MECHANISMS AND MODULATIONS OF PLATELET ACTIVATION: LIGAND-INDUCED CONFORMATIONAL CHANGE OF A HEMOPROTEIN AS A BIOCHEMICAL SIGNAL FOR ACTIVATION

A thesis presented
by

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in the subject of

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SREE CHITRA TIRUNAL INSTITUTE
FOR
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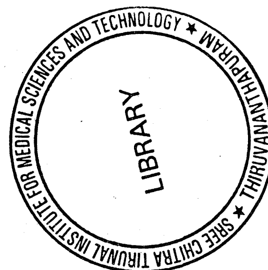
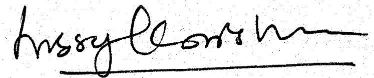


January 1989

CERTIFICATE

I, Lissy K. Krishnan hereby certify that I had personally carried out the work depicted in the thesis entitled " MECHANISMS AND MODULATIONS OF PLATELET ACTIVATION: LIGAND-INDUCED CONFORMATIONAL CHANGE OF A HEMOPROTEIN AS A BIOCHEMICAL SIGNAL FOR ACTIVATION" except where external help sought are acknowledged.



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DECLARATION

This is to certify that Smt. Lissy K.Krishnan in the division of Thrombosis Research of this institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the Ph. D. degree of Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The work relating to her thesis entitled "MECHANISMS AND MODULATIONS OF PLATELET ACTIVATION: LIGAND-INDUCED CONFORMATIONAL CHANGE OF A HEMOPROTEIN AS A BIOCHEMICAL SIGNAL FOR ACTIVATION" was carried out under my direct supervision.

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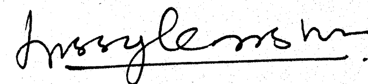


LISSY K. KRISHNAN

SYNOPSIS

This thesis comprises results of the author`s experiments to delineate the role of a dimeric hemoprotein reported from this laboratory as a putative prostaglandin endoperoxide (PGH_2) receptor (67), in platelet aggregation. The thesis comprises of 6 chapters.

In the introductory chapter, current ideas regarding mechanisms of stimulus-response coupling in platelets are briefly but critically reviewed focusing on arachidonate liberation and metabolism and the dawn of the idea of a hemoprotein reeptor leading to its purification in this laboratory (67).

Chapter II documents the Materials and Methods employed in the study.

In chapter III, the modification of the purification procedure to improve the yield along with some of the properties of the protein are described.

HPLC analysis of hydrolysates of dansylated protein revealed the presence of lysine as the sole aminoterminal residue testifying to the homogeneity of the protein. Amino acid analysis showed a preponderence of hydrophobic amino acids in the protein. The protein was found by Ellman`s reagent, to possess two sulfhydryl groups per molecule. Spectral properties of its pyridine hemochrome derivative established the prosthetic group as heme b. Extraction of the heme into $CHCl_3$ after acidification of the protein gave absorption spectra charecteristic of 2,4-ethyl substituted chloro protoporphyrin IX. Quantitation of the heme from

heme per mol of the protein.

In chapter IV, results of difference spectral measurements of the binding parameters of a number of ligands are presented together with consequences of such interactions on the protein conformation studied by chromatographic, immunodiffusion and electrophoretic experiments.

Among the ligands studied, PGH_2 showed swift and large spectral shifts with maximum affinity (half maximal saturation concentration, $S_{0.5}$ =1-2 μM) and lowest molar excess (2) at saturation, followed by 5,8,11,14,17-eicosa pentaenoic acid, hydrogen peroxide, oleic acid, arachidonic acid and several other unsaturated fatty acids. The binding interactions of these compounds exhibited positive cooperativity (h>1) in Hill plots and brought about conformational changes of the protein.

Large-zone gel-exclusion chromatographic data showed that the protein existed in association-dissociation equilibria which the ligands could modify. The charge charecteristics of the protein was changed by specific ligands as detected in ion-exchange chromatography and poly acrylamide gel electrophoresis.

The binding parameters of a given ligand depended on the protein concentration, on the conformational state of the protein, determined by the A_{405}:A_{280} ratio which was around 4 for the native protein and it decreased upon ligand binding or storage under aerobic conditions.

Binding of common heme ligands like cyanide or imidazole did not lead to chromatographically or

In chapter V, results of immunological experiments, spectral measurements and ion-exchange chromatographic methods were used to draw a parellelism between the ligand-induced conformational changes of the purified protein and conformational changes of the same protein during activation of gelfiltered calf platelets (GFP) by different agonists.

Immunofluorescence experiments employing anti-IgG (rabbit) showed the presence of an antigenically similar protein on GFP. The importance of this protein for platelet activation was established by showing that anti-IgG but not normal-IgG at equivalent protein concentration, dose-dependently inhibited rates of aggregation of GFP by ADP, hydrogen peroxide and the calcium ionophore A23187, assayed by the spectrophotometric method (68). Time-course of conformational change of the protein during A23187 induced platelet activation followed by immunodiffusion experiments showed that it occured even at 7 seconds i.e. before any aggregation had begun.

Similarly to the binding interaction of PGH_2 with the purified protein, the kinetics of aggregation by A23187 followed an apparently cooperative pattern but the kinetics of ADP- induced aggregation followed an apparently positively hyperbolic pattern (68) as analysed by the sequential shape change and interaction model of platelet aggregation proposed earlier (66). The spectral and immunodiffusion patterns obtained with extracts of GFP activated by various stimuli were also similar to those obtained upon ligand binding to the purified protein .

Gel filtration of 50-90 % ammonium sulphate fraction

column, showed the emergence of an earlier eluting 410 nm band protein, whose proportion increased with the dose of ADP. The 280 nm absorbance of the protein from ADP-activated GFP was higher after gelfiltration, compared to that of the protein from control GFP, at the same Lowry's protein(80).

Comparison of protein yields after the DEAE Sephadex A-50 column step from equal numbers of the same batch of GFP before and after activation by ADP, showed that the charge charecteristics of the protein changed after activation. 70-80 % of the loaded A_{405} units was recovered in the case of normal GFP whereas the recovery was 20% and 10%, respectively, when platelets activated by 2 μM or 8 μM ADP were employed.

In the final chapter VI, the results are summarised, discussed in the light of currently accepted views of platelet activation, and the hypothesis is advanced that ligand-induced conformational change of the hemoprotein in question is an essential step in the biochemical mechanism of platelet activation and that similar mechanisms might operate in other cell types.

Projections for future lines of research are made.

INTRODUCTION

CHAPTER I

Platelets play a central role in haemostasis and thrombosis by responding to appropriate stimuli. Under physiological conditions they respond to collagen, thrombin or ADP, resulting in release and aggregation. The released components and the substances present on the activated platelet surface contribute, along with many other substances in blood plasma and blood vessel wall, to the formation of haemostatic plug, that seals a break in the vessel wall. In different circumstances, or in some pathological situations, a similar mechanism leads to the formation of a thrombus that can obstruct circulation and cause a heart attack or stroke. The number of components involved in thrombus formation is enormous, but platelets are rated as a major one. The functioning of platelets alone involves a large number of components which interact in a mutually dependent manner. In Part A of this introductory chapter, a general review of the participation of all the components involved in haemostasis and thrombosis is discussed briefly, with special emphasis on the role of platelets. In Part B the molecules involved in the stimulus- response coupling in platelets and the current ideas about the mechanisms of signal generation and transduction are discussed in detail.

Part A: MECHANISMS IN HAEMOSTASIS AND THROMBOSIS:

Haemostasis is an important physiological defence mechanism to arrest bleeding from an injury. The formation of the so-called haemostatic plug is complex, involving vascular

responses, activation of blood coagulation, activation of fibrinolysis and adhesion and aggregation of platelets. Thrombosis has been described as ' haemostasis in the wrong place'(84). However, thrombus formation is even more complex, involving variability in the relative contributions of the vessel wall responses, blood coagulation and platelet aggregation, and the mutually dependent reactions occur simultaneously within the various components. A thorough knowledge of the mechanisms involved in thrombosis is necessary for the pharmacological manipulations in the prevention and treatment of thrombosis. A brief outline of the participation by each component is given below (95).

I.A.1. Role of Vessel wall:

In all blood vessels, a continous monolayer of endothelial cells is in contact with blood. It serves as the first line of defence against the process of thrombosis. It also has a subendothelium containing basement membrane, collagen fibrils, microfibrils rich in fibronectin and glycosaminoglycans.

Of the elements described , collagen fibres are highly reactive to both platelets and the coagulation system. Platelets readily adhere to collagen even in the absence of calcium ions, the latter being necessary for platelet aggregation. Collagen fibrils are known to activate the coagulation system by means of Factor XII activation.

I.A.2. Blood coagulation:

Two pathways are known to operate in the activation of blood coagulation, namely, intrinsic and extrinsic.

The initiation of intrinsic pathway starts with the activa-

tion of Hageman Factor (FXII) to FXIIa by collagen, or a foreign surface or by kallikrein and high molecular weight(HMW) kininogen is a cofactor at this step as well as at the subsequent step where FXI is converted to FXIa by FXIIa. The conversion of prekallikrein to kallikrein acts as a feedback that hastens conversion of FXII to FXIIa. FXIa converts FX to FXa in the presence of protein cofactor FVII, phospholipid and calcium.

