was simulated by applying activity in the form of M-PLAP alone. Total conversion (100%) was simulated by applying no M-PLAP, but rather 50% A-PLAP and 50% B-PLAP. Intermediate extents of conversion (35% and 70%) were also reconstructed with appropriate mixtures of the three isoenzymes. Since we had established a "protein effect", and since we hoped to be able to use the assay to determine conversion activity in serum protein fractions, the assays were performed in either 5mg/ml, 20mg/ml or 30mg/ml BSA.

Appropriate mixtures of A-, B- and M-PLAP and BSA were loaded onto filter paper inserts and subjected to electrophoresis. The papers were then placed in 0.5% sodium deoxycholate and washed at 37°C for one hour with frequent vortexing. Results were then compared to the control, for which activity was detected when an aliquot of each mixture was added directly to the detergent wash (Table 3.5). Activity was added directly in this control to eliminate possible artifacts due to the differential binding to the filter paper identified as the "protein effect" above.

Table 3.5: Reconstruction of degrees of conversion of M-PLAP to A- and B-PLAP.

Conversion Simulated		Measured Activities			Mean Activities	Recovered Activity (%)
In 5mg/ml BSA:						
0%	-	0.524	0.532	0.564	0.540	
	+	0.242	0.224	0.293	0.253	47%
35%	-	0.674	0.697	0.640	0.671	
	+	0.145	0.138	0.133	0.139	21%
70%	-	0.785	0.767	0.744	0.766	
	+	0.099	0.083	0.081	0.088	11%
100%	-	0.862	0.811	0.846	0.840	
	+	0.048	0.026	0.043	0.039	5%
In 20mg/ml BSA:						
0%	-	0.532	0.498	0.529	0.520	
	+	0.133	0.096	0.148	0.126	24%
35%	-	0.611	0.555	0.642	0.603	
	+	0.114	0.066	0.129	0.103	17%
70%	-	0.715	0.732	0.648	0.699	
	+	0.080	0.053	0.053	0.071	10%
100%	-	0.862	0.826	0.816	0.834	
	+	0.036	0.027	0.030	0.031	4%
In 30mg/ml BSA:						
0%	-	0.510	0.491	0.515	0.506	
	+	0.078	0.133	0.155	0.122	24%
35%	-	0.579	0.599	0.648	0.609	
	+	0.103	0.105	0.134	0.114	19%
70%	-	0.764	0.841	0.802	0.803	
	+	0.084	0.063	0.080	0.076	9%
100%	-	0.870	0.854	0.875	0.867	
	+	0.042	0.032	0.037	0.037	4%

\- Alkaline phosphatase added directly to detergent solution.

\+ Filter papers subjected to electrophoresis, then placed in detergent solution.

These results indicate that at each protein concentration, the percentage activity recovered in the detergent wash was proportional to the amount of M-PLAP

applied to the filter, and hence proportional to the extent of conversion simulated.

3.2.6 Use of the Assay to Monitor Conversion of M-PLAP

At this stage it was decided to use the assay system to monitor actual conversion of M-PLAP under the normal conditions reported in Chapter 2. However, the PLAP material used routinely in the filter paper assay was too dilute for me to be able to independently monitor the conversion in the PLAP material by electrophoresis into detergent gels and subsequent staining (as described in Chapter 2). For this reason, it was necessary to perform the conversion reaction with undiluted M-PLAP, after which a 20ul aliquot was removed and assayed by electrophoresis and staining. The incubated material was then diluted into the appropriate range for the filter paper assay.

A second modification was aimed at eliminating the "protein effect". Incubations were performed in either undiluted serum or in serum diluted 1/10 or in no serum. Overall protein concentrations were standardised by making up any shortfall with heat-inactivated serum (incubated at 53°C for 60 minutes. These conditions had previously been shown to remove conversion activity from serum (see Figure 2.9)).

In this experiment, M-PLAP was incubated with an

equal volume of buffer or serum proteins for zero, four or sixteen hours. An aliquot was removed and assayed for conversion by electrophoresis and staining, and the remaining solution diluted 1/25. Aliquots of this material was spotted onto filter papers and subjected to electrophoresis in a detergent-free starch gel at 4°C for one hour. Alkaline phosphatase activity was then washed from the filters in 0.5% sodium deoxycholate buffer at 37°C in one hour. Activity in the wash solution was then assayed, and compared with activity detected when an aliquot of each AP solution was added directly to the wash solution (Table 3.6).

Table3.6: Final experimental system. Assay of AP activity following incubation of M-PLAP with serum proteins.

Incubation Conditions		Conversion Status*		Measured Activities			Mean Activities	Recovered Activity (%)
zero time:								
zero	S	-	-	0.527	0.500	0.442	0.490	
		+		0.172	0.234	0.210	0.206	42%
1/10	S	-	-	0.551	0.570	0.521	0.548	
		+		0.146	0.250	0.234	0.211	39%
1/1	S	-	-	0.484	0.511	0.541	0.513	
		+		0.186	0.211	0.151	0.183	36%
Four hours:								
zero	S	-	-	0.498	0.499	0.459	0.486	
		+		0.123	0.157	0.167	0.150	31%
1/10	S	-	-	0.546	0.569	0.497	0.538	
		+		0.150	0.232	0.203	0.196	36%
1/1	S	-	+	0.480	0.501	0.497	0.493	
		+		0.164	0.219	0.199	0.195	40%
16 hours:								
zero	S	-	-	0.504	0.515	0.477	0.493	
		+		0.109	0.107	0.156	0.125	25%
1/10	S	-	+	0.547	0.584	0.563	0.565	
		+		0.199	0.206	0.196	0.201	36%
1/1	S	-	+	0.516	0.537	0.463	0.506	
		+		0.148	0.199	0.180	0.176	35%

- Alkaline phosphatase added directly to detergent solution.

+ Filter papers subjected to electrophoresis, then placed in detergent solution.

* Conversion Status as assessed by electrophoresis in detergent starch gels and staining.

These results were expected to show a decreasing recovery of AP activity with increasing serum concentration and increasing time. In particular, material demonstrated to contain conversion activity, and hence alkaline phosphatase activity in the form of A- and B-PLAP, as well as M-PLAP, were expected to have a

corresponding decrease in the recovery of AP activity from the filter papers.In reality, no trends were observed.

Despite being sensitive to M-PLAP and linear in reconstitution experiments, the assay, in its present form, could not be used in a real situation.

3.3 Discussion

These experiments set out to design and develop a quantitative assay for the conversion of M-PLAP to A- and B-PLAP. Such an assay could be used to examine possible enzymatic activities of the conversion phenomenon. Furthermore, the assay could be used to monitor the purification of the conversion factor from serum, and to provide data on yields of the factor and relative purification.

The assay is based on the observation that M-PLAP was retained at the origin in starch gel electrophoresis in the absence of detergent. In contrast, A- and B-PLAP migrates into the gel. Subsequent leaching of AP activity from the filter paper insert in detergent would allow the determination of AP activity retained on the filter following electrophoresis.

During the pilot experiments, it was demonstrated that the assay was considerably more sensitive to M-PLAP than to A- or B-PLAP (Table 3.4). Furthermore, the activity recovered from a filter paper insert was shown to be proportional to the relative proportion of M-PLAP in a mixture of PLAP isoenzymes (Table 3.5). However, when tested on conversion products, no proportionality or linearity was detected between the amount of conversion that had taken place (as assessed by electrophoresis and

staining) and the recovered AP activity. Indeed, no significant difference was detected between the experimental and control incubations.

We are unable to resolve the conflict between the model and final experimental systems. The model system worked, whilst the experimental system failed. It is not possible to be sure why the assay did not perform as hoped, however it was evident from the pilot experiments (Table 3.4) that protein concentration affected elution from filter papers. It may be possible that the mixture of proteins presented in serum (Table 3.6) affects elution in a different manner to the BSA present in the model system (Table 3.5). It would have been informative to have repeated the reconstruction experiments using heat-inactivated serum as carrier, though this in itself would not have affected the data in Table 3.6.

Due to the failure of the experimental system, it was clear that much additional work would have been necessary to develop a quantitative assay. Time did not permit these experiments to be done, and work proceeded to the purification of the conversion factor using the qualitative assay of electrophoresis and staining.

As this research came to a close, Malik and Low (1986) published a method for the discrimination of hydrophobic and hydrophilic alkaline phosphatases. This procedure is a modification of Bordier (1981) and

involves the phase separation of hydrophobic AP into Triton X-114 out of an aqueous mixture of hydrophobic and hydrophilic species.

CHAPTER 4

PURIFICATION AND IDENTIFICATION OF THE CONVERSION FACTOR

Chapter 4

Purification and Identification of the Conversion Factor

4.1 Introduction

The work presented in Chapter 2 amply supports the hypothesis that M-PLAP is converted to A- and B-PLAP by a factor present in normal human serum. I now proceeded to devise a purification scheme for the isolation and identification of this factor. There being no quantitative assay for conversion, the purification of the conversion factor was detected by by gel electrophoresis as described in Chapter 2.

4.2 PURIFICATION PROTOCOL AND RESULTS

4.2.1 Purification of Conversion Factor

Ion-exchange chromatography of 50ml of fresh whole human serum was performed on DEAE-Sepharose CL-6B using a salt concentration gradient of 0.0-0.15M NaCl, and 155 fractions of 11ml were collected. Conversion activity was detected in a single peak of elution between 0.09M and 0.14M NaCl (fractions 118-124), with the peak corresponding to 0.09M NaCl (fraction 118). Selected fractions were resolved on a 10% acrylamide SDS-PAGE gel (Figure 4.1). Two species were detected that did not bind to the column at 0.0M NaCl, and these had the approximate molecular weights of immunoglobulin heavy and light chains (track 5).

Fractions 118-122 containing the peak of conversion activity were pooled, dialysed against tris-succinate buffer (pH 7.0), concentrated by freeze-drying and applied to a gel filtration column of Sephadex G200. Fractions from this chromatography system were collected and run on SDS-PAGE together with the pooled fractions from the ion-exchange fractionation. The pattern of polypeptides eluting from G200 can be seen in Figure 4.2 and conversion activity eluted as a single peak in fractions 19-21. The major polypeptides corresponding to serum albumin peaked in fractions 16-22, indicating that

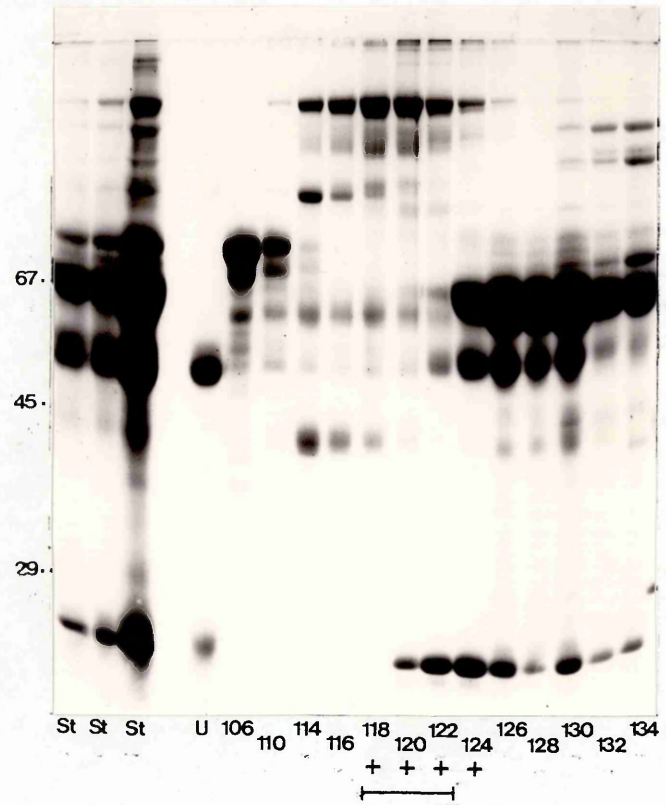


Figure 4.1. SDS-PAGE (10% acrylamide) analysis of normal human serum fractionated on DEAE-Sepharose CL-6B. The gel was stained for protein. Tracks contain unfractionated serum (St), or unbound protein (U), or selected fractions (as numbered). Fractions positive for conversion activity (+) which were selected for further fractionation are indicated by the heavy bar below the tracks. Molecular weight calibration is provided from standard proteins (albumin, ovalbumin, and carbonic anhydrase) run in the same gel.

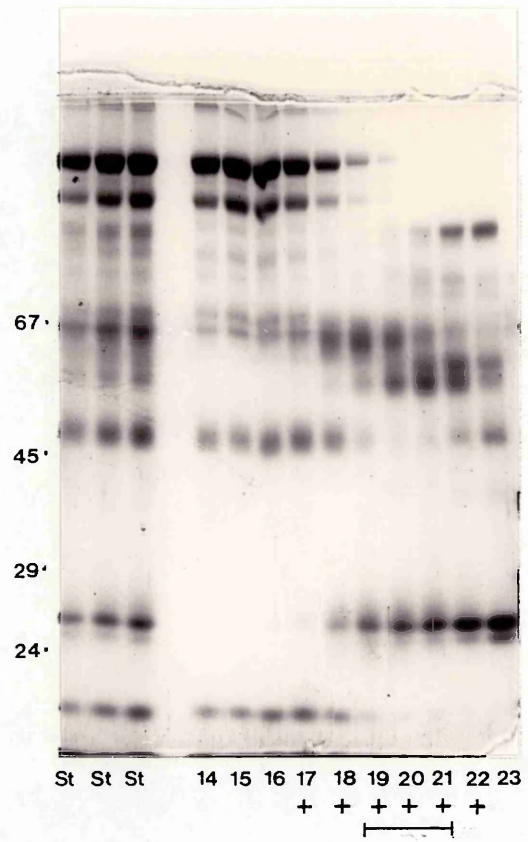


Figure 4.2. SDS-PAGE (10% acrylamide) analysis of selected pooled ion-exchange fractions further fractionated by gel filtration on Sephadex G200. The gel was stained for protein. Tracks contain pooled ion-exchange fractions (St), or selected gel filtration fractions (as numbered). Fractions positive for conversion activity (+) which were selected for further fractionation are indicated by the heavy bar below the tracks. Molecular weight calibration is shown to the left of the gel, and was generated using the standard proteins albumin, ovalbumin, carbonic anhydrase and trypsinogen.

the conversion activity has a slightly lower molecular weight in the region of 60,000 daltons.