The activation of extrinsic pathway involves exposure of blood to tissue factor, a lipoprotein, and calcium and FVIII is also required for the conversion of FX to FXa. The intrinsic and extrinsic pathways provide alternative mechanisms of activating FX to FXa. There are many positive feedback steps operating to amplify the formation of FXa.

Once FXa is formed it converts prothrombin to thrombin, a reaction which is greatly accelarated by phospholipids, at the platelet surface, by calcium and a large protein cofactor FV. Once thrombin is formed it splits off fibrinogen to fibrin monomers, which undergoes spontaneous polymerization to the fibrin clot. Thrombin also catalyzes the conversion of FXIII to FXIIIa , a plasma transamidase, which crosslinks the fibrin clot, thereby promoting its resistance to lysis and increasing its mechanical stability.

The clot which forms a localized seal at the injury is eventually dissolved, to maintain patency of the vessel, by the fibrinolytic system. Many of the pathways that promote clotting are accompanied by parellel pathways that promote fibrinolysis. For e.g. Hageman factor, in addition to activating FXI, also activates prekallikrein to form kallikrein which in turn splits

plasminogen to the fibrinolytic enzyme, plasmin, which then causes lysis of fibrin.

I.A.3. Role of platelets in haemostasis:

a) Morphology:

Platelets are anucleated, disc-shaped cells, 2-3 μm in diameter with a concentration in normal blood of 150-400x10^{9} l^{-1}. The electron microscope reveals a complex organization of subcellular structures (37, 55). Inside the platelet membrane lies a circumferential band of microtubules. The cytoplasm contains mitochondria and a number of granules which include, dense bodies, glycogen granules and α-granules. The dense bodies contain seratonin (5-hydroxy tryptamine), calcium, adenine nucleotides and pyrophosphate and these contents are intimately concerned with platelet aggregation. α-granules contain a number of platelet protein including platelet factor 4, β-thromboglobulin, platelet derived growth factor and fibrinogen. The contents of the cytoplasm include microfilaments, probably the morphological representation of the contractile protein, thrombosthenin. An open canalicular system, for communicating to the surface of the cell, and providing an excretory pathway for the products of cell metabolism, is also present, in human platelets.

b) Platelet function:

Platelets interact with a number of agonists resulting in shape-change, release and aggregation. The formation of haemostatic plug depends on the adhesion of platelets to the damaged blood vessel wall, through their affinity for subendothelial collagen, then the accumulation of more platelets on the initial adherent layer (aggregation). In physiological situations, the

platelet stimuli may include: ADP that can be derived from injured tissues (including red blood cells) which can affect the platelets in the vicinity; collagen exposed by injury to the vessel wall to which platelets adhere; microfibrils around elastin, also exposed following vascular injury, which can activate platelets in presence of von Willebrand factor; and thrombin that can be generated when the blood comes into contact with tissues other than endothelium in the injured vessel wall. In addition a number of non-physiological agonists has been identified which stimulates platelets among which are tumor promoters, such as phorbol myristate acetate, ristocetin, concanavalin A, phytohemaglutinins and other plant lectins from a variety of sources, cationic polymers such as polylysine, zymosan with adsorbed components of the complement system, latex and other particulate matter, tumor cells and divalent cation ionophore A23187. As a result of the interaction of these agents with platelets, a series of events can be initiated at the plasma membrane, within the cytoplasmic matrix of platelets and at the level of platelet granules. However different agonists activate different pathways within the platelets that are linked through a common effector path.

b.i. Shape change:

A circumferential microtubule ring maintains the disc shape of platelets(97). At this stage, the cytoplasm is dense and no filamentous material is seen (7). When the platelets are stimulated, the platelet granules become concentrated in the central area of the cell(134) and cell shape changes from disc to sphere and narrow, long psuedopods are extended (2). Such

psuedopod formation results in an increase of effective surface area, facilitating contact with neighbouring platelets. Studies with the calcium ionophore A23187 suggest that both pseudopod formation and granule centralization result from a flux of intracellular calcium(47,78). Following the initial shape change there is a reorganization of the cytoplasmic contents, internal contraction within the cytoplasm sweeps all the intracellular organelles to the centre, still surrounded by the microtubule ring and sometimes by a band of microfilaments (136)

The contraction within the cytoplasm brings the secretory granules into close proximity with the surface connected canalicular system into which the granules are emptied (45). Further contraction then squeezes the secreted components into the plasma.

Several lines of evidence suggest that pseudopod formation results at least in part, from an interaction of actin, actin-binding protein and α -actinin(121) and this interaction may be controlled by phosphorylation of actin-binding protein. Granule centralization is currently believed to occur as a result of actin-myosin interaction which produces tension (24,44). Actin-myosin interaction in platelets appears to be primarily controlled by calcium interacting with calmodulin and a protein kinase to initiate phosphorylation of myosin light chain(1, 26,59,74). Actomyosin appears to provide the force for the contractile system, although the molecular mechanism by which it acts is still unclear(7,22,23,110,).

b. ii. Release reaction:

Many substances provoke degranulation of platelets, which

include ADP, adrenalin, collagen, thrombin, immune complexes and metabolites of arachidonic acid and platelet activating factor(21,64,81,83,93). The nonphysiological agonist, the calcium ionophore A23187 causes degranulation of many granule types in the platelets of many species(47) but some degranulating agents are effective on platelets of some species only; e.g. acetyl-choline in dog (93). Some agents, e.g. ADP induce the secretion of certain categories of granules only(dense bodies and α-granules but not lysosomal granules). Thrombin on the other hand induces secretion of all types of granules including lysosomes(64).

Heterogeneity of platelet granules has been described by many authors. Dense bodies can vary in composition in different species, in different subjects and even within the same platelet (125). There are unknown numbers of different types of α-granules which contain fibrinogen, fibronectin, factor VIII -related antigen, FV & FVa, and low affinity platelet factor 4 (124). At least 3 types of lysosome is present in platelets(124).

Though the reasons for the existance of several types of granules in platelets are not clear, it may be that, certain granule components may not be packed together on account of their structure or chemistry. It is also clear that different granule types are discharged in different situations, for e.g. platelets can act as carriers of growth factors and deliver them to a site of injury with great precision and without releasing lysosomal enzymes(71).

Exposure of platelets to release-inducing agents causes a change in shape that is correlated with the movement of granule

to the centre of the platelet(135). However it is not clear whether this movement is causally related to secretion; if centralised granules are those about to be discharged, those that will not be discharged or some of both.

Much evidence suggests that secretion is initiated as a result of a raised cytoplasmic calcium level (20,32,33,34,137,138). Secretion probably results from a combination of contraction(granule centralization), and fusion of organelle membranes to membranes of the surface connected canalicular system (granule labilization) (45). The mechanisms inducing granule labilization are at present unknown but it may be significant that membrane fusion is facilitated by calcium(102).

b.iii. Aggregation:

Stimulation of platelets with an agonist results in the exposure of receptor systems on the surface of the cell. These receptors, once exposed can interact with selected proteins (ligand) present in the subendothelial matrix or released from platelet stores. These ligands include fibrinogen, fibronectin and von Willebrand factor, and the binding of these `adhesive proteins' imparts specific adhesive properties to the platelet (109). Two independent groups demonstrated that ^{125}I labelled fibrinogen interacts in a specific and saturable manner with platelets exposed to ADP but not with non- stimulated platelets (10,86,90). Other than ADP, a variety of platelet agonists can induce the platelet fibrinogen receptor, including epinephrine(107), thrombin (106), collagen (105), arachidonate (56), prostaglandin (PGH_2) and stable analogues(11), calcium

ionophore and platelet activating factor (88). Thus the existance of a common fobrinogen- dependent pathway of aggregation has been postulated (88,105).

Synergistic effects between ADP and other stimuli have been observed. Collagen epinephrine or thrombin at concentrations that fail to induce fibrinogen binding, do so in the presence of ADP concentrations as low as 10- 50 nM. With other agonist combinations, such as collagen plus calcium ionophore, additive rather than synergistic effects are observed (88). These observations may be of physiological significance in vivo, as platelets may be exposed to subthreshold concentrations of stimuli during the haemostatic response.

A large body of evidence suggests that Glycoprotein (GP) IIb/IIIa complex plays the role of fibrinogen receptor in platelets(87). The component of the complex which interacts directly with fibrinogen may be GP IIIa. However an unidentified membrane component may be present which associates stoichiometrically with the GPIIb/IIIa complex and which interacts directly with fibrinogen.

A sequence of reactions is implied in the process of platelet aggregation including

1. Interaction of an agonist with the cell.
2. Induction of fibrinogen receptor on the platelet surface.
3. Binding of fibrinogen to its platelet receptor (which is associated with platelet aggregation)
4. Stabilization of fibrinogen-receptor complex.

These four basic reactions are postulated and is illustrated in Fig.1. to describe the relationship between fibrinogen and

platelets in the pathway of fibrinogen dependent aggregation(86,88,108).

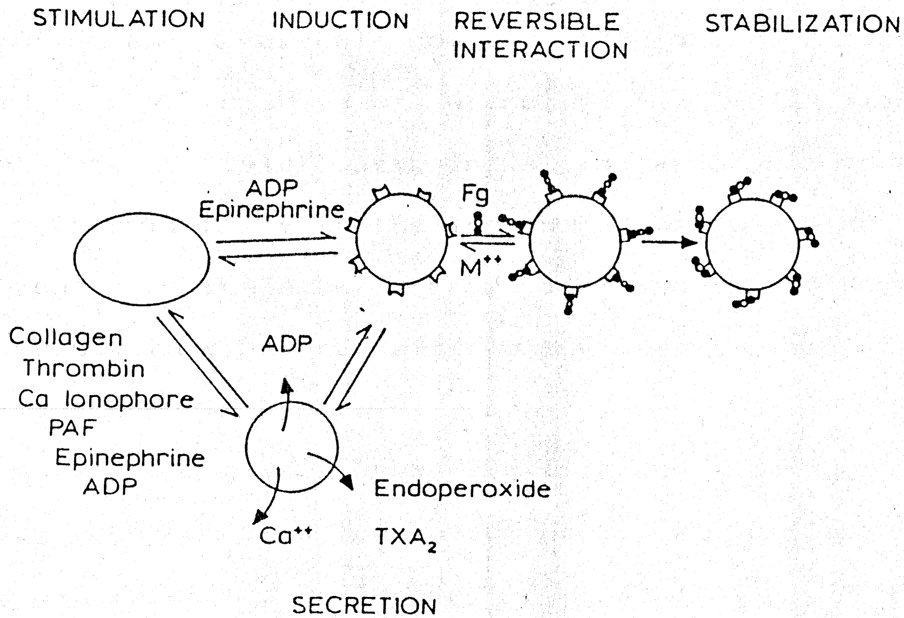


Fig.1.1. Sequence of reactions in fibrinogen-dependent aggregation.

Since binding of fibrinogen is induced by an array of platelet agonists, it is likely that the reaction 1 can involve multiple cell surface events and or receptors which activate a variety of different biochemical events. Mobilization of platelet associated ADP, arachidonate metabolism, changes in cAMP levels and calcium translocation are possible signals responsible for initiating the induction of fibrinogen receptor.

This reaction involves subtle rearrangement of membrane associated glycoproteins or thier microenviornment, and divalent cations may play a role in this reaction. The association of fibrinogen with its induced receptor is dependent on the divalent

cations and is the rate-determining step of the overall reaction sequence (89).

It has been reported that the endogenous lectin of human platelets is an α-granule component(38) and fibrinogen is its receptor(39). The studies of Gartner et al.(41,42) also suggest that platelet- platelet stickiness is analogous to a lectin-receptor interaction, with the lectin on one platelet and the lectin receptor on the other. From later studies of Gartner (40) it would appear that several different platelet-platelet bridges may occur as shown in Fig.2.

Bridge no.1 shows glycoprotein IIb/IIIa and fibrinogen bound to it, acting together as a lectin receptor, while fibrin binds to platelets and functions as lectin.

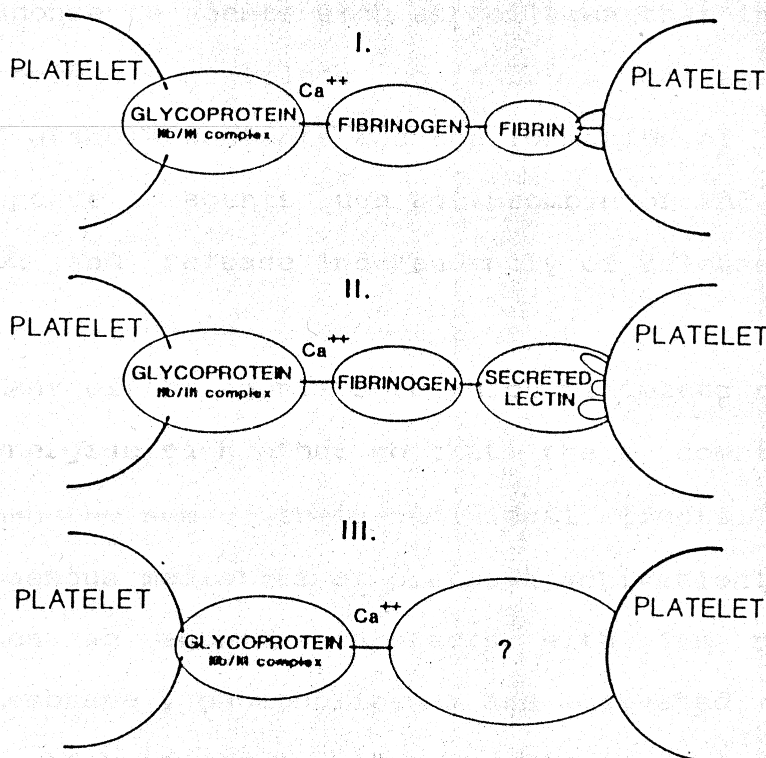


Fig.1.2. Platelet-platelet bridge formation during aggregation.

From studies suggesting that a secreted substance (not fibrin) can bind to the platelet membrane and function as the lectin, the second interplatelet bridge is postulated. Other studies suggest that neither of these concepts applies to ADP-induced primary aggregation, so the existence of a third inter platelet bridge is postulated. Fibrinogen may also be involved in this bridge (96).

B: STIMULUS-RESPONSE COUPLING IN PLATELETS

Platelets can be activated by a variety of agonists. Different agonists , however, seem to activate different pathways within the platelets that are linked through an apparently common final effector path.

Response of platelets to stimuli may be grouped into three broad categories;

1. The response to ADP in the absence of release reaction;
2. The response to agents such as collagen that involves the initial adherence of platelets to fibrous collagen followed by release of granule contents and the formation of thromboxane A_2
3. The response to agents such as thrombin or PAF that can induce aggregation and release independently of released ADP or formation of thrombaxane A_2.

Many of the agonists to which platelets respond may augment or synergise each other so that their combined effect is greater than the sum of their individual effects.

I.B.1.Endogenous mediators of platelet activation:

When an agonist interacts with its receptor on the platelet membrane , phospholipases are activated. Phospholipase C activation which can occur without an increase in cytosolic

calcium leads to hydrolysis of phosphatidyl inositol 4,5 biphosphate (PIP_2) to inositol 1,4,5- triphosphate (IP_3) and 1,2 diacylglycerol which are regarded as important second messengers.

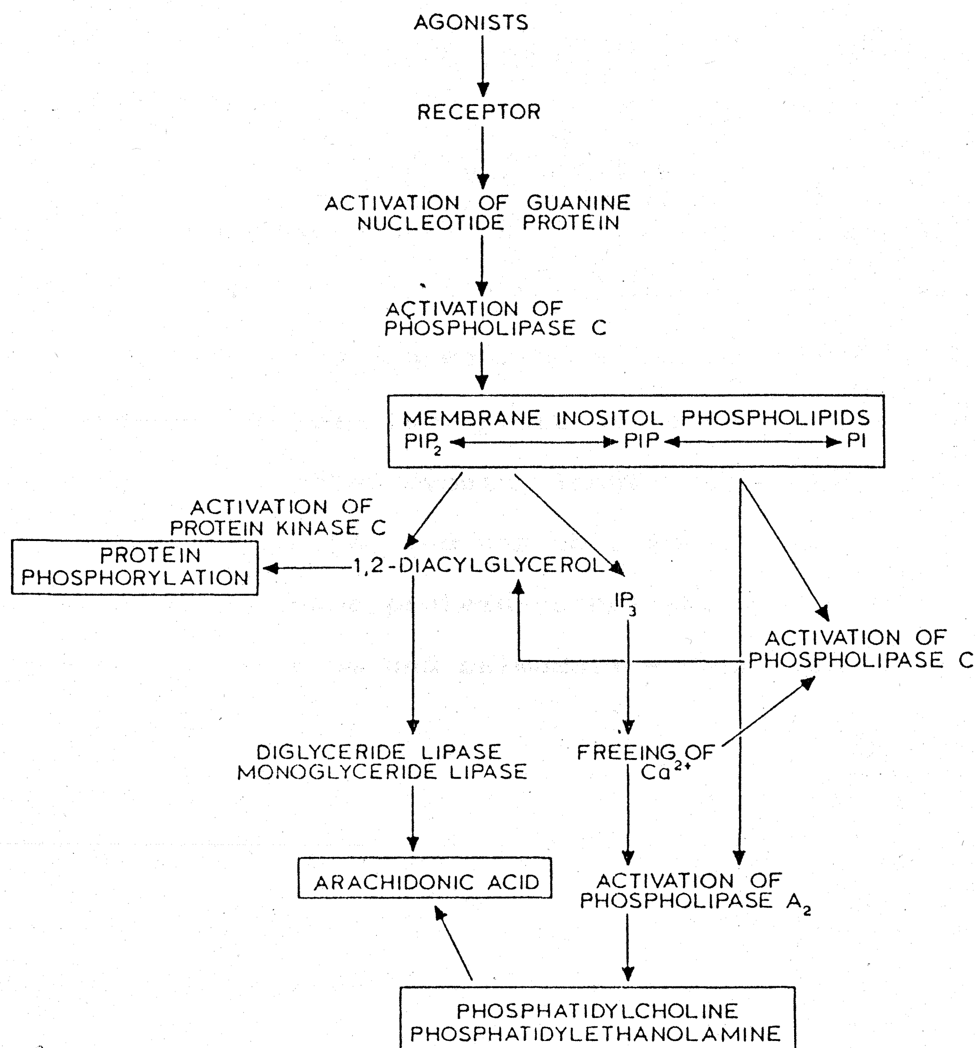
The diacylglycerol can be acted upon by diacylglyceride lipase and subsequently by monoglyceride lipase , and arachidonic acid can be freed (8,77).