Fractions 19-21 were pooled, filtered at 0.2um and applied to an ion-exchange column of Mono Q for FPLC (Fast Protein Liquid Chromatography). The column was then developed with an increasing salt gradient of between 0.0M and 0.4M NaCl; 5ml fractions were collected. Conversion activity was detected as a single peak in fractions 20-24, corresponding to a salt concentration of 0.19-0.26M NaCl. The polypeptide patterns in these fractions can be seen in Figure 4.3 as resolved by SDS-PAGE (10% acrylamide). The major polypeptide species present in the fractions positive for conversion activity has the approximate molecular weight of 27kD; minor species can also be seen corresponding to albumin and transferrin.

Fractions 20-24 from the FPLC ion-exchange were pooled, filtered at 0.2um, brought to a final concentration of 1.0M $(NH_4)_2SO_4$ and applied to an FPLC Phenyl-Superose column for hydrophobic interaction chromatography. The salt concentration was rapidly reduced to 0.2M, then a shallow salt gradient was produced from 0.2-0.0M salt. Conversion activity was detected as a single peak in fractions 20-23, corresponding to 0.0M $(NH_4)_2SO_4$. Elution of protein was analysed by on-line detection at 280nm, and part of the trace is shown in Figure 4.4. Selected fractions were

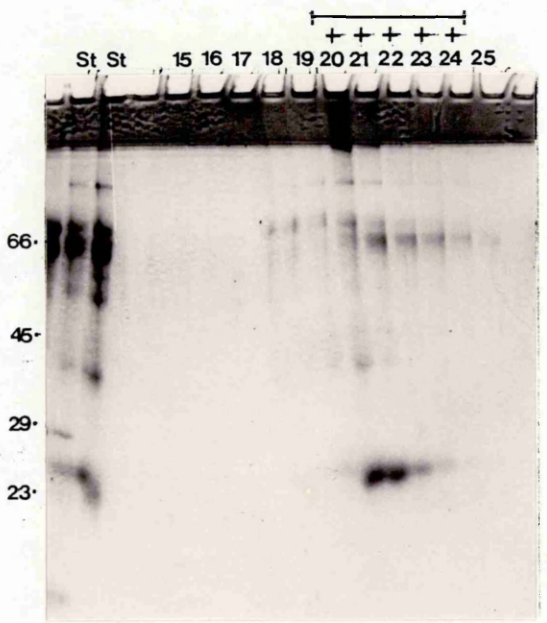


Figure 4.3. SDS-PAGE (10% acrylamide) analysis of selected pooled gel filtration fractions further fractionated by FPLC ion-exchange on MonoQ. The gel was stained for protein. Tracks contain pooled gel filtration fractions (St), or selected ion-exchange fractions (as numbered). Fractions positive for conversion activity (+) which were selected for further fractionation are indicated by the heavy bar above the tracks. Molecular weight calibration is shown to the left of the gel, and was generated by running standard proteins (albumin, ovalbumin, carbonic anhydrase and trypsinogen) in the same gel.

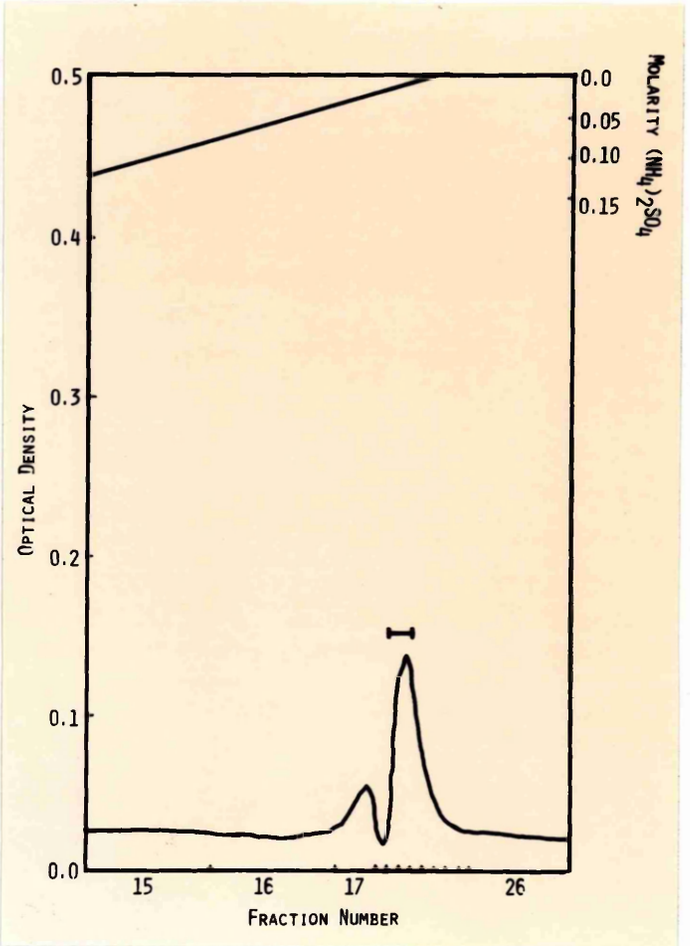


Figure 4.4. Elution profile of selected pooled FPLC ion-exchange fractions further fractionated by FPLC hydrophobic interaction chromatography on Phenyl-Superose. Optical density at 280nm and concentration of $(NH)_2SO_4$ are plotted against fraction number. The horizontal bar represents elution of conversion activity, and those fractions pooled for further fractionation.

resolved on a 12.5% acrylamide gel under reducing and non-reducing conditions (Figure 4.5). A 27kD band comprised about 90% of the Coomassie-Blue stainable components under reducing and non-reducing conditions. In addition to this major component, there was a species present in the non-reduced panel having a mobility corresponding to a molecular weight of 18kD. Faint bands were observed higher up the gel under both conditions, and these had the expected mobilities of albumin and transferrin.

Table 4.1 shows the results of the overall purification scheme. The table presents amounts of protein applied to the column, total protein recovery presented both as an absolute amount and as a percentage of that applied, and protein present in fractions containing conversion activity presented as absolute amount and as a percentage of total recovered protein. No figures can be given for total protein recovery from the FPLC columns, as protein determinations were only made for those fractions containing conversion activity.

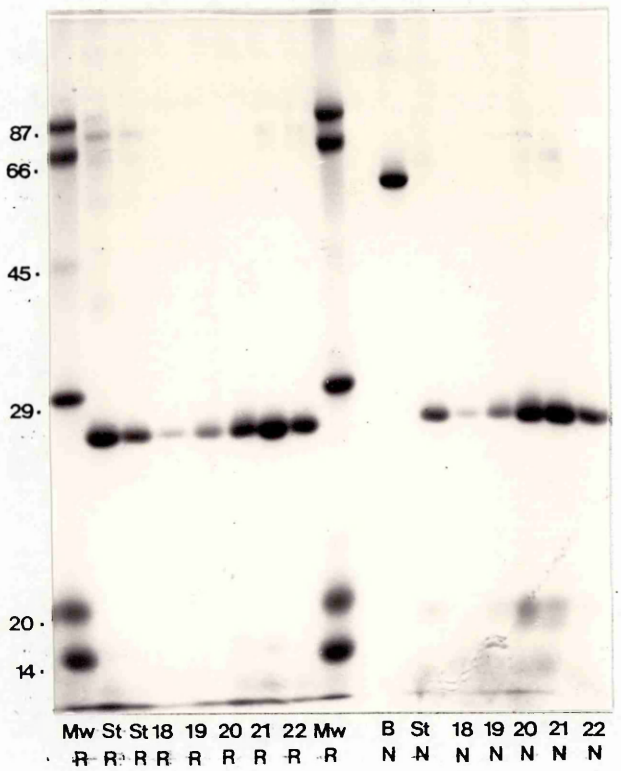


Figure 4.5. Resolution of the polypeptide components of FPLC hydrophobic interaction chromatography fractions containing conversion activity (PSCA) on 12.5% acrylamide gel electrophoresis under reducing and non-reducing conditions, stained for protein. Tracks contain molecular weight markers (Mw), pooled FPLC ion-exchange fractions (St), selected fractions (as indicated), or bovine albumin (B). Tracks were run under reducing conditions (R) or non-reducing conditions (N). Molecular weight calibration is shown to the left of the gel, and was generated by running the following standard proteins: transferrin, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and alpha-lactalbumin.

TABLE 4.1
Purification and Recovery of Protein

Scheme	Total Applied mg	Protein Recovered mg	%	Protein Recovered in CA Peak mg	%
DEAE-Sepharose	1370	1300	95	91	7
G200	63	38	60	7.5	7
FPLC Mono Q	5.7	ND	--	2.3	40
FPLC Phenyl-Superose	2.3	ND	--	0.5	22

CA = Fractions where conversion activity was detected.
ND = Not Determined.

4.2.2 Analysis of Purified Conversion Factor

The results of fractionation of conversion activity by ion-exchange on DEAE-Sepharose, G200 gel-filtration, FPLC ion-exchange and FPLC hydrophobic interaction chromatography was a preparation containing a major polypeptide of about 27kD, plus a 18kD component detected latterly in 12.5% acrylamide gels under non-reducing conditions. In addition, there are small amounts of protein species in the 55-80kD range. This material was termed PSCA (for Phenyl-Superose derived Conversion Activity).

It was hoped that the components of PSCA could be analysed and identified using the commercially available antisera raised against many of the components of human serum. However, it first had to be demonstrated that the PSCA polypeptides were immunogenic. This was tested by using a multivalent antisera against whole human serum in Western blotting. In this experiment, the first antibody

was rabbit anti-human serum diluted 1/100. The second antibody was 1/1000 peroxidase conjugated goat anti-rabbit immumnoglobulin heavy chain. Peroxidase activity was detected by chloro-napthyl staining.

Figure 4.6 shows a Western blotting filter stained for protein to demonstrate transfer of protein from the SDS-PAGE gel onto the nitrocellulose. The 27kD band is too faint to be seen on the photograph but was present on the blot. Panels 2 and 3 were stained after application of the second antibody to detect peroxidase activity. Panel 2 is stained without application of the first antibody, and demonstrates that only the rabbit immunoglobulin heavy chain present in the rabbit serum track is stained. The third panel was stained following incubation with the first and second antibodies, and demonstrates that antibodies are present that bind a number of human proteins in serum (track 2), and that there is little binding to rabbit proteins (track 4). Track 3, containing PSCA, was heavily labelled in the region of albumin, demonstrating that the minor component of this mobility is a major antigen. Further staining is observed at a location corresponding to mobility of the 27kD polypeptide, determined by comparison to the location of the 27kD band on the original SDS-PAGE.

Further controls in this experiment, namely chloro-napthyl incubation in the absence of second antibody both

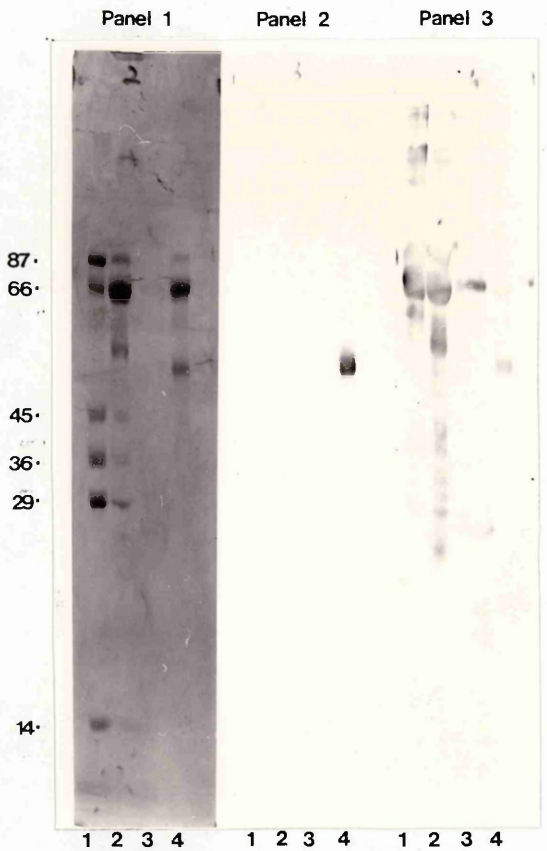


Figure 4.6. Western Blot analysis of PSCA. Each of the three panels contains the following four tracks: molecular weight markers (Track 1), human serum (track 2), PSCA (Track 3), and rabbit serum (Track 4). Panel 1 shows a nitrocellulose panel stained for total protein. Panel 2 is a control for non-specific binding of second antibody. Panel 3 shows specific binding of antibody to protein components. Molecular weight markers were transferrin (87kD), albumin (66kD), ovalbumin (45kD), glyceraldehyde-3-phosphate dehydrogenase (36kD), carbonic anhydrase (29kD) and alpha-lactalbumin (14kD).

before and after first antibody incubations, were negative and are not shown.

In order to conserve PSCA, it was decided to increase the ability to detect very small amounts of PSCA protein by labelling the material with radioactive iodine according to the Chloramine T protocol, and unlabelled human serum albumin was used as carrier protein. This material was fractionated from the unbound counts by exclusion chromatography on Sephadex G15. The excluded material was resolved on a 15% non-reducing SDS-PAGE and counts were detected by autoradiography (Figure 4.7). A number of clear bands of radioactivity were detected. Label running on the gel front was named Band 1, while material with mobilities corresponding to molecular weights of 18kD and 27kD were called Band 2 and 3 respectively. In addition a smear was observed running from the top of the resolving gel down to a position corresponding to a molecular weight of approximately 55kD. Alongside the non-reduced panel was run a single track containing the same material resolved under reducing conditions. The 27kD species is still present, while the 18kD species is resolved on 15% acrylamide as a species of lower molecular weight. Label is still observed at the gel front, while the high molecular weight smear is resolved as a band with a molecular weight corresponding to albumin.