The IP_3 can by an unknown mechanism, cause a rise in cytosolic calcium thought to be required for the activation of phospholipase A_2. Phospholipase A_2 can cleave arachidonic acid from membrane phospholipids, in particular phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine(9,16,17, 18,116,118,119,120).

Phospholipase A_2 activation seems to be the major pathway for arachidonic acid release in the platelet and availability of arachidonic acdi is the rate-limiting step in its metabolic conversion(15).

Arachidonic acid once liberated is acted upon, primarily, by cyclooxygenase(52) and its site of action is localized as platelet dense tubular system(48). This results in its conversion to short-lived prostaglandin endoperoxide intermediates, PGG_2 and PGH_2 (85,94) which are acted upon by a variety of enzymes depending on the cell types. In platelets the membrane bound thromboxane synthetase converts these endoperoxides to a short-lived product thromboxane A_2 which is rapidly converted to an inactive end product, thromboxane B_2 (53). The formation of these end products of arachidonic acid metabolism appears to play a major role in the synergism between pairs of aggregating and release-inducing agents to which platelets may be exposed.

The relationship between phospholipid metabolism , activation of arachidonate pathway and protein phosphorylation is summarised in scheme I.



Scheme I.

Thrombin-induced aggregation and release are accompanied by phosphorylation of two platelet proteins one having an Mr 40,000-47,000 and another myosin light chain, having an Mr 20,000 (58). Nishizuka and his colleagues (99) have shown that phosphorylation of 20kDa protein occurs through a reaction that is mediated by a calcium and calmodulin- dependent protein kinase

and phosphorylation of the 40-47 kDa protein is mediated by a calcium and phospholipid-dependent kinase, protein kinase C that is activated by 1,2 diacylglycerol.

I.B.2.Second messengers of platelet activation:

The action of stimuli on their respective specific surface receptors activates coupling mechanisms which allow the signal to be carried (transduced) through the membrane to the cytosol, often leading to formation or liberation of intracellular second messengers which in turn act on targets inside the cell. The second messengers may influence effector molecules such as enzymes, transporters or cytoskeletal proteins, or they may cause the generation of yet other intracellular second or third messengers acting on intracellular targets. The details of the operation of these coupling mechanisms are not clearly understood at present.

The second messengers most widely recognized as being important in platelet function are calcium ions and cAMP although cGMP and H^+ ions have also recieved attention . Nishizuka(99) has assigned second- messenger roles for diacylglycerol and inositol phosphates. Calcium and diacylglycerol are proposed to be important in platelet activation, and cAMP is thought to be the key for inhibition. The proposed role of each second messenger molecule may be summarised as follows:

i. calcium:

In platelets calcium is located in the dense granules(63,123) and dense tubular system (25). When platelets are stimulated to secrete their granule contents the calcium con-

tained in platelet granules and dense bodies is also released to the outside of the cell(27).

The evidence for calcium being the second messenger is indirect and can be summarised as follows: Ca^{2+} ions do activate platelets, though the response remains below the basal level, obtained with agonists(114). Calcium ionophores can produce near normal platelet responses(36). By using graded concentrations of calcium ionophore and external calcium, one can produce stable graded rises in calcium and then construct $[Ca^{2+}]_i$/response curves for shape-change, myosin phosphorylation, aggregation, secretion, arachidonic acid liberation and thromboxane production(50,51,115). Calcium ions are required for platelets to aggregate, but shape change in response to ADP and thrombin occurs even in the absence of calcium. The inability of platelets to aggregate in the absence of external calcium has been attributed to the important role played by calcium ions in:

(a) maintaining the glycoprotein IIb/IIIa complex and

(b) the binding of fibrinogen to the activated glycoprotein IIb/IIIa both of which are necessary for aggregation.

Calcium ions may also contribute to the stability of platelet aggregates.

ii. Diacylglycerol:

Diacylglycerol is formed independently of $[Ca^{2+}]_i$ elevation, when agonist-receptor interaction stimulates the hydrolysis of inositol lipids by action of phospholipase C. Nishizuka and his colleagues showed that diacylglycerol can activate protein kinase C and proposed it as an important part of activation cascade, particularly in the release reaction, in platelets and many

other cells(70). Protein kinase C can be activated by diacylglycerol at or below basal $[Ca^{2+}]_i$ in many cell types. At higher $[Ca^{2+}]_i$, but below the threshold to cause secretion or aggregation, phorbol ester or exogenous diacylglycerol give a much more rapid and physiological- looking response (114). Probably natural response reflect the synergistic actions of calcium and diacylglycerol.

iii.cAMP:

Agents that increase cAMP antagonize platelet response to stimulation. If the various platelet reactions to cell stimulation are all activated by a common initiating factor, namely Ca^{2+} (62), cAMP regulates the level of free Ca^{2+} in the cell, or control the Ca^{2+} dependent reactions which are essential for the responses. The enzyme adenylate cyclase is activated by prostaglandin I_2, E_1 and D_2 and is inhibited by 2 adrenergic agents, ADP and thromboxane A_2 (35). These actions are apparently transduced through the coupling G-proteins, Ns and Ni. Inhibition of c AMP phosphodiesterase can also elevate cAMP in platelets and increase the effect of cyclase activators. Elevation of cAMP in platelets produces a wide-spread suppression of stimulation by most agonists. A major effect of cAMP may be to stimulate Ca^{2+} sequestration into the dense tubular system and to prevent or frustate the second messenger role of Ca^{2+} ions(72). cAMP also reduces stimulated hydrolysis of inositol lipids (70,117). cAMP thus interferes with the formation or mobilization of excitatory second messengers.

iv.Other messengers:

Inositol 1,4,5-triphosphate can release Ca^{2+} from

sequestering organelles and can promote thromboxane formation, perhaps by calcium activation of PLA_2, in permeabilised platelets(101,5). $InsP_3$, therfore, may be acting as an agent for mediating the internal discharge of calcium ions. It may also promote phosphorylation of myosin light chains (76). Stimulation is followed by a rise in cGMP, but it is not known if it is an important second messenger.

In summary it is apparent that no clear picture is available on the mechanism of càlcium release or the role of this ion in arachidonic acid release and other platelet activation reactions.

I.B.3. Approach to the proposed problem and its relevance:

As mentioned earlier, the agonist- receptor interaction of many ligands at the platelet- membrane results ultimately in the rapid liberation of arachidonic acid from membrane lipids. Once liberated, but not when it is bound to the lipid , arachidonic acid is converted by the enzyme cyclooxygenase to the prostaglandin endoperoxide PGG_2 and PGH_2. These compounds are then converted to thromboxane A_2 by the enzyme thromboxane synthetase. The calcium ionophore A23187 has also been reported as a potent inducer of this conversion (73). The endoperoxides and TxA_2 are found to be powerful aggregating agents(53,54).

Platelet-activating factor (PAF,1-O-alkyl-2-acetyl-Sn-glycerol-3-phosphoryl choline) is generated when cells are appropriately challenged. It appears to be formed from phosphatidyl choline containing plasmalogen that is acted on by phospholipase A_2 and subsequently acetylated by acetyl coA (13). The platelets respond to PAF independently of released ADP or the formation of

TxA_2 (133).

In summary, all these intermediates of platelet activation, including PAF, PGG_2, PGH_2 and TxA_2 are liberated secondary to cell stimulation, but they are not second messengers.

The arachidonic acid metabolites PGG_2, PGH_2, and TxA_2 are potent inducers of platelet aggregation and secretion(53,54). In view of the pathophysiological importance of arachidonate metabolites in haemostasis and occlusive vascular deseases(126), the mechanisms whereby these compounds induce platelet activation are of considerable interest. All three compounds appear to activate platelets by combining with the same plasma-membrane located receptor(4,82,). The mechanisms that link receptor occupancy to platelet activation also have been investigated and it has been reported that these compounds might decrease cAMP (49,92), induce an efflux of calcium from the platelet dense tubular system to the cytosol(46) and stimulate the phosphoinositide hydrolysis and elevation of the cytosolic free calcium concentration(111). But it has been observed that shape-change can be stimulated by PAF or the stable analogue of prostglandin endoperoxide U46619 without any measurable change in calcium (122). These observations suggest an as yet unidentified transduction pathway . Rink *et al.* (113) have speculated that the conformational change induced in the cytoplasmic part of the receptor molecule following ligand binding could directly induce a cytoskeletal change such as the initiation of actin polymerisation. This would provide a membrane transduction process not relying on the generation of a soluble or membrane-retained second messenger.

The charecterization of such receptors for PGG_2, PGH_2

and TxA_2, and the study of their structure activity relationships seems to be a highly prospective area in thrombosis research. Though these ligands are highly unstable, the availability of more stable synthetic analogues helped to design mimics of the biological response. It was thought to be likely that the receptor for the endoperoxides could be a hemoprotein because peroxide binding proteins are generally hemoproteins. For e.g. the proteins involved in arachidonic acid metabolism, prostglandin synthetase and thromboxane synthetase which have different affinities for the endoperoxides, possess heme as their prosthetic group. A similar protein without catalytic activity but endowed with ligand-induced conformational change could form an appropriate receptor molecule. This idea led us to look for such a hemoprotein in platelets which culminated in its purification from calf platelets in 1985(67).

This thesis enumerates the results of experiments designed to sustaniate the idea that this protein could be a putative receptor of PGH_2 (67) and to define more precisely the role of the protein in platelet activation.