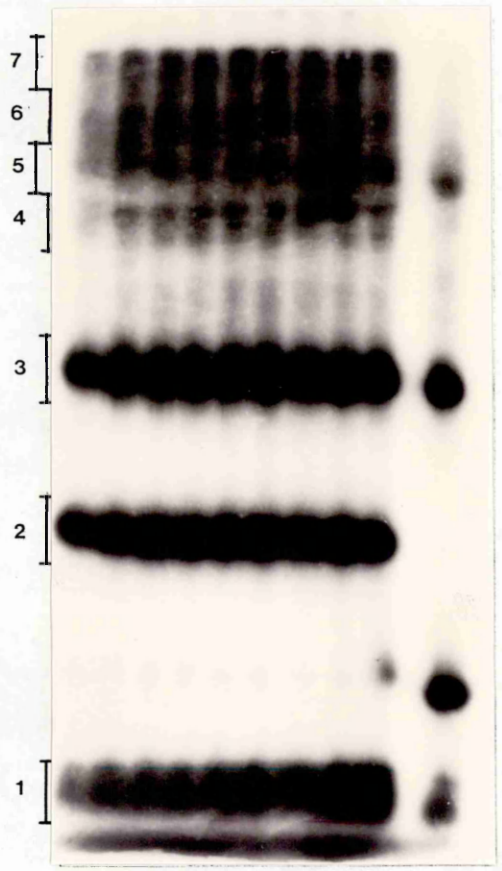


Figure 4.7. Autoradiograph of iodinated purified material (PSCA) run on a 15% acrylamide SDS-PAGE under non-reducing conditions. Areas of gel containing counts have been arbitrarily assigned the numbers shown on the left of the figure to ease description in the text. Shown to the right of the main panel is a track of identical material run under reducing conditions.

Gel fragments containing non-reduced Bands 1-3 were excised, as were four sections of the high molecular weight smear corresponding to 55-65kD (Band 4), 65-75kD (Band 5), 75-85kD (Band 6), and 85kD and above (Band 7). Gel fragments were then electroeluted in dialysis tubing in an electric field of 200V. Elution proceeded for 4 hours and each hour the bags were opened and soluble counts measured. The results of this procedure are shown in Table 4.2, in which the size of each band under reducing and non-reducing conditions are shown alongside the initial and final activity for each gel fragment, measured counts in solution every hour, and the resulting percentage recovered in solution after four hours. The table shows that as the polypeptide size increased, so the percentage recovery of activity declined, suggesting that larger proteins may have been trapped in the gel and would have benefited from a longer electroelution time. Also note that Band 1 soluble counts reached a peak very quickly, but fell off towards the end of the electroelution by as much as 30%, suggesting that counts were escaping through the dialysis bag.

TABLE 4.2
Electroelution of Gel Fragments

BAND No.	MOLECULAR SIZE kD	FRAGMENT ACTIVITY INITIAL	FINAL	SOLUBLE COUNTS 1	2	3	4	% RECOVER AFTER 4h
1	NR	480*	78	32	448	541	401	84%
2	18	930	204	23	127	260	558	73%
3	27	890	196	11	240	352	666	77%
4	55-65	410	79	10	143	211	243	75%
5	65-75	630	390	8	99	166	246	39%
6	75-85	360	118	3	26	48	55	32%
7	>85	350	92	3	9	15	20	18%

NR = not resolved from dye front in 15% acrylamide.
* = all measurements of radioactive decay are presented as counts per minute per 100ul of electro-elution buffer.

Solutions containing more than 200 counts per second per 100ul (electroelution of Bands 1-5) were acetone precipitated to concentrate the counts in a smaller volume. The results are presented in Table 4.3. Band 1 did not precipitate under the conditions employed, suggesting that it contained unbound counts.

TABLE 4.3
Acetone Precipitation of Soluble Counts

Band	1	2	3	4	5
Pellet	149	2506	2468	2857	1203
Supern^{t}	1328	327	159	67	250
1st Wash		1548	854	1270	855
Tube	ND	587	972	178	279
2nd Wash	ND	1669	1052	ND	ND
Tube	ND	418	875	ND	ND
Recovery	0%	83%	65%	94%	77%
Activity per ul		33cps	26cps	64cps	21cps
Con^{c} factor		11x	4x	19x	18x

ND = not done

Concentrated aliquots of Bands 2-5 were run on a 15% SDS-PAGE under non-reducing conditions to assess the quality and purity of the products (Figure 4.8). Band 2 is seen at approximately 18kD molecular weight, Band 3 is between 27-29kD molecular weight, Band 4 contains two species between 48 and 60kD, while band 5 shares the higher molecular weight band at 60kD. There is no cross contamination between the purified bands. Starting material is also shown and contains more intense counts in locations corresponding to the purified components.

The antigenic behaviour of PSCA and isolated Bands 3-5 were analysed by antibody-antigen crossed electrophoresis (AACE), using whole human serum as both marker and carrier protein. Aliquots (0.08uCi) were placed in the sample well of AACE plates together with 3ul of human serum. After electrophoresis in the first

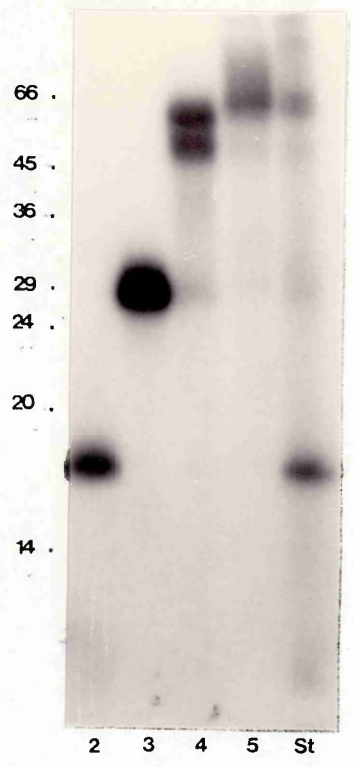


Figure 4.8. Autoradiograph of SDS-PAGE (17.5% acrylamide) on which was resolved iodinated PSCA (St), and concentrated iodinated bands 2, 3, 4 and 5 (as indicated beneath the tracks). Tracks were run under non-reducing conditions. Molecular weight calibration is shown to the left of the gel and comprised the following standard proteins: albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibitor, and alpha-lactalbumin.

dimension, the second dimension gel was prepared containing 5% rabbit anti-whole human serum. Figures 4.9 to 4.12 show the immunoprecipitation arcs of each AACE plate stained for protein, while the autoradiograph of the plate is shown inset and indicates the location of the labelled protein. Figure 4.9 contains labelled PSCA, and ^{125}I counts were detected in precipitation arcs corresponding to albumin, transferrin and alpha-mobility lipoprotein. Figure 4.10 contains labelled Band 3 (the 27kD species), and label was detected with the expected mobility of alpha-lipoprotein, and more faintly in the albumin arc. The AACE plate containing labelled Band 4 (Figure 4.11) contained label in the albumin and alpha-mobility lipoprotein arcs, while the Band 5 plate (Figure 4.12) was labelled in the albumin and transferrin arcs.

In each of the AACE plates containing isolated, homogeneous bands (as shown in Figure 4.8) there were a number of precipitation arcs containing small amounts of label. This suggested that there were some protein-protein associations which were not overcome by electrophoresis under the conditions used. This implies that only major arcs should be regarded as significant in drawing conclusions from these results.

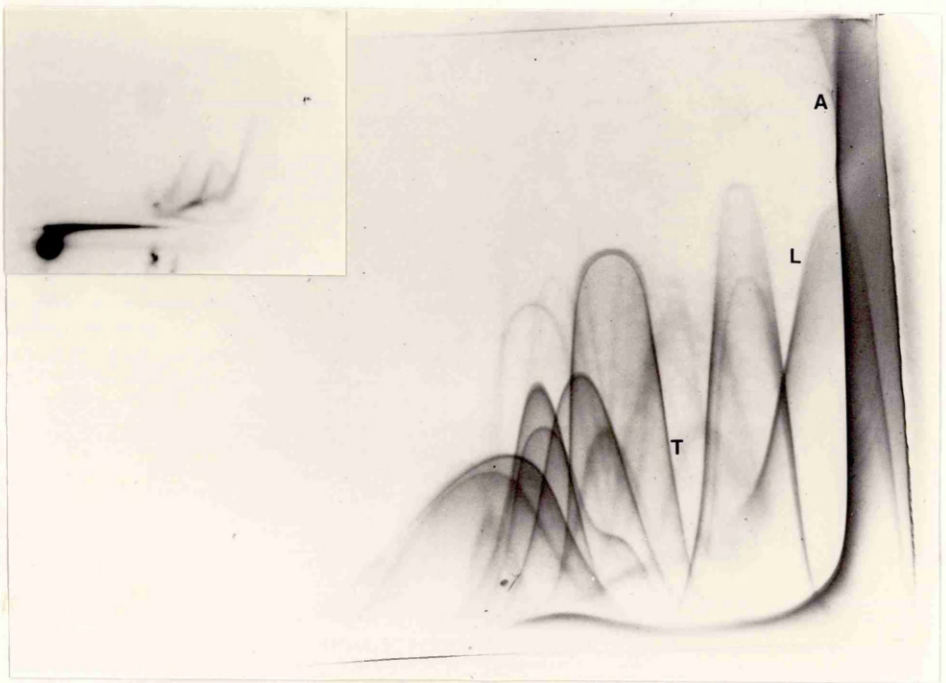


Figure 4.9. AACE plate in which serum plus labelled PSCA was run against 5% anti-whole human serum in the second dimension. Inset is shown the autoradiograph of the plate indicating those arcs containing precipitated counts. Major labelled arcs are identified as albumin (A), alpha-lipoprotein (L), and transferrin (T).

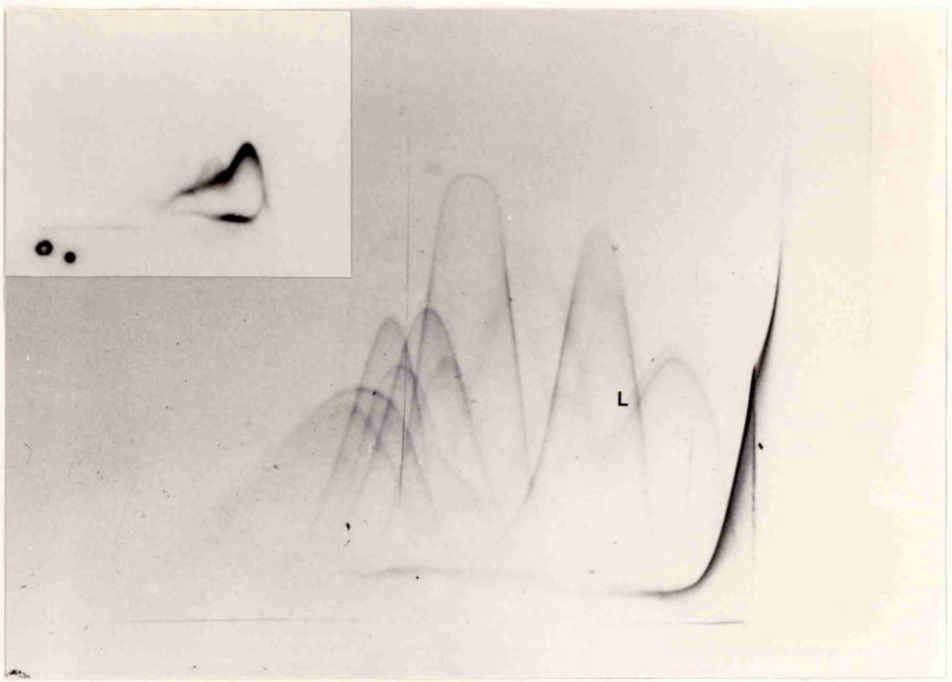


Figure 4.10. AACE plate in which serum plus labelled isolated Band 3 was run against 5% anti-whole human serum in the second dimension. Inset is shown the autoradiograph of the plate indicating those arcs containing precipitated counts. The major labelled arc is identified as alpha-lipoprotein (L).

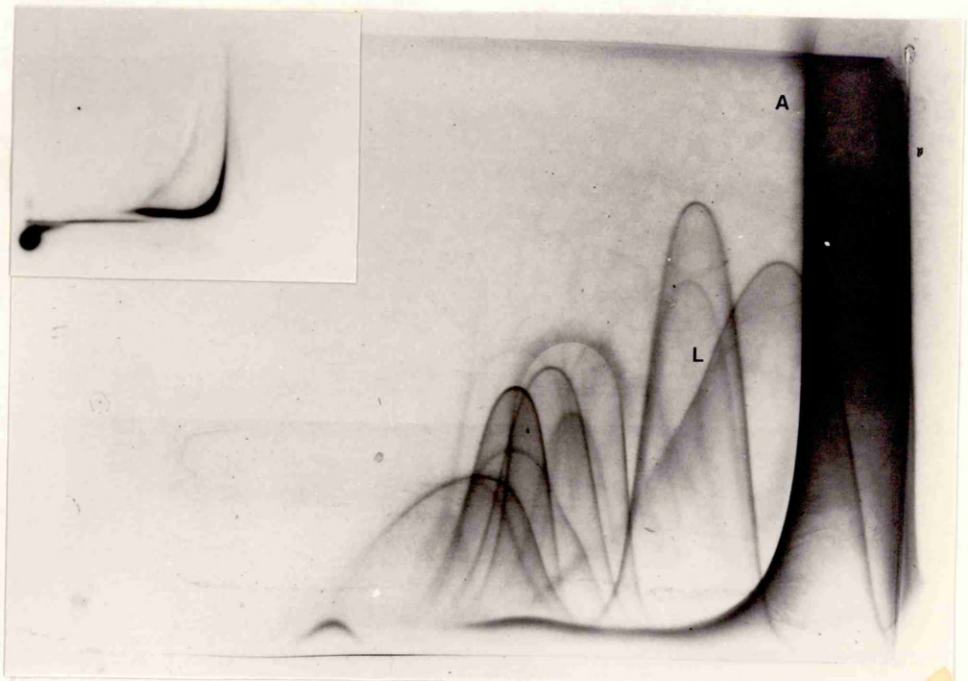


Figure 4.11. AACE plate in which serum plus labelled isolated Band 4 was run against 5% anti-whole human serum in the second dimension. Inset is shown the autoradiograph of the plate indicating those arcs containing precipitated counts. Major labelled arcs are identified as albumin (A) and alpha-lipoprotein (L).

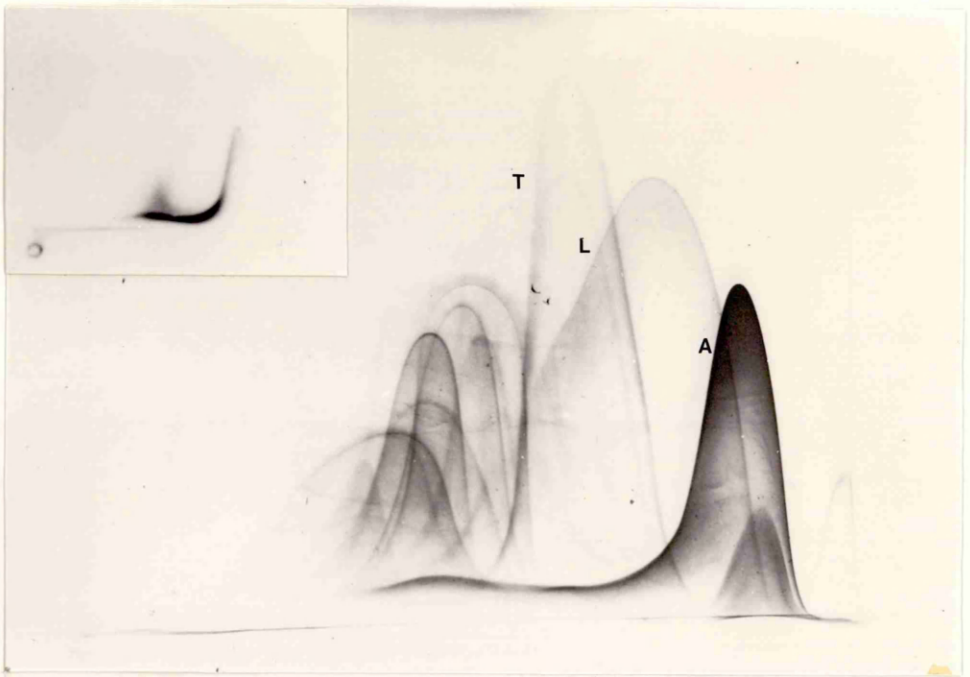


Figure 4.12. AACE plate in which serum plus labelled isolated Band 5 was run against 5% anti-whole human serum in the second dimension. Inset is shown the autoradiograph of the plate indicating those arcs containing precipitated counts. Major labelled arcs are identified as albumin (A), alpha-lipoprotein (L) and transferrin (T).

4.2.3 Identity of the 18kD Polypeptide Species

Evidence for the identity of the 27kD species as being a polypeptide component of alpha-lipoprotein has been documented above. It has been shown to have the expected molecular weight of apolipoprotein A-I under reducing and non-reducing conditions, and has been shown to precipitate in the alpha-lipoprotein arc in AACE. However, the identity of the 18kD species is less clear since it was only resolved in 12.5% acrylamide gels under non-reducing conditions, and since it was not isolated in sufficient amounts to be used in the AACE experiments. Thus, it was necessary to demonstrate that this low molecular weight component of PSCA had the mobility of apolipoprotein A-II under reducing and non-reducing conditions. PSCA was run in a 10% acrylamide gel containing 6M urea alongside purified apolipoprotein A-II (a gift of the Glasgow Royal Infirmary). The results (Figure 4.13) indicate that the mobility of the low molecular weight component of PSCA is indistinguishable from that of apolipoprotein A-II, and lend weight to the hypothesis that apolipoprotein A-II is indeed present in PSCA.

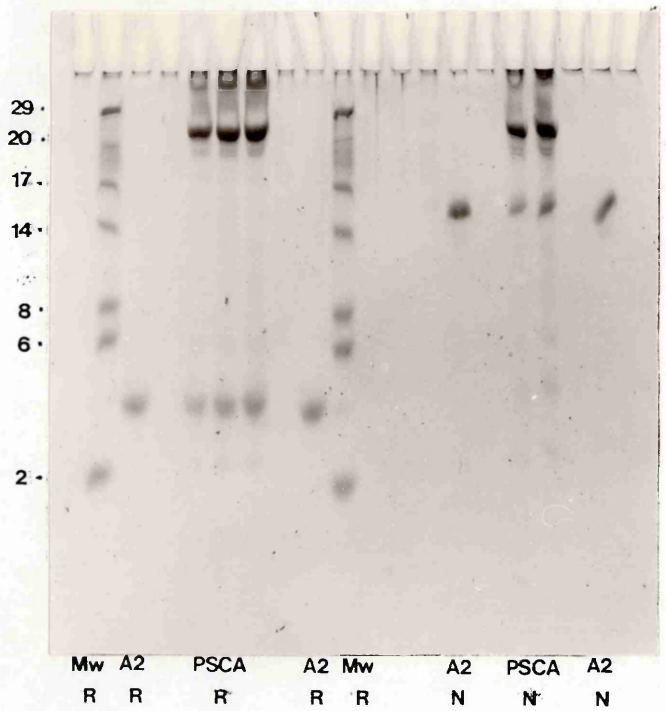


Figure 4.13. SDS-PAGE (10% acrylamide, 8M urea) analysis of the low molecular weight component of PSCA. The gel was stained for protein. Tracks contain molecular weight markers (Mw), reference apo-lipoprotein A-II (A2), or purified conversion activity (PSCA). Tracks are run under reducing conditions (R) or non-reducing conditions (N) as indicated. Molecular weight calibration is shown to the left of the gel, and was generated from the following standard proteins and peptides: carbonic anhydrase (29kD), trypsin inhibitor (20kD), myoglobin (17kD), plus peptides derived from specific cleavage of myoglobin with cyanogen bromide (14kD, 8kD, 6kD, and 2kD).

4.2.4 Affinity Chromatography of Alpha-Lipoprotein

To summarise, PSCA contained proteins which, when labelled with ^{125}I and run on AACE together with whole human serum, precipitate at the expected mobilities of albumin and alpha-lipoprotein and, to a smaller extent, transferrin. That the material fractionated on SDS-PAGE under reducing and non-reducing conditions, at the expected mobilities of transferrin and albumin supports the hypothesis that these species are indeed present. Furthermore, since alpha-lipoprotein is classically well known to be mainly comprised of the proteins apo-lipoprotein A-I and A-II, having the molecular weights of 28,500 and 17,500 respectively, it is possible that major components of PSCA are indeed those proteins. To test the hypothesis that a component of alpha-lipoprotein is responsible for conversion activity, rabbit antiserum specific to the apo-lipoproteins of alpha-lipoprotein was conjugated onto a Sepharose 4B column and used to remove apolipoproteins A-I and A-II from whole human serum by affinity chromatography. Whole human serum (1.5ml containing 63mg protein) was applied to the column in tris-succinate buffer containing 0.1M NaCl. Unbound protein was then collected in 1.0ml fractions. Bound protein was then eluted using 3.0M KSCN, and 1.0ml fractions were collected. Fractions were dialysed against tris-succinate buffer and the protein concentration

estimated by optical density at 280nm.

Figure 4.14 shows a 12.5% SDS-PAGE on which has been resolved selected fractions from an affinity chromatography experiment. The 27kD band was absent from fractions 1-28 and was present in fractions 29-33. The material bound to the column and eluted with 3M KSCN contained a strong band at 27kD plus a minor species which ran close to the gel front. There was also some minor species in the molecular weight range 50,000-66,000 daltons.

Equivalent amounts of protein from selected fractions were applied into the sample wells of a 1-dimensional rocket immunoelectrophoresis plate and electrophoresed into an agarose bed containing a 1% concentration of the above antiserum. The relative concentration of apolipoproteins in each fraction was then estimated by observing the height of the precipitation rocket. This is shown in Figure 4.15, and confirms the SDS-PAGE analysis of the fractions, namely that flow-through of apolipoproteins (and saturation of the column) began in fraction 29. Bound protein eluted from the column was enriched in the apolipoproteins relative to the initial serum.

Fractions from the affinity chromatography were grouped into pools A, B and C. Pools A and B contained serum protein (adjusted to the original protein

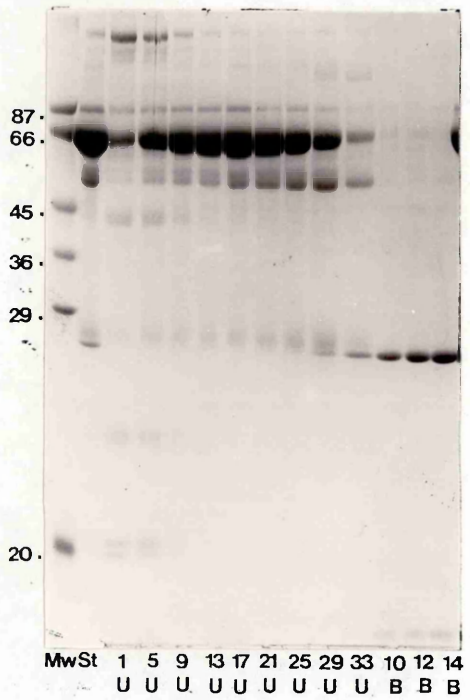


Figure 4.14. SDS-PAGE of selected fractions from the affinity chromatography of normal human serum on anti-alpha lipoprotein-conjugated Sepharose 4B. The gel was stained for protein. Tracks (labelled U) contain selected fractions of unbound protein (as numbered). Tracks (labelled B) contain selected fractions of bound and eluted protein (as numbered). Molecular weight calibration is shown to the left of the gel, and was generated using the following standard proteins: transferrin, albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, and trypsin inhibitor.

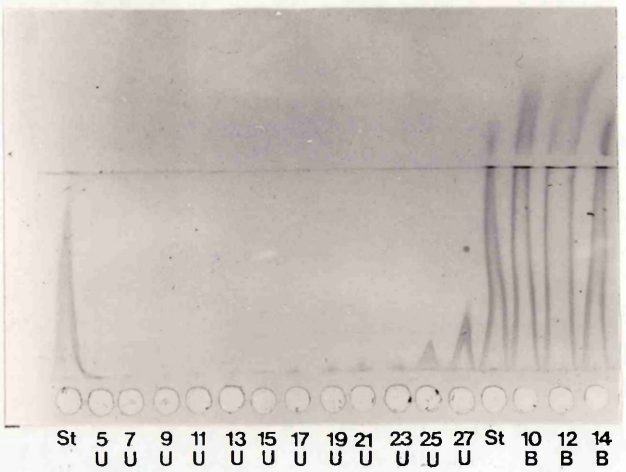


Figure 4.15. Rocket immunoelectrophoresis plate (stained for protein) of selected affinity chromatography fractions in agarose containing 1% anti-alpha lipoprotein. Tracks contain either whole serum (St), selected unbound fractions (as numbered, labelled "U"), or selected eluted fractions (as numbered, labelled "B"). All tracks contained 0.85mg protein.

concentration of the applied serum) which had passed through the affinity column unbound. Pool A was composed of early fractions (pre-fraction 29) which were depleted in apolipoproteins, while Pool B was composed of later fractions (post-fraction 29) which contained apoliproteins. Pool C contained dialysed fractions of serum proteins eluted from the column in 3M KSCN, adjusted to the original volume used in the fractionation. These Pools were used to test whether apolipoproteins were effective in the conversion of M-PLAP (Figure 4.16). In this experiment, M-PLAP was incubated with control tris-succinate buffer, control sera, and with affinity pools A, B and C. Negative controls of tris-succinate buffer with and without incubation (tracks 1 and 2) indicated that PLAP substrate was predominately M-PLAP with some small contamination by A-PLAP. The positive controls of reference serum with and without dilution and reconcentration (tracks 3 and 4) contain more A- and B-PLAP and less M-PLAP, and indicate the maximal extent of conversion under the conditions used. Tracks 5 and 6 contain M-PLAP incubated with Pool A and demonstrate that in the absence of apolipoproteins, no conversion is observed. Pool B, which was not depleted for apolipoproteins showed conversion activity (track 7), as did Pool C, which contained the desorbed apolipoproteins.

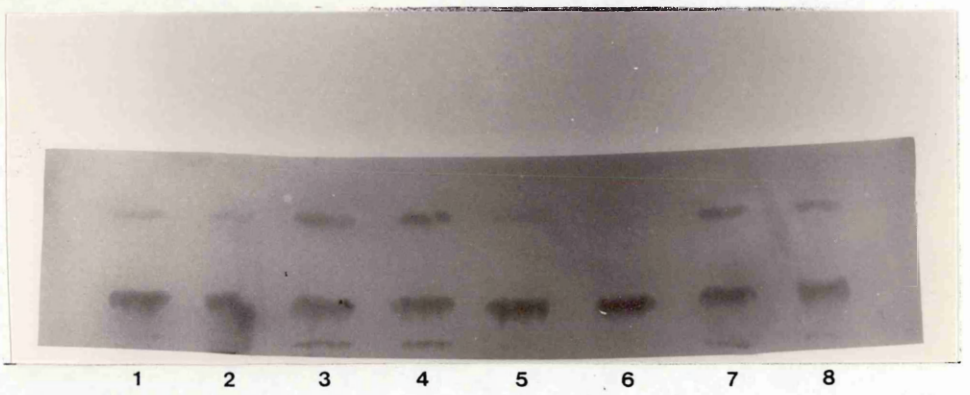


Figure 4.16. Electrophoresis (in a detergent starch gel subsequently stained for enzyme activity) of M-PLAP following incubation with selected pools of fractions from affinity chromatography. Material was treated as follows: Track 1, M-PLAP in buffer without incubation. Track 2, M-PLAP in buffer incubated at 37°C overnight. Track 3, M-PLAP incubated with undiluted serum. Track 4, M-PLAP incubated with serum diluted then reconcentrated. Track 5, M-PLAP incubated with reconcentrated fractions depleted in alpha-lipoprotein (Pool A). Track 6, as track 5. Track 7, M-PLAP incubated with reconcentrated fractions not depleted in alpha-lipoprotein (Pool B). Track 8, M-PLAP incubated with reconcentrated dialysed fractions desorbed from the column.