MATERIALS AND METHODS

CHAPTER II

II.1. Materials:

II.1.1. Blood source:

Swiss Brown-cross-bred steers aged 6 to 18 months, wiegh-ing 40-125 kg were used. The animals were maintained in cement floored sheds, and fed chiefly on fodder and Gold Mohar cattle feed supplied by Hindustan Lever (Lipton), Bangalore.

II.1.2. Chemicals:

All unsaturated fatty acids, imidazole, sodium azide, 5,5`- dithiobis-(2-nitrobenzoic acid), N-ethyl maleimide, dithiothreitol, Triton X-100, fluorescein isothiocyanate, Coomassie brilliant blue, adenosine di phosphate , thrombin, the calcium ionophore A23187, the chromatographic gels(Sephadex G-25, Sephadex G-200, DEAE Sephadex A-50, Sepharose 2B) Agarose, the components of polyacrylamide gel(acrylamide, N,N`-methylene-bis-acrylamide), Sigmacote and Sucrose were from Sigma Chemical Co. USA. The Freund`s complete adjuvant was from DIFCO Laboratories, USA. The prostglandin endoperoxide PGH_2, 9,11, methanoepoxy-15-hydroxy- prosta-5,13-dienoic acid (U46619) and pinane thromboxane A_2 were from Cayman Chemicals, USA. Sodium cyanide was from May & Baker, England. All the other chemicals were the best quality available in India.

II.1.3. Equipments:

A Hitachi model SCR 20 BA high-speed centrifuge and Hitachi model SCP 55H ultra centrifuge were used.

A Shimadzu UV-VIS 240 microprocessor-controlled double-

beam recording spectrophotometer equipped with thermostated cuvette holder was employed.

Other equipments employed were as mentioned at the appropriate places.

Double quartz-distilled deionized water was used throughout.

II.2. Protein purification:

The protein purification described earlier (67) was modified to improve the yield.

II.2.1. Blood collection:

Blood (425 ml) was collected into 75 ml ACD (Appendix A, i) as anticoagulant in polyethylene bottles.

II.2.2. Preparation of platelet rich plasma (PRP):

The blood collected was taken in poly allomer tubes and centrifuged at 25° C in a Hitachi model SCR 20 BA centrifuge at 93 *g* for 20 min. The plasma was drawn out using a polyethylene Pastuer pipette and was centrifuged again at 60 *g* for 15 min to eliminate the contaminating RBCs and WBCs. The packed cells obtained (RBCs) after first centrifugation was suspended in Tris-HCl buffer (Appendix A,ii) and centrifuged at 60 *g* for 20 min and the supernatent was cleared off the contaminating RBCs by centrifugation at 40 *g* for 15 min. The washing was pooled with PRP and thus ~85 % of the total platelets present in whole blood was recovered in the platelet rich plasma.

II.2.3. Preparation of gelfiltered platelets(GFP):

The PRP was gel filtered as described by Tangen *et al.* (131) in a column of Sepharose 2B. Glass columns (C26/40) obtained from Pharmacia Chemical Co., Sweden, with attachments like polyethylene tubing, bed support screen and snap-on bed

support net were employed. The columns were siliconized with Sigmacote. Sepharose 2B washed and suspended in Tris- HCl pH 7.4 (130)(Appendix A,ii) was packed to get a bed volume of 150 ml and a flow rate of 100-120 ml per h. 50 ml PRP was loaded each time and GFP were collected in the void volume. The gelfiltration was done at 32° C. The column was washed and reused.

II.2.4. Preparation of crude extract:

The gelfiltered platelets were sonicated in 30 ml batches using an MSE Soniprep 150 ultrasonic disintegrater at amplitude 14 microns. The temperature was maintained 25° C by keeping the container in cold water and 1 min interval was given after 30 s sonication which was repeated 4 times. The sonicated platelet suspension was centrifuged in a Hitachi model SCP 55H preparative ultracentrifuge at 34,400 *g* for 30 min at 4° C The supernatent (crude extract) was processed for protein purification.

II.2.5. Ammonium sulphate fractionation:

The crude extract was treated with solid $(NH_4)_2SO_4$ (31.3 gm per 100 ml) added gradually with stirring at 4° C. The precipitation was allowed to proceed for 16 h in a 4° C cold room. The precipitate was removed by centrifugation at 34,400 *g* for 30 min at 4°C and the supernatent was treated with solid ammonium sulphate (29.2 gm per 100 ml) and the precipitation was allowed to proceed for 6-8 h at 4° C. The precipitate was collected by centrifugation at 34,400 *g* at 4° C for 20 min and dissolved in 3 ml phosphate buffer (Appendix A,iii) and clarified by centrifugation at 5,500 *g* for 10 min.

II.2.6. Gelfiltration of the protein:

Sephadex G-200 was boiled in distilled water for 6 h, the

fines were removed, and resuspended in phosphate buffer (Appendix A,iii). Columns(1x55 cm), were packed and equilibrated with the buffer and the protein from step (II.2.5) was loaded and eluted with the same buffer at flow rates of 5-6 ml per h. One ml fractions were collected using an LKB 2111-002 MultiRac fraction collector operating in the drop mode. The elution of the hemoprotein was monitored by measuring the absorbance of the fractions at 405 nm.

II.2.7.Ion-exchange chromatography:

DEAE Sephadex A-50 gel was soaked in phosphate buffer (Appendix A iii) for 24 h ,was packed into columns (1.5x30 cm) and equilibrated with the same buffer. The Sephadex G-200 eluants with A_{405}> 0.05 were loaded on the ion-exchange column, fraction by fraction without mixing, in the order in which they emerged from the column. The elution of the hemoprotein was followed by the A_{405} of the 1 ml fractions collected. The percentage yield was estimated by determinig the Lowry's protein(80).

The protein fraction with high 405 nm absorbance were pooled, sealed in N_2 atmosphere and stored at -20` C.

II.3. Preparation of antibodies:

II.3.1. Antibodies agaist the purified protein were raised in rabbits aged 1-2 years, weighing 1.2-1.5 kg. The first dose was 0.25 mg protein in 2 ml buffer, mixed with an equal volume of Freund's adjuvant.

Two booster injections were given on days 15 and 28 after the first injection, with 0.1-0.15 mg of protein in buffer. All injections were given subcutaneously. Blood was collected on the fifth day after last injection by puncturing the marginal ear

vein. Boosters were given as and when more antibodies were required and blood was collected on the fifth day after injection. Serum was allowed to separate at room temperature (30` C) and the separated serum was clarified by centrifugation at 5,500 *g* for 30 min at 4` C and then by filtration through a Millipore (Millex-GS, 0.22 µm) filter. Normal serum was used as the control. The serum was stored at -30` C, dispensed in small aliquots.

II.3.2. Purification of IgG fraction:

Purification of IgG fraction from normal and anti-serum was done by ammonium sulphate fractionation and DEAE cellulose chromatography as described (43). After DEAE cellulose chromatography the peak fractions obtained were pooled and concentrated using immersible Millipore ultrafilters. In all experiments with IgG, the Lowry`s protein of the control and anti- IgG was adjusted to be the same.

II.4. Charecterization of the protein:

II.4.1. N-terminal analysis:

The N-terminal aminoacid residue of the protein was identified by HPLC analysis of acid hydrolysates of the dansylated protein. The dansylation was done as described by Hartley(57). 200 µg of the protein was treated with dansyl chloride, dried and dissolved in 6N HCl and hydrolysed for 1 h. The hydrolysate was then dried and dissolved in distilled water (100 µl) and 10 µl was injected. The HPLC was done in an LKB HPLC system using Lichrosorb RP 18 column at a run rate of 0.5 ml per min. The solvant used was a gradient (Appendix A,iv) formed from methanol and 0.6% acetic acid-0.008% triethylamine in water. Monitoring of the

dansyl fluorescence was done in a Shimadzu model RF-540 recording spectrofluorimeter with excitation maximum at 333 nm and emission maximum at 510 nm.

II.4.2. Amino acid composition:

Acid hydrolysate of the protein was dansylated as described (91) and the dansyl derivatives were then analysed by the same experimental system described for N-terminal analysis. The percentage composition was calculated from the area under the peaks.

II.4.3. Identification and estimation of cysteine residues:

The cysteine content was obtained after unfolding the native protein in a denaturant and treating the protein with 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB)(30). Here the absorbance of the protein at 412 nm was determined and then 0.5 ml of the Urea-DTNB reagent (Appendix A,v) was added to the sample and reference cuvettes containing 0.5 ml of protein and 0.5 ml of phosphate buffer respectively. After mixing well, the increase in absorbance at 412 nm was noted. The molar concentration of -SH group (c) was calculated from the expression $c = \Delta A_{412}/13,600$ where 13,600 is the molar extinction coefficient of 5' dithiobenzoic acid. The -SH content per mol protein was calculated with respect to the Lowry's protein(80) used for the experiment.

II.4.4. Test for the presence of disulphide bonds:

The presence of cystine was tested after treating the protein with dithiothreitol(DTT) in the presence of a denaturant and then estimating the cysteine content. The unreacted DTT was removed by passing the protein- DTT mixture through a small column (1x10 cm) of Sephadex G-25 prior to -SH determination.

II.4.5. Spectral properties:

The complete spectrum of the protein was recorded in a Shimadzu UV-Vis 240 spectrophotometer from 700-250 nm.