In a separate experiment, serum was applied to the column in 0.5M NaCl, and the rest of the experiment repeated as described above. Although rocket immunoelectrophoresis and PAGE gel analysis results were indistinguishable from the above, no conversion activity could be detected in any of the three pools. To test the hypothesis that the conversion activity was irreversibly inhibited by prolonged incubation in high salt buffers, serum was dialysed overnight into tris-succinate buffer containing either 0.1M or 0.5M NaCl, followed by further overnight dialysis into tris-succinate buffer alone. Conversion activity was not subsequently detected in the serum dialysed into 0.5M NaCl, but was present in the positive control. This result led to the proposal that high salt concentrations can irreversibly inhibit conversion activity, and suggests that the conventional lipoprotein preparation method of salt density centrifugation may not be entirely appropriate for studies of this kind.

4.3 DISCUSSION

4.3.1 Purification Strategy

The object of the present study was to purify and investigate the serum factor responsible for the *in vitro* conversion of M-PLAP to A- and B-PLAP. The purification scheme was devised to separate the conversion factor from other serum components most efficiently. Following the ion-exchange chromatography (Figure 4.1), fractions were selected to maximise the separation of conversion activity from the major protein species serum albumin, which had already been demonstrated not to be responsible for conversion activity (see Figures 2.2 and 2.3, where the presence of contaminating albumin did not convert M-PLAP to A- and B-PLAP).

Gel filtration was chosen for a second round of fractionation to demonstrate a previous undocumented observation that conversion activity had a molecular radius corresponding to a molecular weight of around 60,000 daltons, and eluted from Sephadex G200 immediately after serum albumin. These observations were confirmed (Figure 4.2), and further fractionation of conversion activity was achieved.

Fast Protein Liquid Chromatography (FPLC) was used in the third round of fractionation (Figure 4.3). Protein species were found to elute from Mono Q at a higher salt

concentration than they had eluted from DEAE-Sepharose. This was an unexpected effect, but was clearly not limited to conversion activity. This result indicates that, in general, elution on Mono Q cannot be predicted from the elution profile on DEAE-Sepharose.

A final step of hydrophobic interaction chromatography on FPLC was chosen on the basis that, since conversion activity has an affinity for the relatively hydrophobic M-PLAP, it was possible that conversion activity would be retained on the Phenyl-Superose column under conditions in which protein contaminants would not. Conversion activity was detected within the the final protein peak observed eluting from the column (Figure 4.5).

4.3.2 Components of the Purified Species

The final purified conversion activity (PSCA) was found to be composed largely of a single polypeptide species of around 27kD molecular weight, together with a smaller species resolved in 15% SDS-PAGE having a molecular weight of around 8,000 daltons under reducing conditions (and 18,000 in native gels), plus minor species in the molecular weight region of 55-80,000 daltons.

Several lines of evidence point to the 27kD polypeptide being apolipoprotein A-I. Firstly, the

polypeptide has a molecular weight and mobility close to that already reported for apolipoprotein A-I under reducing and non-reducing conditions (Baker et al, 1974; Brewer et al, 1978). Secondly, the 27kD species co-precipitates in the precipitin arc of alpha-mobility lipoprotein in AACE (Figure 4.10). Thirdly, column affinity chromatography with anti-alpha-lipoprotein specifically binds conversion activity and a 27kD polypeptide.

Less strenuous evidence has been provided for the nature of the 8,000 / 18,000 dalton species. In part, this was due to the unfortunate choice of 10% SDS-PAGE during the purification of conversion activity, preventing this low molecular weight species from being resolved. Immunological characterisation of this species was prevented by the low yield of the ^{125}I labelled band. Nevertheless, it has been shown that the low molecular weight species is indistinguishable from apolipoprotein A-II on SDS-PAGE under reducing and non-reducing conditions (Figure 4.13).

The high molecular weight, low abundance material resolved at 55-80,000 daltons has been shown to be located in the albumin and transferrin precipitin arcs in AACE (Figure 4.11 and 4.12), and are not considered to be responsible for conversion activity (see argument above).

4.3.3 Size Considerations of Conversion Activity

The major polypeptide species co-purifying with conversion activity are apolipoproteins A-I and A-II. These species are normally associated in a complex lipoprotein array of high density lipoproteins (HDL), which gel filters in the region of 200,000 molecular weight. Gel filtration of serum indicates that no conversion activity is detected in that molecular weight range, and purified HDL has no conversion activity (Chapter 2). Thus, the apo A-I and A-II species implicated in conversion cannot be associated with HDL. This activity of a small population of apo A-I and A-II molecules may represent the known heterogeneity amongst lipoprotein polypeptides (Nestruck *et al*, 1980), or be simply due to steric hindrance in those polypeptides already associated with lipid.

4.3.4 Lipoprotein Function in Conversion

The results presented in this chapter have shown that conversion activity can be irreversibly abolished by prolonged incubation in 0.5M NaCl. This suggests that the routine preparation of apolipoproteins by salt density centrifugation may be inappropriate in functional studies of apo A-I and apo A-II. This may help to explain why no functions have been reported for apolipoproteins which suggest a role in M-PLAP conversion. Apo A-I is a known

activator of lecithin-cholesterol acyl transferase (LCAT), though no conversion of M-PLAP was evident on incubation with a purified sample of LCAT (Chapter 2).

The products of M-PLAP conversion are not uniform, but consist of two products, A- and B-PLAP. This suggests that there must be molecular heterogeneity within M-PLAP and/or the conversion factor functions in two mutually exclusive ways. Recent evidence bearing on both the structure of M-PLAP and on the nature of the conversion activity will be discussed in the following general discussion.

CHAPTER 5

GENERAL DISCUSSION

CHAPTER 5

GENERAL DISCUSSION

5.1 Three Classes of Membrane Binding Proteins

Since M-PLAP is associated with lipid in the microvillous membrane (Abu-Hasan et al, 1984), it is of interest to review the general mechanisms by which protein-lipid association is thought to take place. All lipid-associated polypeptides known to date can be classified as having an hydrophobic anchorage, or covalently bound to fatty acid via thioester and/or amide bonds, or covalently associated with a complex glycolipid via phosphodiester linkage.

The first class of membrane proteins is characterised by the presence, in the mature polypeptide, of sequences of hydrophobic amino acids which anchor the protein in the membrane (Capaldi, 1982). These amino acids are in some cases located at one or other terminus of the protein, while in other cases the polypeptide chain is believed to traverse the membrane with substantial domains on both sides of the bilayer.

Another common form of anchorage is widespread in viruses, bacteria, and eukaryotes, in which fatty acids are covalently bound to cysteine side chains via thioester bonds or to the amino-terminal residue by amide linkage (Magee and Courtneidge, 1985). The former linkage

involves the most abundant acyl chains i.e. palmitate, stearate, and oleate, while amide linkage is exclusively to myristate. Most of these proteins are membrane bound as might be expected for proteins modified with a hydrophobic acyl chain. However, a number of myristylated proteins fractionate in the cytosol fraction. Recent reports of Rous sarcoma virus transforming protein pp60 suggest that amino-terminal myristylation is required, but not sufficient, for the plasma-membrane localisation of the protein (Cross et al, 1984; Buss et al, 1984). Thus some myristyl proteins may be able to move between the cytosol and cell membranes, perhaps after a conformational change involving exposure of the acyl chain. Alternatively, myristylation may have a role in the interactions of one protein with another or with a substrate.

By contrast, a number of eukaryotic proteins are currently believed to be covalently bound to fatty acids as components of complex glycosyl-inositol phospholipids, and the diacylglycerol moiety of phosphatidylinositol has been shown to be the sole means of membrane attachment (Low et al,1986; Cross, 1987). In general, inositol proteins have been identified by their specific and non-disruptive release from the cell membrane by phosphatidylinositol-specific phospholipase C. However, details of the chemistry and synthesis of the hydrophobic

linkage have been gleaned from studies of a few well characterised inositol proteins.

5.1.1 Trypanosome Variant Surface Glycoproteins

The parasitic protozoan *Trypanosoma brucei* is protected from the host immune system by the sequential expression of genes encoding immunologically distinct variant surface glycoprotein (VSG), which form a dense, protective surface coat (Cross, 1984; Turner, 1984). In viable trypanosomes, the VSGs are firmly anchored to the plasma membrane; upon lysis, by either mechanical shear or osmotic shock, the VSG coat is rapidly released in a water-soluble form (sVSG). Amino acid sequencing of this form has revealed that sVSG lacks a C-terminal hydrophobic peptide extension of 17 or 23 amino acids (depending on the variant), predicted from cDNA sequences. The mode of membrane attachment of mature VSG remained unclear until work by Cardoso de Almeida and Turner (1983) revealed that lysis of trypanosomes in boiling detergent solutions resulted in the recovery of an amphiphilic membrane form of the VSG (mfVSG). The conversion of mfVSG to sVSG was shown to be due to an endogenous phospholipase C and to correlate with the presence or absence of myristic acid (Ferguson *et al*, 1984; Ferguson and Cross, 1984). Further analysis revealed that the C-terminal glycolipid also contained

ethanolamine (Holder, 1983a), phosphate (Baltz et al, 1982; Cardoso de Almeida and Turner, 1984), sugars (Holder and Cross, 1981; Holder, 1983b), and glycerol (Duvillier et al, 1983).

Purification and analysis of the myristylated pronase fragment of mfVSG has revealed that the myristic acid is covalently attached to the glycan in the form of s,n-1,2-dimyristyl glycerol, via a phosphodiester linkage (Ferguson et al, 1985a,b).

5.1.2 Other PI-Linked Membrane Proteins

Consistent with the evidence for VSG precursor polypeptide tails, the DNA sequence of the rodent Thy-1 antigen predicts a thirty-one amino acid tail that is absent from the mature protein (Seki et al, 1985), and can be released from the membrane by a highly purified phosphatidylinositol-specific phospholipase C (Low and Kincade, 1985).

The chicken neural cell adhesion molecules (N-CAMs) represent an intriguing situation: two forms of N-CAM are typical transmembrane proteins with different cytoplasmic domains, but a third is PI-linked (He et al, 1986). All three polypeptides are derived from transcripts of a single gene by alternative RNA splicing (Hemperly et al, 1986). It remains to be established whether addition of PI is associated with cleavage of its predicted

hydrophobic terminal amino acid sequence.

5.1.3 Structural Features of PI-Protein Anchoring Domains

Although the structural features of other hydrophobic membrane anchoring domains in other proteins have not been characterised in as much detail as VSG, certain features seem to be shared. In acetylcholinesterase (Dutta-Choudhury and Rosenberry, 1984) and Thy-1 (Tse *et al*, 1985) these domains are located at the carboxy-terminus. Rat brain Thy-1, thymus Thy-1 and erythrocyte acetylcholinesterase (AChE) have been shown to contain relatively few amino acids, none of which are hydrophobic. Thy-1 membrane anchoring domain contains ethanolamine, glucosamine, myo-inositol and fatty acids. The membrane anchoring domain of erythrocyte AChE also contains glucosamine, ethanolamine and fatty acids, but interpretation is complicated by the observation that only a small proportion of the AChE in human erythrocytes is released by PI-PLC (Low and Finean, 1977; Shukla and Hanahan, 1982). In view of these unusual shared structural features, it seems likely that these other proteins are also linked to PI by a structure similar to that identified for VSG. A pictorial representation of the predicted carboxy-terminal membrane-binding domain of phosphatidylinositolated (glypiated) proteins is shown in Figure 5.1.

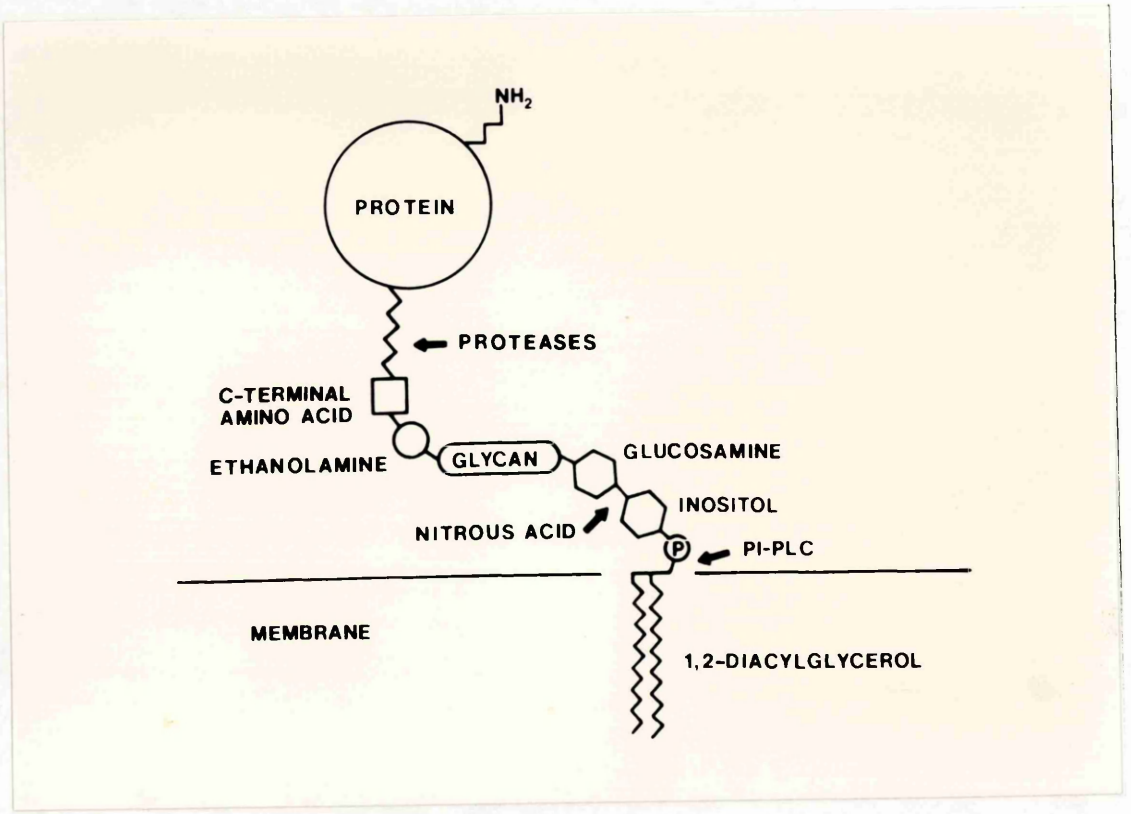


Figure 5.1. Proposed general structure of the C-terminal membrane anchoring domain of a PI-PLC sensitive protein. The structure is based on the evidence available for VSG of trypanosomes. It is likely that other phosphatidylinositolated proteins are bound by a similar structure, although the actual composition and arrangement of the sugars in the glycan is likely to be variable. Figure from Low et al (1986).

Conceivably, any eukaryotic membrane protein that terminates in a short hydrophobic segment is a candidate for glypiation. While there is yet no identifiable consensus sequence for glypiation, such a signal may become evident as more PI-linked proteins are added to the repertoire of predicted protein sequences.

5.1.4 Functional Significance of Covalently Attached PI

The evidence that the diacylglycerol moiety of PI is solely responsible for membrane anchoring in AChE, VSG and Thy-1 is very strong and is based on three principle lines of evidence. Firstly, there is an absence of any substantial hydrophobic amino acid sequence in the membrane anchoring domain isolated from mature VSG (Cross, 1984), Thy-1 (Tse *et al*, 1985), or human erythrocyte AChE (Roberts and Rosenberry, 1985). Secondly, there is non-disruptive release of these proteins from the plasma membranes to which they are attached, and/or conversion from an amphiphilic form to a non-aggregating hydrophilic form (Ferguson *et al*, 1985b). Thirdly, membrane bound VSG can be converted to a water soluble form by an endogenous phospholipase (Ferguson *et al*, 1985a).

The fact that an apparently adequate, hydrophobic, membrane-anchoring peptide is removed from the carboxyl terminus of VSG immediately after synthesis suggests that

covalently attached lipid may, in general, serve some other purpose in addition to membrane attachment. It may relate to the intracellular transport and targetting of the polypeptide from the endoplasmic reticulum to the outer surface of the plasma membrane. The fact that all proteins currently believed to be covalently attached to phosphatidylinositol are also predominantly located on the outer surface of the plasma membrane seems to support this suggestion. Alternatively, it may provide a means of selectively mobilising, *in vivo*, proteins so anchored. Glypiation, in combination with specific PI-PLCs, permits rapid and specific release of membrane-bound enzymes or receptors, thus generating soluble activities or down-regulating receptors. Again, it may be significant that diacylglycerol, an activator of protein kinase C, is a product of the protein-releasing reaction. Signaling specificity could be introduced if individual members of the protein kinase C family displayed differing sensitivities to diacylglycerols having different acyl groups. The possibility that diacylglycerols can be converted in the inner plasma membrane leaflet to phosphatidic acid introduces another putative regulatory mechanism (Moolenaar *et al*, 1986).

5.2 Membrane Anchorage in Alkaline Phosphatases

Slein and Logan (1962; 1963) reported that a factor present in Bacillus cereus culture filtrates, when injected in rabbits, produced a large increase in serum alkaline phosphatase activity, and also released alkaline phosphatase from kidney and bone slices. The factor later turned out to be a phosphatidylinositol-specific phospholipase C, suggesting that tissue-unspecific alkaline phosphatase was bound to the membrane via interactions with a phosphatidylinositol molecule (Slein and Logan, 1965; Low and Finean, 1977). Since this tissue-unspecific alkaline phosphatase was released in a soluble non-aggregating form which was not associated with membrane vesicles nor with other lipid-protein complexes, and by analogy with other phosphatidylinositolated proteins, it was believed that PI was specifically involved in the attachment of these proteins to the membrane, and that 1,2-diacylglycerol was serving as a membrane anchor which was largely or entirely responsible for membrane attachment. Further analysis suggested that the association between tissue-unspecific alkaline phosphatase and phosphatidylinositol was a covalent one (Low and Zilversmit, 1980).

The mechanism of membrane binding of mammalian intestinal alkaline phosphatase is less clear. Seetharam et al (1987), found that rat intestinal brush border

alkaline phosphatase was not released from the brush border membranes by phosphatidylinositol-specific phospholipase C. Further studies led to the proposal that rat intestinal alkaline phosphatase was bound to the membrane by tight hydrophobic interactions rather that a covalent association. However, conflicting work by Low et al (1987) showed that treatment of bovine intestinal alkaline phosphatase with nitrous acid resulted in release of the enzyme in a soluble form and free myo-inositol, suggesting that the protein contained a phosphatidyl moiety attached through a glycosidic linkage to a sugar residue bearing a free amino group.

5.2.1 Models of Membrane Binding of Placental AP

Since the work described in this thesis was concluded there have been a number of published reports from the Udenfriend group which together strongly indicate the mechanism of membrane binding in placental alkaline phosphatase.

Berger et al (1987b), working on simian (COS) cells transfected with a eukaryotic expression vector (pBC12) bearing a cDNA encoding human placental alkaline phosphatase, found that the cells transiently expressed membrane-bound alkaline phosphatase. The enzyme could be specifically released from the cell surface in a hydrophilic form by treatment with phosphatidylinositol-

specific phospholipase C, a property strongly indicative of the presence of an inositol-glycan membrane anchor. Furthermore, the transiently expressed alkaline phosphatase was heat-stable and resistant to inhibition with l-homoarginine but sensitive to inhibition with l-phenylalanine, and thus had properties characteristic of the PLAP isoenzyme. A criticism of this work, however, is that the monkey cell line does not normally express placental alkaline phosphatase, and therefore the post-translational glypiation of the enzyme may not reflect the situation in the human trophoblast.

In parallel with this work, Howard et al (1987) characterised the alkaline phosphatase expressed in WISH, a transformed cell line from human amnion. When cell suspensions were incubated with purified phosphatidylinositol-specific phospholipase C, alkaline phosphatase activity was released in a hydrophilic form. Also, when the cell were cultured in the presence of [^{14}C]ethanolamine, [^{14}C]myristic acid, or myo-[^{3}H]inositol, each was incorporated into the carboxyl terminus of the alkaline phosphatase. The alkaline phosphatase expressed in the WISH cell line was reported as heat-stable, resistant to catalytic inhibition by l-homoarginine and susceptible to inhibition by l-phenylalanine. This led the workers to the belief that they were studying placental alkaline phosphatase.

However, precise identity had not been established between this placental-like alkaline phosphatase expressed by transformed cells, and tissue-extracted placental alkaline phosphatase (see the introductory chapter of this thesis for a general review of this subject).

Although the above reports are limited in the extent to which the results can be seen as models of the mechanism of membrane binding of normal trophoblast expression of placental alkaline phosphatase, they complement each other and together suggest that placental alkaline phosphatase may be covalently associated with glycolipid. However, to further examine this possibility it is important to study placental alkaline phosphatase as extracted from the placenta.

5.2.2 Membrane Attachment in Tissue-Extracted PLAP

Evidence for the mechanism of membrane attachment of tissue-extracted placental alkaline phosphatase came from the Udenfriend group as this thesis was being prepared for submission. Micanovic *et al* (1988) purified and characterised the C-terminal chymotryptic polypeptide from a commercial preparation of PLAP. Their investigation revealed that the carboxy-terminal amino acid residue of mature PLAP is an aspartic acid residue, to which is attached a phosphatidylinositol-glycan

moiety. The aspartic acid residue was demonstrated to be Asp-484, suggesting, by analogy to VSG protein processing, that a 29-residue polypeptide predicted from the cDNA sequence is cleaved out during glypiation. Consistent with other examples of inositolated proteins, this cleaved polypeptide is highly hydrophobic and may be involved in anchoring the nascent polypeptide to the endoplasmic reticulum prior to inositolisation.

The results of Micanovic et al (1988) were derived from study of only those PLAP molecules demonstrated to be hydrophobic by their behaviour in Triton X-114 phase-separation. The remaining 90% of the PLAP molecules were found to be hydrophilic in behaviour, indicating to the workers that the diacylglycerol portion of the phosphatidylinositol-glycan had been lost during preparation of the enzyme. An alternative interpretation, that is not raised in their report, is that the hydrophilic PLAP is not a degradation product, and thus that the commercial preparation corresponds to the condition of PLAP in vivo. This interpretation reflects the findings of the Sutcliffe group, in that our preparations of PLAP contain A-PLAP, B-PLAP and M-PLAP. However, no attempt was made by Micanovic et al (1988), nor by any of the Udenfriend group, to electrophoretically characterise the PLAP molecules. The question therefore remains open as to whether M-PLAP is

inositolated and whether removal of this "converts" M-PLAP to A-PLAP.

Conversion of PLAP from a hydrophobic to a hydrophilic form has also been investigated by Malik and Low (1986), who have been particularly interested in the possible conversion *in vivo*. They have approached this study by attempting to characterise the necessary conditions for hydrolysis, firstly on butanol extraction and secondly in normal human plasma.

5.2.3 Butanol Extraction of Placental AP

Malik and Low (1986) have suggested that solubilisation of human placental alkaline phosphatase by butanol involved the activation and action of an endogenous factor. On extraction of placental membranes at pH 5.4, the alkaline phosphatase released into the aqueous phase was found to be indistinguishable from that released on incubation with a phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus* i.e. both forms were relatively hydrophilic and had molecular radii of around 170,000. By contrast, butanol extraction at pH 8.3, or at low pH in the absence of calcium ions, released an aggregated form of approximately 600,000 molecular size which was relatively hydrophobic. Proteinase inhibitors had no effect. Further investigation of the effect indicated that the

phosphoinositide-specific phospholipases C from cytosol and lysosomes were unlikely to be responsible for conversion. Malik and Low (1986) also detected a phosphatidylinositol-hydrolysing activity in the particulate fraction. However, under conditions in which the PI-hydrolysing activity was completely abolished, PLAP-conversion activity was unaffected. Characterisation of this factor in the absence of butanol, lipids and enzymes was not attempted.

The above report provides a close parallel to the work described in this thesis, and hence deserves closer examination and comparison with this work. At no stage of the present work, or of the work of Abu-Hasan (thesis), was the pH of butanol extraction controlled or measured, except that it was performed in a low-buffered solution of pH 7.0 (namely 25mM succinic acid, 4mM Tris). The relative quantities of M-, A- and B-PLAP recovered were variable, however, and seemed to be dependent on the efficiency of washing of the microvillous membrane pellet. This uncontrolled observation is consistent with both the leeching out of the Malik and Low factor prior to extraction, and with the removal of the conversion factor examined in this thesis.

It is important to note that this butanol-stimulated conversion phenomenon need not have occurred in our hands, since Malik and Low prepared their placental

membranes from whole homogenised placenta as contrasted with our enriched microvillous membranes, a difference which is likely to alter the co-purifying contaminants.

It must also be stressed, that any such conversion during butanol extraction was effectively invisible to the Sutcliffe group, since we disregarded, to a large extent, the relative proportions of A- and M-PLAP with a view to fractionating the isoenzymes by chromatography.

The conversion phenomenon reported by the Sutcliffe group is of the butanol extracted products, which were stable in the absence of serum, and was not calcium dependent. It may be the case, therefore, that there are two independent mechanisms of conversion.

5.2.4 Conversion of PLAP Isoenzymes in Plasma

As this thesis was being prepared for submission, a report was published by Low and Prasad (1988) describing an activity abundant in mammalian plasma which removed the phosphatidylinositol membrane anchor from hydrophobic human placental alkaline phosphatase. The activity could be inhibited by EGTA and 1,10-phenanthroline, and was stimulated at low pH. The workers suggested that the agent responsible for the activity was the same as that described by Malik and Low (1986), and proposed that the presence of the activity in the Malik and Low particulate fraction was due to contamination by the plasma enzyme.

The specificity of the agent was examined and the results indicated that conversion was due to a phospholipase D activity.

5.3 Conversion and Apolipoproteins

Part of the present work was the discovery of a factor present in normal human serum that mediated conversion of hydrophobic, membrane-bound M-PLAP to the soluble forms A- and B-PLAP following butanol extraction. This conversion is not affected by the addition of antiproteases, and does not involve loss of subunit size detectable on SDS-PAGE. This, together with the evidence of Abu-Hasan, Davies and Sutcliffe (1984) that M-PLAP remained hydrophobic on incubation with a range of organic solvents and ionic and non-ionic detergents, implies that M-PLAP is tightly associated with a hydrophobic moiety. The above work of Micanovic *et al* (1988) indicates that commercially available hydrophobic PLAP is covalently associated with a phosphatidylinositol glycan moiety. It is likely, though not proven, that the hydrophobic PLAP characterised by Micanovic *et al* is at least very similar to the hydrophobic PLAP studied in this thesis, namely M-PLAP. Work by Low and Prasad (1988) has shown that normal plasma contains a phospholipase D activity which cleaves the phosphatidylinositol tail from hydrophobic PLAP *in vitro*, although purification and

identification of the factor has not yet been reported. The present work has purified and characterised the M-PLAP converting activity from normal human serum, and it has been demonstrated as associated with lipoprotein A.

5.3.1 Functions of Lipoproteins and their Components

The study of lipoproteins and apolipoproteins has essentially focused on a number of inter-related areas. Plasma lipoproteins have attracted the attention of investigators interested mainly in lipid transport (for reviews see Morrisett et al, 1975; Scanu et al, 1982), both in the normal condition, and in relation to diseases e.g. atherosclerosis (Goldstein and Brown, 1977), coronary thromboses, alcoholism, Tangier Disease. Other workers have, however, focused on the fact that plasma lipoproteins participate in the activation or inhibition of lipid-metabolising enzymes. For example, apolipoprotein A-I is a specific activator of lecithin cholesterol acyltransferase (Yokoyama et al, 1978; Furukawa and Nishida, 1979), and apolipoprotein C-II enhances the triglyceridase and phospholipase A_1 activity of rat heart lipoprotein lipase (Groot et al, 1978). The mechanism of action of these apolipoproteins is not fully understood, but appears to involve enhancement of the transfer of the enzyme between vesicles (Nishida et al, 1986). It has also been shown that plasma lipoproteins

play important roles in lymphocyte proliferation and immunologic homeostasis (Morse et al, 1977; Chisari, 1977). Thus, it is becoming increasingly evident that circulating lipoproteins are dynamic structures and recognise a series of structural changes which are controlled by enzymatic and physicochemical phenomena.