The reduced spectrum of the purified protein was recorded after mixing with a few grains of sodium dithionate. The difference spectrum of the reduced protein was recorded after correcting the baseline with protein in both the cuvettes and then reducing the protein in the sample cuvette.

II.4.6. Identification and quantitation of heme:

Heme was identified from spectral charecteristics of alkaline pyrichrome of the protein. After adjusting the pH of the protein (0.8 ml) to 9.5 with 0.002 g NaOH, 0.2 ml of pyridine was added and mixed. The spectrum of the pyrichrome was recorded against the buffer and a few grains of sodium dithionate were added to the sample cuvette, mixed well and the reduced pyrichrome spectrum was recorded.

The heme was also quantitated after extracting into chloroform. The protein was acidified to pH 3.5 with acetic acid and mixed with an equal volume of chloroform. The mixture was centrifuged at 5,500 *g* for 20 min and the chloroform layer was drawn out using a Pasteur pipette. The spectrum of the cloroform extract was recorded against chloroform, from 600-350 nm. Molar absorbance values of 106 at 379 nm, 8.4 at 507 nm, 9.0 at 534 nm and 4.4 at 635 nm were used (19) to quantitate the heme content.

II.4.7. CO binding spectrum:

The carbon monoxide binding spectrum was recorded after bubbling CO through the protein solution. CO was prepared by adding slowly, strong formic acid from a dropping funnel to con

centrated sulphuric acid placed in a distilling flask. The impurities were removed by passing the gas through KOH.

II.4.8. Effect of pH on the spectral properties of the protein:

Effect of pH on the protein spectrum was tested after raising the pH gradually with dilute NaOH or reducing the pH gradually with dilute HCl and recording the spectrum at different pH values. The reversibility of the effect was tested by gradually bringing the pH back to the original by adding HCl or NaOH.

II.5. Ligand binding properties:

Ligand binding to the protein was studied by difference spectral measurements and the data were analysed using double rciprocal and Hill plots.

1 ml (~0.7 μg) of the freshly purified protein was taken in both sample and reference cuvettes and the baseline was corrected from 450- 350 nm. Small aliquots (1 μl) of the ligand in an appropriate solvant was stirred into the sample cuvette and an equal volume of solvant to the reference cuvette. The spectrum was recorded after each addition of the ligand. The spectrum showed peaks at 420-425 nm and dips at 405-402 nm, depending on the ligand. The difference in absorbance between the maximum and minimum (Δ A) was taken as a measure of binding. The addition of ligand was continued till there was no appreciable change in A between the last two additions. The double reciprocal plot of ΔA vs ligand concentration was done to get the ΔAmax and the Hill plot to get half maximal saturation of binding($S_{0.5}$) and the Hill coefficient (h) indicated whether or not ligand interaction is cooperative (h >1 positive cooperativity, h <1 negative

cooperativity and h =1, no cooperativity).

II.6. Detection of change in molecular size due to ligand binding:

Two methods were employed.

i. Large-zone gel- exclusion chromatography:

Small Sephadex G-200 columns (0.9x10 cm) equilibrated with 0.05 M phosphate, pH 7.4 were used. 1 ml blue dextran was loaded and eluted with the same buffer. Fractions (0.5 ml)were collected to determine the void volume(VO) of the column. Then 1 ml of control, and ligand-treated, protein samples were loaded and eluted as before. The elution volume (Ve) of each fraction was determined from its A_{405} and plotted against Ve/VO. The schematic representation of the elution pattern of the ligand treated-protein samples were compared with that of the control protein.

ii) Immunodiffusion:

Double diffusion in Agar gel was done by the Ouchterlony`s method(100). 0.8% agar gel in borate buffered saline(Appendix A, v) was poured onto microscopic slides and was refrigerated for 1 h before cutting the well patterns. Antiserum was placed in the central well and the native protein and ligand -bound protein samples were placed in alternate peripheral wells. The gel was kept in a moist atmosphere at 4` C for 48- 72 h for the development of precipitin lines. The gel was then washed with saline for 48 h, with 3 changes of saline. The gel was then dried placing it inverted on several layers of filter paper. The dried gel was stained with Coomassie brilliant blue R-250.

II.7. Detection of change of net charge of the protein due to ligand binding:

Two techniques were used.

i) Ion-exchange chromatography:

DEAE Sephadex A-50 columns (1x8 cm) equilibrated with 0.05 M phosphate buffer pH 7.4 was used. The control protein and the protein samples treated with saturating concentrations of various ligands, were passed through a small column (0.9x10 cm) of Sephadex G-25 to remove the unreacted ligands and the protein eluted from this column was loaded onto the ion exchange column (1x8 cm). The column was regenerated after each experiment by washing with 0.5 M NaCl, deionized water and then equilibrated with the running buffer. The elution of the protein was monitored by measuring both A_{405} and A_{280}. The percentage recovery of each sample from the ion exchange column was calculated from the sum of the 405 nm absorbance values of their column fractions and the initial value at the time of loading.

ii) Polyacrylamide gel electrophoresis:

Electrophoresis of native protein and the ligand-bound forms were done with fresh concentrated protein. The protein was treated with saturating concentrations of the ligands for 5 min. Ten per cent rod gels(Appendix A, vii) were used and the experiment was run in 0.1 M phosphate at pH 7.2 employing a current of 5 mA / tube. The gels were stained with 0.1 g % Coomassie blue R-250, destained (Appendix A, vii) and photographed.

II.8. Detection of modifications of the protein in intact platelets on cell activation:

Whole platelets were isolated and activated with various

agonists. The properties of the protein from activated platelets were compared to those of the protein from normal platelets.

II.8.1. Spectral effects:

The spectra of the supernatants prepared from normal, ADP- activated and thrombin activated platelets were recorded and compared.

II.8.2. Immunodifusion:

Ouchterlony's immunodiffusion was done with the whole platelet lysates obtained by lysing normal, ADP-, thrombin-, the calcium ionophore A23187- and cold-activated platelets with 1% Triton X-100. The experimental conditions were as described for immunodiffusion with purified protein.

II.8.3. Gel exclusion chromatography:

Platelets isolated for protein purification were divided into 2 equal parts. One part was stimulated with various concentrations of ADP prior to sonication and the purification steps were carried out. The elution pattern of the protein from Sephadex G-200 column was analysed by plotting 405 nm absorbance against Ve/V0 of the fractions. The spectral property of each hemoprotein fraction from normal and ADP-activated platelets were also recorded to see the modifying effects of ADP activation, on the spectral properties of the protein.

II.8.4. Detection of change in net charge of the protein:

The yield of protein after ion exchange chromatography, from ADP- activated platelets was compared with that from normal platelets. Three batches of ADP-activated platelets with 3 different concentrations were used for protein purification and compared to the protein purified from normal platelets.

II.9. Localization of the protein on platelets by immunofluorescence:

The accessibility of the protein for exogenous ligands were detected with flurescein isothiocynate (FITC) conjugated anti-IgG.

II.9.1. FITC conjugation of the immunoglobulin:

1 mg of FITC was dissolved in 1 ml of 0.15 M $Na_2HPO_4.2H_2O$(pH 9.0). To 0.5 ml IgG (2.0 mg) 0.5 ml of the dye solution was added and the pH was adjusted to 9.5 with 0.1M $Na_3PO_4.10H_2O$ while stirring. The reaction was allowed to continue in a 4°C cold room for 16 h. The unreacted dye was removed by gelfiltration through a small column of Sephadex G25(1x10 cm) in 0.05 M phosphate buffer pH 7.4.

II.9.2. Immunofluorescent labelling of platelets:

Platelets were isolated from calf blood as described earlier(II.2.3) and to 5 ml of the platelet suspension 1 ml (0.6mg) of the FITC conjugated anti-IgG for the test, and normal IgG for the control, was added. After half an hour the treated platelets were again gelfiltered through a 1.5x10 cm column of Sepharose 2B to remove the unreacted antibodies.

The labelled platelets were then concentrated by centrifugation at 100 *g* and a smear was prepared on a microscopic slide, air dried and fixed with acetone and mounted in DPX.

The preparation was then examined under an 'OLYMPUS' fluorescence microscope with a blue-green filter.

II.10. Localization of protein on the subcellular fractions of platelets:

Platelet membrane was prepared by a modified method of

Barber and Jamieson(6). GFP were concentrated by centrifugation at 100 g . The resuspended platelet concentrate ($\sim 3x10^{9}$ platelets per ml) was sonicated for 15 s employing an MSE Soniprep disintegrator. The sonicated suspension (5 ml) was loaded on a 20 ml cushion of 27% sucrose at 4° C and centrifuged at 190,000 g for 1 h in a Hitachi model SCP 55H preparative ultracentrifuge employing the RPV 50 T vertical rotor. The sediment containig granules and debri was preserved for immunodiffusion experiment.

The membrane fraction found at the junction of sucrose solution was collected, diluted 1:1 with 0.01 M Tris-HCl buffer pH 7.4 and 5 ml of it was loaded on a sucrose step gradient : 40% (4ml); 35% (3 ml); 31% (3 ml); 23% (3 ml); and 15% (4 ml) and centrifuged at 190,000 g for 4 h at 4° C in the same rotor and equipment as that used for the previous centrifugation. The sediment which contained the membrane, and the granule obtained in II.10 were lysed with 1% Triton X-100 and used as antigen in immunodiffusion experiments. In parellel experiments membranes from ADP-activated platelets were also prepared and used in immunodiffusion experiments.