Most recently, lipoprotein vesicles have been demonstrated to be crucial in a number of cellular activities, including release of cell surface proteins from tissue explants. LDL binding to plasma membrane receptors appears to suppress cholesterol synthesis through modulation of the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (Goldstein and Brown, 1977). Further, the apolipoproteins of high density lipoprotein (HDL), namely apoA-I, apoA-II and apoC-I, have been shown to stimulate the release of human placental lactogen (hPL) by placental explants (Handwerger et al, 1987). Other recent work by Darbon et al (1986) demonstrated that HDL apolipoproteins stimulated phosphorylation of a 27kD protein in quiescent bovine endothelial cells, strongly supporting a role for apolipoproteins in stimulating protein kinase C.

5.3.2 Lipoproteins or Apolipoproteins in PLAP Conversion

Despite intensive and extensive research into the roles played by lipoproteins and their protein components, there appear to have been no reports of any directly induced changes in other proteins caused by apolipoproteins. More specifically, given the hypothesis that M-PLAP is covalently bound to phosphatidylinositol, there have been no reports of a phospholipase activity directly associated with apolipoproteins or lipoproteins. A recent (17/11/87) computer literature search on Data-Star (copyright Biological Abstracts, Inc., Philadelphia/Penn, USA) searching for English-language reports on lipoproteins or apolipoproteins in association with phospholipases and in association with plasma, serum or blood published between 1985 and 1987 revealed no relevant reports. This leaves the author to consider a number of possible hypotheses. As raised in the discussion of the relevant chapter, it is likely that the conversion activity is associated with apolipoproteins A-I and A-II. However, this association may be direct, in the form of a previously undiscovered activity of the apolipoproteins, or indirect by means of other protein factors. These possibilities are discussed in more detail below.

The action of a factor associated with lipoprotein A on M-PLAP could be due to an as yet undetected activity

of the apolipoproteins comprising 95% of the purified conversion factor. This activity has remained "hidden" due to the routine method of isolation of serum lipoproteins i.e. salt density centrifugation. The work herein reports that 0.5M NaCl completely and irreversibly removed the conversion activity. This hypothesis might at first appear to be unlikely given the predicted secondary structure of apolipoprotein A-I. According to the analyses of Segrest et al (1974), Segrest and Feldman (1977) and of Fitch (1977) apolipoprotein A-I has a secondary structure of limited complexity, having at least 11 alpha-helical regions, two beta-sheets, and 13 cycles of 11 repeating units. However, this reasoning does not take into account the high degree of microheterogeneity of apolipoprotein molecules, both at the level of primary structure and at the level of function. A subset of apoA-I molecules, namely apolipoprotein A-I_4, have already been shown to have atypical properties with respect to the activation of LCAT, and may contain a different amino acid sequence (Nestruck et al, 1980). It is therefore possible that a small percentage of apoA-I contains M-PLAP conversion activity.

A second hypothesis to explain the dependence of conversion activity upon the presence of a lipoprotein-associated factor is that the apolipoproteins activate an

endogenous phospholipase. This phospholipase may be present as a minor species of the purified conversion activity (PSCA), or as a contaminant of the butanol-extracted microvilli used as the source of M-PLAP. Given the range of chromatography systems employed in the fractionation of serum to give PSCA, any phospholipase activity co-purifying with apoA-I is also associated with apoA-I either in the phospholipid vesicles or in apolipoprotein aggregates or the apolipoprotein in free solution. This hypothesis is thus indistinguishable from the previous hypothesis of the direct action of the apolipoproteins, and has again never been observed by other workers. That the phospholipase activity originates in the M-PLAP substrate is also unlikely since purified M-PLAP, which contained two PLAP bands plus albumin, was a good substrate for conversion with normal human serum.

5.4 Summing Up

This project was aimed at the elucidation of the differences between the A-, B- and M-PLAP forms, and at the nature of the serum conversion activity. The recent reports of Low and Prasad (1988) and of Micanovic *et al* (1988) indicate that mammalian placental alkaline phosphatase contains a covalently attached phosphatidylinositol-glycan at its carboxy-terminus, and that this can be cleaved from the molecule *in vitro* by a

phosphatidylinositol-specific phospholipase D activity abundant in normal mammalian plasma. It is likely that the hydrophobic PLAP molecules found by these workers is partly or wholly analogous to the hydrophobic PLAP described in this thesis and by Abu-Hasan (thesis). It follows therefore that there is a likelihood that at least M-PLAP contains an inositol anchor, and that A-PLAP does not contain the anchor. The nature of B-PLAP is less clear. In order to determine the substrate/product relationships between the phospholipase D activity and the isoenzymes of PLAP, the experiments of Micanovic need to be repeated demonstrating the relative proportions of A-, B- and M-PLAP.

This thesis has described the purification and characterisation of a factor present in normal human serum that mediates the conversion of M-PLAP to A- and B-PLAP. The activity has been shown to be associated with apolipoproteins A-I and A-II. Whether this conversion phnomenon is similar to that described by Low and Prasad (1988) remains to be demonstrated, but the possibility has been raised that apolipoproteins may have other functions and activities other than those already characterised and associated with lipid metabolism. Purification of apolipoproteins by more gentle methods than salt density centrifugation may reveal the nature of these activities.

CHAPTER 6

GENERAL METHODS

CHAPTER 6

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6.1 Preparation of Placental Alkaline Phosphatase

6.1.1 Purification of Placental Microvilli

Placental microvilli were prepared according to the method of Smith et al (1974), as described by Abu-Hasan et al (1984), as follows:

1) Fresh placentae (less than 4 hours old) were given an initial wash in 0.85% saline and 1.22% CaCl.

2) Amnion and chorionic plate disected off, and remaining material chopped into pieces of approximately $1cm^3$.

3) Material washed in 150-180ml ice-cold saline for 30 mins at 4°C with rapid and continuous agitation.

4) The wash was then poured off and spun at 12,000g for 10 mins at 4°C in a Beckman JA-14 (13cm radius). The supernatant was taken and subjected to ultracentrifugation at 145,000g in a Beckman Ti50 rotor (7cm radius) for 30 mins at 4°C.

5) If the resulting pellet was still red, then the pellet was resuspended using a tight fitting glass homogeniser in Tris-succinate buffer (25 mM succinic acid, 4 mM Tris, pH 7.0) and ultracentrifugation step

repeated. Once the pellet was creamy-white, it was resuspended and stored at -20°C, until a stock of phenotyped placentae had built up.

6.1.2 Butanol Extraction of Microvilli

Syncytiotrophoblast plasma membrane preparations were extracted using the method of Boyer (1963).

1) To each suspension of microvillous membranes was added an equal volume of ice cold butan-1-ol, and the mixture shaken vigourously for 1 hour at 4°C.

2) The mixture was subjected to centrifugation at 12,000g for 10 mins at 4°C, to separate the aqueous and organic phases.

3) The aqueous phase (bottom) was drawn off and dialysed against Tris-succinate buffer (pH 7.0) overnight to remove excess butanol.

6.2 Electrophoresis

6.2.1 Starch Gel Electrophoresis

Horizontal starch gel electrophoresis was performed at pH 6.0 (Robson and Harris, 1965) as previously described (Abu-Hasan et al, 1984).

1) 25g starch was suspended in 290 ml Tris-succinate

buffer (pH 6.0), and heated to boiling point with rapid agitation to keep the starch in suspension until dissolved.

2) Approximately 1.25ml Triton X-100 (0.5%) was added to 10ml Tris-succinate buffer (pH 6.0), and the mixture heated to dissolve the detergent.

3) Both mixtures were added together and swirled to mix.

4) Before the starch had begun to set, the solution was poured into a perspex former. A thin plastic sheet was added as a lid, taking care to exclude air bubbles, and this was topped by a heavy glass plate to expel excess starch solution. This was then placed at 4°C for one hour for the starch to set.

5) Once cold, the glass plate and the plastic sheet were removed, and the detergent-starch block was cut lengthwise twice, once 1cm from a long edge, and once 5cm further up and parallel to the first cut. The 1cm wide piece of gel was removed from the perspex former and kept aside. The 5cm wide slice was then slid down, revealing a cut surface within the body of the gel onto which 5mm x 7mm fragments of 3MM blotting paper were applied, impregnated with 20ul of PLAP solution. These fragments were inserted upright onto the cut surface of the starch block, alternating with inserts wetted with Tris-succinate buffer alone. Once complete, the gel was

reclosed by sliding the gel up and replacing the 1cm slice.

6) The block was then placed in an electrophoresis tank containing 0.41M citric acid, pH 6.0. Paper wicks were applied, ensuring that the sample papers were towards the cathode, and 8v/cm was applied for 5 hours at 4°C.

7) After this time, the block was removed from the perspex former and cut horizontally to reveal a representative cross-section of the gel.

8) This was stained using a solution of Fast Blue RR salts (25mg) and beta-napthyl phosphate (25mg) in 50ml borate buffer (60mM sodium tetraborate, 10mM $MgSO_4.7H_2O$, pH 9.7).

6.2.2 Acrylamide Gel Electrophoresis

SDS-PAGE was performed by the method of Laemmli (1970), modified to run slab gels. Gels were routinely made up to 10% acrylamide, although 12.5%, 15% and 17.5% gels have been used.

1) Glass plates were washed in acetone and 100% ethanol, and and 2mm spacers washed in ethanol alone. The plates were assembled and made watertight with tape.

2) The acrylamide stock solution was 30% acrylamide, 0.8% bisacrylamide.

3) Stock resolving gel buffer comprised 3.0M Tris-HCl (pH 8.9).

4) Stock stacking gel buffer comprised 0.5M Tris-HCl (pH 6.7).

5) Stock detergent solution was 10% SDS.

5) 1.5% ammonium sulphate solution was made up fresh daily.

6)(i). For a 10% gel, 15ml acrylamide was added to 5.6ml resolving gel buffer, 0.3ml SDS, 21.7ml dH_2O, and 1.5ml persulphate.

(ii) For a 12.5% gel, 18.75ml acrylamide was added to 5.6ml resolving gel buffer, 0.3ml SDS, 18ml dH_2O, and 1.5ml persulphate.

(iii) For a 15% gel, 22.5ml acrylamide was added to 5.6ml resolving gel buffer, 0.3ml SDS, 14.2ml dH_2O, and 1.5ml persulphate.

7) 25ul TEMED was added to the appropriate mixture, and quickly poured between the glass plates, leaving room to add the stacking gel.

8) Since air inhibits the polymerisation of acrylamide, the solution was overlayed with 0.1% SDS or butanol.

9) 5% stacking gel solution was made up by adding 2.5ml acrylamide to 5.0ml stacking gel buffer, 0.2ml SDS, 11.3ml dH_2O, and 1.0ml persulphate.

10) When the resolving gel had polymerised, the gel

plates were wrapped in cellophane and stored at 4°C for up to 48 hours until ready for use. Stacking gel was not formed on top of the resolving gel until within 2 hours of running the gel, in order to maintain the pH step at the gel interface.

11) The top of the gel was washed with a small volume of the stacking gel solution. 15ul TEMED added to the remaining solution and the gel plates filled to the top. The gel comb was inserted which would form the wells, making sure that no air bubbles were left, and the gel left to polymerise.

12) Meanwhile, double concentration sample buffer was made up containing 0.12M Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.004% bromophenol blue. To the reducing sample buffer was added 10% beta-mercaptoethanol.

13) 10-50ul of sample containing 1-10ug protein per protein species was added to an equal volume of sample buffer in 0.5ml eppendorf tubes. Reduced samples were boiled for 3mins in steam, while non-reduced samples were kept on ice. Finally the samples were subjected to microcentrifugation to collect all the solution at the bottom of the eppendorf tubes.

14) In addition to the sample tracks, marker proteins were included in the gel in order to calibrate the gel. Typical proteins included ovalbumin (45,000), bovine serum albumin (66,000), transferrin (87,000) and beta-

galactosidase (116,000).

15) Electrophoresis buffer was made up to contain 0.025M Tris-HCl (pH 8.8), 0.192M glycine, 0.1% SDS. 16) The gel comb and bottom tape were removed and the gel wells washed quickly in dilute stacking buffer before assembly into the electrophoresis tank. Samples were loaded in appropriate wells and gently overlayed with electrophoresis buffer. The tank was then filled with electrophoresis buffer and 40mA applied for approximately 4 hours, or until the bromophenol blue front had reached the bottom of the gel.

17) Following electrophoresis, the gel was carefully removed from the gel plates and submerged in 10% acetic acid, 30% methanol, 0.3% Coomassie brilliant blue for approximately 1 hour. The gel was then destained in 10% acetic acid, 30% methanol until the protein bands showed blue against a clear background. The gels were either photographed immediately, stored in 10% acetic acid, or washed for 1 hour in 30% ethanol, 1% glycerol and dried down under vacuum.

6.2.3 2-D Antibody-Antigen Crossed Electrophoresis

Antibody antigen crossed electrophoresis was performed as described below.

1) Glass plates measuring 9cmx9cmx2mm were used

throughout, and were pre-coated as follows. 10ml of molten 1% agarose dissolved in distilled H_2O was poured onto and spread evenly over each plate. Plates were left to cool at room temperature. The plates were then incubated at 37°C until dry, leaving an agarose film on the surface. This film helped in anchoring the agarose gel to the glass plate.

2) Barbitone buffer was made up with 58mM (12g per litre) 5,5'-diethylbarbituric acid, sodium salt, 24mM (4.4g per litre) 5,5'-diethylbarbituric acid, pH 8.6. At this stage, 0.2g thiomersal as preservative was added per litre buffer.

3) 1% agarose in barbitone buffer was melted and 15ml poured onto and spread evenly over each plate, avoiding air bubbles, and left to cool. The remaining agarose was kept at 50°C in a water bath, as were a number of cut tip 10ml pipettes and test tubes.

4) A well was punched in a corner of each plate using the cut tip of a pasteur pipette, such that the 2mm diameter hole was 1.5cm from one edge and at least 1cm from the other edge.

5) The sample, normally 4ul containing approximately 150ug normal serum proteins, was loaded into the wells with a Hamilton syringe, as was 1ul of 0.01% bromophenol blue as a front marker.

6) The plate was subjected to electrophoresis at 20mA

at 4°C in barbitone buffer, such that the antigen migrated parallel to the edge of the plate.

7) Examination of the migration of the bromophenol blue revealed two spots of differing mobilities. This was interpreted as bromophenol blue-bound albumin lagging unbound bromophenol blue.

8) When the albumin was judged to have migrated to about 1cm from the far edge of the plate, electrophoresis was stopped, and the plates removed to room temperature.

9) A cut was made across each plate 2mm from, and exactly parallel to, the track of the sample. Further cuts were made from this line at 1cm from the edge following the perimeter of three sides, thus forming a rectangle in the middle of the plate. This rectangle of gel was carefully excised, and the glass plate underneath cleaned with a tissue.

10) 6ml of 1% agarose in barbitone buffer cooled to 50°C was transferred to a warmed test tube using a warmed cut tip pipette. To this was added antibody to a known final concentration, usually 5% rabbit anti-whole human serum, and the mixture vortexed.

11) Before the agarose began to set the 6ml was transferred onto the rectangular well avoiding air bubbles, and spread evenly within the well.

12) Once cool, electrophoresis was performed at right angles to the first dimension at 5mA at 4°C overnight,

such that the sample migrated into the antibody bed.

13) Following electrophoresis, the plates were submerged in physiological saline. The plates were gently agitated at room temperature for at least 48 hours with at least 3x1 litre changes of saline, thus allowing the unprecipitated sample and antibody remaining in the gel to leach out during this time.

14) The gel was then overlayed by a square of blotting paper cut to size, and incubated at 37°C until completely dry.

15) The plates were then stained with coomassie blue stain as described for acrylamide gels.

6.2.4 Rocket Immunoelectrophoresis

Rocket immunoelectrophoresis was performed as 2-dimensional AACE with the exception that electrophoresis was performed only in one dimension directly into the antibody bed.

6.3 Assay Systems

6.3.1 Estimation of Protein Using Folin Reagent

Protein assay was carried out according to the method of Lowry et al (1951), with the modification of Bonsall and Hunt (1971) for solutions containing Triton X-100.

1) A standard solution of 1mg/ml bovine serum albumin was made up in the same buffer as the unknown sample, and was used to provide a range of protein amounts, all in 200ul. The unknown sample was diluted over a broad range, and 200ul taken at a number of dilutions.

2) Solution A was made up as 2% Na_2CO_3 in 0.1M NaOH.

3) Solution B contained 1% $CuSO_4.5H_2O$

4) Solution C was 2% KNa tartrate.

5) Solution D was made up as 25ml solution A, 250ul solution B and 250ul solution C.

6) 1ml solution D was added to 200ul of each standard and diluted sample. Each tube was immediately vortexed, and left to stand at room temperature for 10 minutes.

7) To each reaction was added 100ul of freshly 50% diluted folin ciocalteu's phenol. Reactions were vortexed again, and left to stand at room temperature for a further 40 minutes. For samples containing Triton X-100, the phenol solution is diluted in a final concentration of 0.25% SDS.

8) Optical densities were measured at 500 nanometres, and a graph drawn of the protein standards. The concentration of the unknown sample was interpolated from the graph.

6.3.2 Protein Estimation By Optical Density

A rough estimation of protein concentration can be quickly gauged by measuring optical densities as detailed below.

1) A range of sample dilutions were made in dH_2O.

2) Optical density measured in 1cm quartz cuvettes at 260nm, 280nm and 320nm, using dH_2O as a blank.

3) The 320nm reading was subtracted from the 260nm and 280nm readings.

4) The ratio of the 280/260 readings was calculated. If the ratio was greater than 1.4, as it was in most cases, then the corrected 280nm reading was assumed to be approximately equal to the protein concentration. Below 1.4, this assay method is increasingly inaccurate and another protein assay system was employed.

6.3.3 Alkaline Phosphatase Assay

Alkaline phosphatase activity was measured using the method of Sutcliffe *et al* (1972), with p-nitrophenolphosphate as substrate.

1) Substrate solution was made up containing 20mg/ml p-nitrophenolphosphate in 6mM $MgCl_2$, 31mM Na_2CO_3, pH 10.7.

2) 30ul substrate solution was added to a range of alkaline phosphatase dilutions, all in 20ul.

3) This mixture was left at room temperature for 30 minutes with frequent vortexing.

4) Enzyme activity stopped by the addition of 1ml stop buffer (0.05M glycine, 50mM EDTA, 50mM potassium phosphate, pH 9.3).

5) Optical density was measured at 400nm, and the alkaline phosphatase activity calculated, given the extinction coefficient of 1.62×10^4.

6.4 Column Chromatography

Details of the protocols used for gel filtration, ion-exchange and FPLC chromatography are given in the relevant results sections.

6.4.1 Activation of Sepharose 4B

Some experiments used commercially activated Sepharose, though on some occasions we used Sepharose that had been activated in the laboratory. Activation was performed as described by Porath *et al* (1958) and as follows:

N.B. CNBr is hazardous, as under acidic conditions can give off HCN fumes, and should therefore be handled in a fume cabinet. All materials coming in contact with CNBr should either be washed immediately afterwards, or protected with foil.

1) On the basis that 1g wet weight Sepharose can bind 15mg protein, the required amount of matrix is weighed out. 10g wet weight Sepharose requires 1g CNBr for activation and a total reaction volume of 25ml.

2) Sepharose 4B slurry weighed out and poured into a clean, dry column. This is washed well with 2-3 column volumes of distilled H_2O.

3) Solid CNBr very carefully weighed out and dissolved in 1/3 total reaction volume of dH_2O.

4) A pH probe was placed in the CNBr solution to monitor pH for remainder of reaction. A peristaltic pump was primed with 2M NaOH, with outlet over the CNBr beaker.

5) Slowly, the washed Sepharose was added. Sufficient NaOH was pumped into the beaker to maintain pH above 11.3.

6) High pH maintained for at least 10 minutes, or until pH stabilised.

7) Once equilibrated, the Sepharose was poured back into the column and washed with ice-cold dH_2O, then cold 0.1M $NaHCO_3$ (at least 4-5 column volumes).

8) Solutions eluting from the column were drained into a beaker containing 10M NaOH to neutralise any remaining CNBr.

9) The Sepharose was now ready to be incubated with the

protein to be bound.

6.4.2 Antibody Coupling to CNBr-Activated Sepharose 4B

Antibody coupling to CNBr-activated Sepharose 4B was performed as recommended by Pharmacia as follows:

1) The required amount of Sepharose was weighed out, allowing 1g per 10mg bound protein.

2) The Sepharose was well washed in 1mM HCL on a sintered glass funnel under vacuum, using at least 200ml HCl per gram matrix.

3) Antiserum was diluted in coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl, pH 8.3), allowing 5ml per gram Sepharose. Sepharose added and the vessel stoppered. Mixed well for two hours at room temperature.

4) Excess ligand was washed away with coupling buffer and assayed for protein concentration. Difference between total soluble protein before and after coupling indicates amount of protein bound on matrix.

5) Remaining active sites were blocked by incubating with ethanolamine (1M, pH 9.0) for two hours at room temperature. Product was then washed with three cycles of acetate buffer (0.1M, 0.5M NaCl, pH 4.0) followed by Tris buffer (0.1M, 0.5M NaCl, pH 8.0).

6) Product poured into column and stored in Tris-succinate buffer (pH 7.4) containing 0.1M NaCl.

6.4.3 Antibody Affinity Chromatography

Many variations in the conditions used for antibody affinity chromatography were used, including concentrations of NaCl in application buffer, presence or absence of ammonium hydroxide, and either sodium phosphate or Tris-succinate buffers. What follows are the details of those parameters under which the control experiments worked.

1) The column used contained 346mg of rabbit antiserum raised against human alpha-lipoprotein bound to 35ml Sepharose 4B.

2) 1.5ml of normal human serum at a protein concentration of 42 mg/ml was applied onto the column in ice-cold Tris-succinate buffer containing 0.1M NaCl at a flowrate of 75ml/hour. Unbound protein was collected in 1.0ml fractions which were assayed for protein by optical density.

3) Protein bound to the column was eluted using 3.0M potassium thiocyanate, and 1.0ml fractions were collected.

4) Selected fractions of unbound protein were pooled, desalted and reconcentrated by ultrafiltration, to a final concentration of 42mg/ml, and assayed for conversion activity. Selected fractions of bound protein

were desalted and reconcentrated to a final concentration approximating to the concentration of this protein in the starting serum. This was calculated by firstly expressing the total bound protein as a fraction of the total recovered protein, which approximates to the proportion of associating protein present in the applied serum, as well as to the starting concentration of such protein.

5) Selected fractions were also analysed on 1-dimensional rocket electrophoresis and on SDS-PAGE.

6) The column was re-equilibrated with Tris-succinate buffer containing 0.1M NaCl, and stored at 4°C until required.

6.5 Miscellaneous Techniques

6.5.1 Western Blotting

Western blotting was carried out as described below. Initial transfer of protein from the gel onto the nitrocellulose sheet was performed according to the method of Towbin *et al* (1979). Antibody probing utilised the modern technique of using 0.1% Tween to prevent non-specific association of antibody to the nitrocellulose.

1) SDS-polyacrylamide gels were run as described above, with tracks arranged to give 6 identical panels divided by one blank track.

2) One panel was removed for Coomassie blue staining as above, while the remaining gel was washed in transfer buffer (25mM Tris base, 192mM glycine, 20% methanol).

3) Nitrocellulose was cut to the dimensions of the remaining gel and washed in transfer buffer, as were four pieces of blotting paper the size of the plastic former.

4) Taking care to exclude air bubbles, a sandwich was made up of the former, two sheets of blotting paper, the gel, the nitrocellulose, more blotting paper, and finally the plastic former.

5) The sandwich was transferred to the transfer tank such that the gel was more towards the cathode than was the nitrocellulose. The tank is filled up, again avoiding the inclusion of air bubbles.

6) Transfer was performed at 200mA overnight with cooling and mixing of the transfer buffer.

7) Following transfer, the sandwich was dismantled and the gel stained with Coomassie blue and compared with the untransferred panel to check transfer out of the gel. The nitrocellulose was washed in TBS-tween (10mM Tris-HCl, pH 7.4, 140mM NaCl, 0.1% Tween 20) to remove any adhering polyacrylamide.

8) To check transfer onto the nitrocellulose, one panel was cut from the nitrocellulose and stained with 0.1% amido black in 45% methanol, 10% acetic acid for 10 minutes, and destained with 45% methanol, 10% acetic

acid.

9) The remaining four panels were then separated, and treated differently as follows.

10) The experimental panel was washed in the first antibody diluted in HST buffer (10mM Tris-HCl, 1M NaCl, 0.5% Tween 20) for one hour with constant shaking. The correct dilution factor for the antibody was assessed *per se*, with the expectation that it was between 1:100 and 1:1000. The polyspecific antibody used in this work was diluted to a final concentration of 1:400. The correct volume of solution to use depends upon the size of the panel, and is calculated as 1ml per $10cm^2$ of nitrocellulose.

11) The panel was then removed and washed at room temperature in TBS-tween. After five minutes, this solution was poured off and replaced with fresh TBS-tween and washed for a further five minutes. The filter was then washed in HST, then a further twice in TBS-tween.

12) The peroxidase-conjugated second antibody was applied diluted 1:500 - 1:1000 in HST buffer, and the filter washed for a further hour at room temperature with constant shaking.

13) The filter was removed and washed three times in TBS-tween, once in HST, followed by three times in TBS-tween. Finally, the filter was washed in TBS.

14) Substrate solution was made up by dissolving 18mg 4-

chloro-1-napthol substrate in 6ml methanol. This was added to 94ml TBS and 25ul H_2O_2 immediately before use. Substrate solution was poured onto the nitrocellulose panel and agitated for approximately 30mins, or until the panel was stained. The panel was kept in distilled water in the dark at 4°C.

15) The other three panels acted as controls and were all stained with 4-chloro-1-napthol following incubation with either first antibody alone, or second antibody alone, or with no antibody.

6.5.2 Radiolabelling of Protein

Proteins were radiolabelled with ^{125}I by the Chloramine T method of Greenwood et al (1963), using the modifications of Hunter (1970).

1) Phosphate buffer made up containing 0.05M sodium phosphate, pH 8.0, and used to make up the following reagents:

3% bovine serum albumin as carrier protein dissolved in phosphate buffer containing 0.02% sodium azide.

0.5mg/ml Chloramine T dissolved in 25ul phosphate buffer.

0.5mg/ml sodium metabisulphate.

1 mCi $Na^{125}I$ dissolved in phosphate buffer.

2) Purified protein to be labelled present in phosphate buffer at a concentration of 0.05mg/ml in 20ul.

3) Added 1mCi of ^{125}I, followed by 25ul of Chloramine T solution, and mixed.

4) After 60 seconds, added 50ul of metabisulphate solution, and the solution was diluted with 1ml of BSA-phosphate.

5) Free iodide was removed by passing solution down a column packed with Sepharose G-15 equilibrated with BSA-phosphate, collecting the unretarded fraction.

6) The resulting preparation was stored at 4°C and used within 4 weeks.

6.5.3 Preparation of Normal Human Serum

1) Fresh blood was taken and left to stand at room temperature for half an hour.

2) Blood then placed at 4°C overnight.

3) Spun in a swing bucket rotor (Beckman SW 7.5) at 900rpm for 5 minutes at 4°C.

4) Supernatant poured off and dialysed overnight in Tris-succinate buffer, pH 7.4. Frozen down and stored in aliquots.

CHAPTER 7

BIBLIOGRAPHY

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