II.11. Detection of antigenic alteration with time due to platelet activation:

Concentrated gelfiltered platelets ($3x10^{9}$ platelets per ml) were activated with 10 nM of the calcium ionophore A23187 and 90 ul of the activated platelet suspension was transfered to 10 ul of 10% Triton X-100 at 7 s, 15 s, 30 s, 60 s 120 s after stimulation and were used in the immunodiffusion experiments with 1% Triton X-100 extract of normal platelets as the control.

II.12. Inhibtion of platelet aggregation by anti-IgG:

Initial rates of platelet aggregation were measured employing the spectrophotometric method(68). 1 ml of gelfiltered calf platelets (A_{540} ~ 0.5) preincubated with anti-IgG or normal IgG (for control) were taken in each of a pair of siliconized glass cuvettes and placed in the reference and sample cuvette positions of a Shimadzu UV-VIS spectrophotometer. After temperature equilibration (32° C), the absorbance reading at 540 nm was set to zero. The agonist was stirred into the sample cuvette and the differential changes in absorbance were recorded as a function of time. The rate of aggregation for anti IgG and normal IgG treated platelets were compared at similar agonist concentrations. The agonists used included ADP, hydrogen peroxide and the calcium ionophore A23187.

RESULTS AND DISCUSSION

CHAPTER III

MODIFICATION OF THE PURIFICATION PROCEDURE OF THE PLATELET HEMOPROTEIN TO IMPROVE YIELD AND CHARECTERIZATION OF THE PROTEIN

The protein purified by the original method (67) resulted in very low yields making structural and functional charecterization of this newly reported protein difficult. Also the protein in dilute solutions was found to be highly unstable and concentration techniques like dialysis against solid Sephadex or ultrafiltration resulted in heme loss and aggregation of the protein. Therefore the purification method was modified to improve yield and stabilty of the protein. This modification and charecterization of the molecular properties of the protein constitute the subject matter of this chapter.

III.i. Modification of purification:

500 ml or more blood was collected instead of the 100 ml in the original method, so that the concentration of the protein would remain high through the purification steps. Originally the hemoprotein containing fractions from the Sephdex G-200 column and the DEAE Sephadex A-50 column were pooled and concentrated by ammonium sulphate precipitation. These steps were dispensed with in the modified method, because of the high amount of protein present in each of the fractions collected. Salt precipitation resulted in the loss of considerable amount of protein due to pH variation. In the modified method fractions from Sephadex G-200 column having A_{405} > 0.05 were loaded on the ion-exchange column, without mixing, in the order in which they emerged. Since the fractions were not pooled the peak fractions eluted from the DEAE Sephadex A-50 column also contained enough amount of protein for further studies and for raising antibodies.

The yield of the protein with respect to the total soluble protein present initially, also increased. The recovery of the Lowry`s protein in the hemoprotein was ~0.15% of the initial, in the original method . It increased to ~0.65% in the modified method.

III.ii. Antibodies to the protein:

The titre of the antiserum collected was determined by double diffusion in agar plates using a serial dilution of 1:2, 1:4, 1:8, 1:20, 1:40. The maximum titre obtained was 1:8 and so for immunodiffusion experiments reported in this thesis, undiluted antiserum was used. The homogeneity of the antigen-antibody system was established by the appearence of a single precipitin line in immunodiffusion experiments with antiserum and the purified protein (Fig.3.1).

Single precipitin line was formed when the protein concentration was 2.1 μg or 2.8 μg which is an indication of the homogeneity of the protein preparation. Previously (67) single band was obtained in the polyacrylamide gel electrophoresis of the native protein.

III.iii. N-terminal analysis: identification of L-lysine as the N-terminal residue.

The chromatogram obtained by the HPLC analysis of the dansylated protein hydrolysate showed fluorescence peaks corresponding to dansyl derivative of L-lysine only, (Fig 3.2), testifying this amino acid to be the sole amino terminal residue. This result confirmed the homogeneity of the protein preparation and lent support to the earlier conclusion that the subunits of the protein may be identical(67).

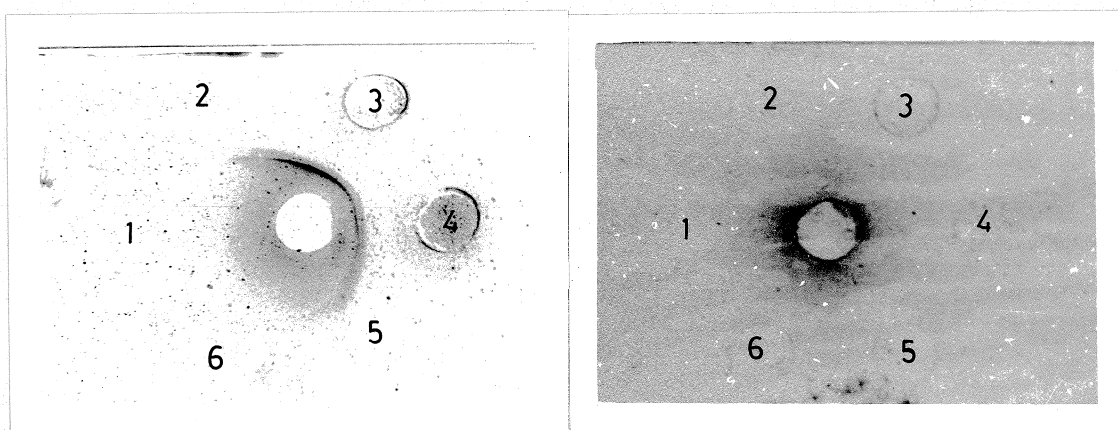


Fig.3.1. Immunodiffusion pattern to show the homogeneity of the purified protein. 0.8% agar in borate buffered saline (appendix A,vii) was used as the diffusion medium. The middle wells in A and B contained antiserum and normal serum, respectively, and the peripheral wells the purified protein. The amount (μg) of protein in wells 1-6 were, 0.7, 1.4, 2.1, 2.8, 3.5 and 4.2 respectively.

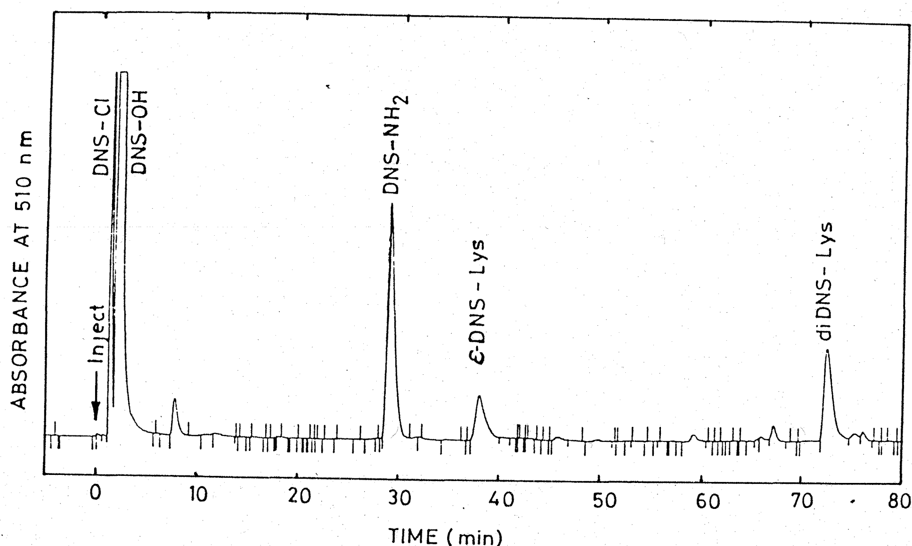


Fig.3.2. Results of HPLC analysis of dansyl amino acid residues of platelet hemoprotein. The purified hemoprotein dialysed exhaustively against distilled deionized water(200 µg in 200 µl) was dansylated, dried, dissolved in 6N HCl (200 µl) and hydrolysed for 1 h. The hydrolysate was dried, dissolved in 100 ul of distilled water, and clarified by centrifugation in Eppendorf tubes at 5000 g for 30 min. Ten µl of the resulting solution was injected and chromatography was done as described in II.4.i.

III.iv. Determination of partial aminoacid composition of the protein:

Presence of unusually high proportions of hydrophobic and charged residues were detected(Fig.3.3) .

The HPLC analysis of the dansyl derivatives of the protein hydrolysate could identify 9 amino acids. There were two unidentified peaks which may represent rare aminoacids or modified residues. Using the total area under the peaks, percentage composition of each of the nine identified residues was calculated as 26% Phe; 12% Val; 8% Ala; 5% pro; 3% Lue; 17% Lys; 2% Arg and 12% (Asp+ Glu). Out of the total identified 54% were nonpolar and 31% charged.

Two mol of sulfhydryl groups per mol of protein was estimated by the DTNB method using the denatured protein. The same number of -SH group found after dithiothreitol reduction under denaturing conditions suggested the absence of disulfides.

III.v. Molecular size, modification of size and stability of the protein by the sulfhydryl reagent N-ethylmaleimide:

The moecular weight of the protein was reported as 40,000 with 2 subunits of molecular weight 20,000 (67). However the large-zone gel-exclusion chromatographic patterns of the purified protein showed sharp ascending boundaries and diffuse trailing boundaries (Fig. 3.4, curve 1). The elution pattern and the area under the peak were modified by N-ethyl maleimide, a sulfhydryl modifying agent (Fig.3.4, curve 2). The elution positions were scattered from that of hemoglobin with an Mr.of 66,000 to that of cytochrome c with an Mr. of 13,400.

The results indicate that the purified protein is in an

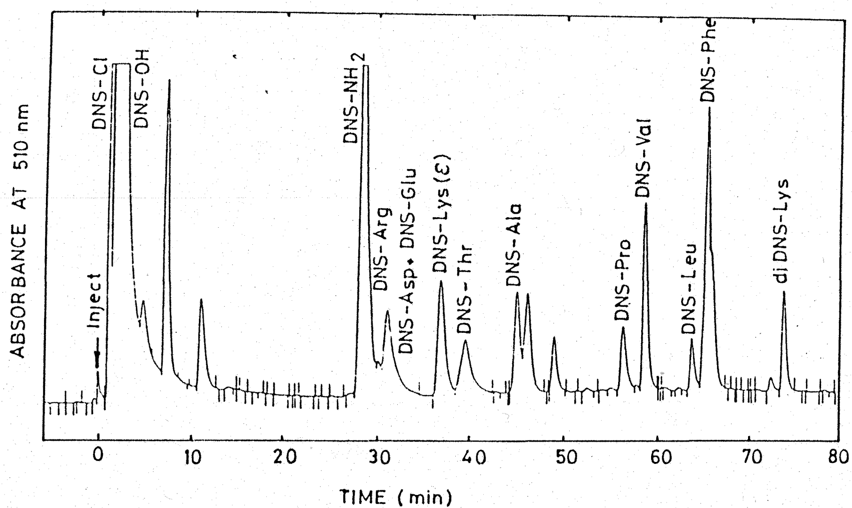


Fig.3.3. Results of the HPLC analysis of the dansyl derivatives of the protein hydrolysate. The experiment is described in II.4.2.

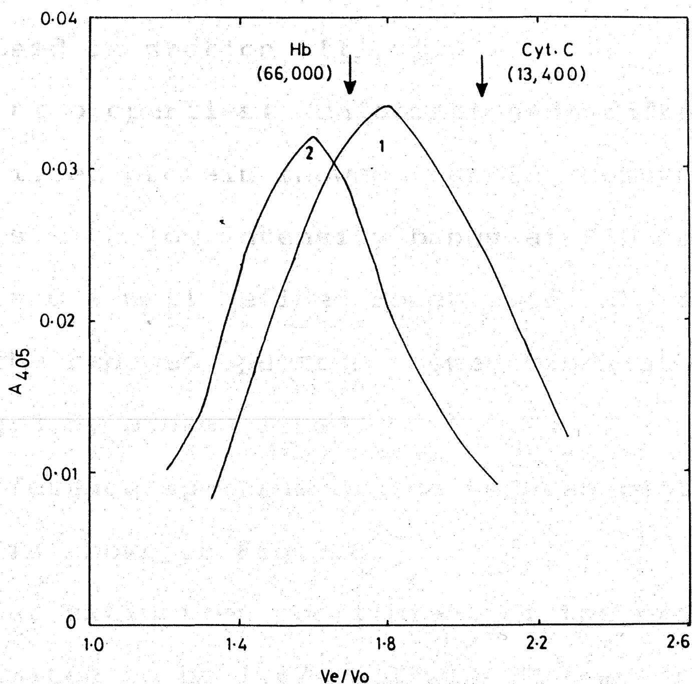


Fig.3.4. Schematic representation of the large-zone gel exclusion chromatographic pattern of the native protein. A small column (0.9x10 cm) of Sephadex G-200 was packed and equilibrated with 0.05 M phosphate buffer at pH 7.4, One ml (~1 μg) of the control protein was loaded and 0.5 ml fractions were collected. 405 nm absorbance of each fraction was plotted against Ve/V0 (Ve, elution volume of each fraction and V0, void volume) (curve, 1). Similarly Ve/V0 of NEM-treated protein after loading 1 ml, and same protein content as the control was plotted (curve 2). The elution peaks of hemoglobin and cytochrome C are marked by the arrows.

association- dissociation equilibrium. Treatment of the protein with NEM shifted its elution peak position to the left so an association of the protein may be expected. However, the NEM treatment was found to stabilize the protein conformation which will be discussed in section (III.vi.)

III.vi. Spectral properties: Conformation-dependent variations.

The purified protein showed ferric hemoprotein spectral charecteristics with low intensity bands at 630 nm, 574 nm, 536 nm and 495 nm and a well defined Soret band at 405 nm(Fig.3.5, solid line). The reduced spectrum showed bands at 620 nm, 550 nm, and 424 nm(Fig.3.5, broken line).

The difference spectrum of the reduced protein against the oxidized form is shown in Fig.3.6.

The molar extinction coefficient of the protein (E_{M}^{405}) at pH 7.4 was estimated to be 1.47$\pm$ 0.12x10^{5} $M^{-1}cm^{-1}$ (mean$\pm$ SE, n=4) based on an Mr. of 40,000 and protein determined by the method of Lowry et al.(80)

The Soret band showed reversible hypochromicity below pH 7.0 and above pH 7.8 (Fig.3.7. A and B).

Towards the alkaline side, when the pH reached 10.06, the Soret peak was shifted to the red , with a reduction in peak height . But these changes could be reversed when the pH was brought back to normal. Similarly at the acidic side, at pH 3.25, the peak was shifted to 395 nm, with hypochromicity, which was also a reversible change.

The protein had a relatively weak UV absorbance band with maximum at 276 nm and the band intensity was variable(Fig.3.8).

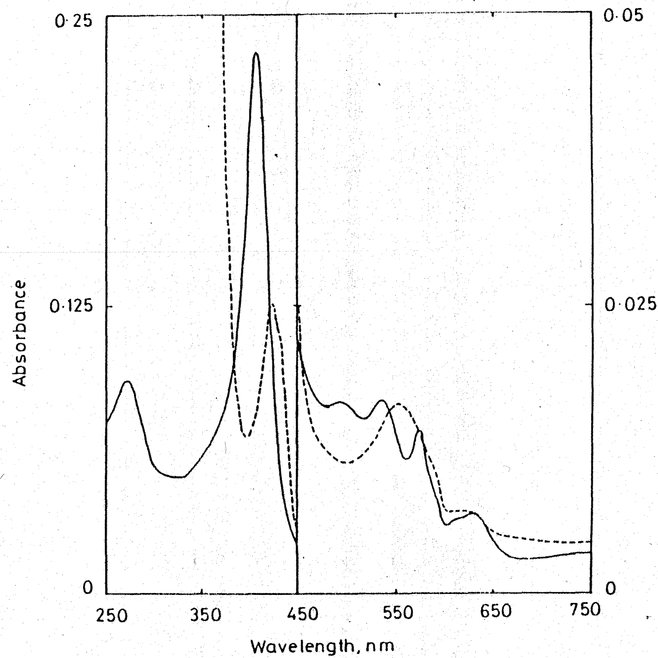


Fig.3.5. Absorption spectra of the oxidized and reduced forms of hemoprotein. Freshly purified hemoprotein in 0.05 M sodium phosphate buffer pH 7.4 (——) and the same protein treated with a few grains of $Na_2S_2O_4$ to get the reduced form (----).

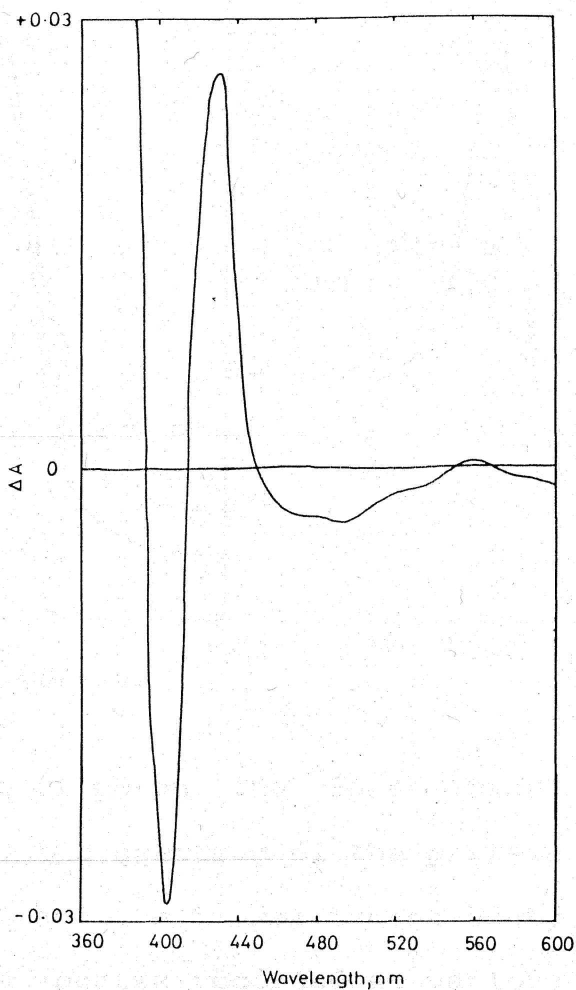


Fig.3.6. Difference spectrum of the reduced vs oxidized protein. 1 ml samples of the protein (~1.0 μg) was taken in both sample and reference cuvettes and baseline was corrected from 700-350 nm. A few grains of $Na_2S_2O_4$ were stirred into the sample cuvette and the spectrum was recorded.

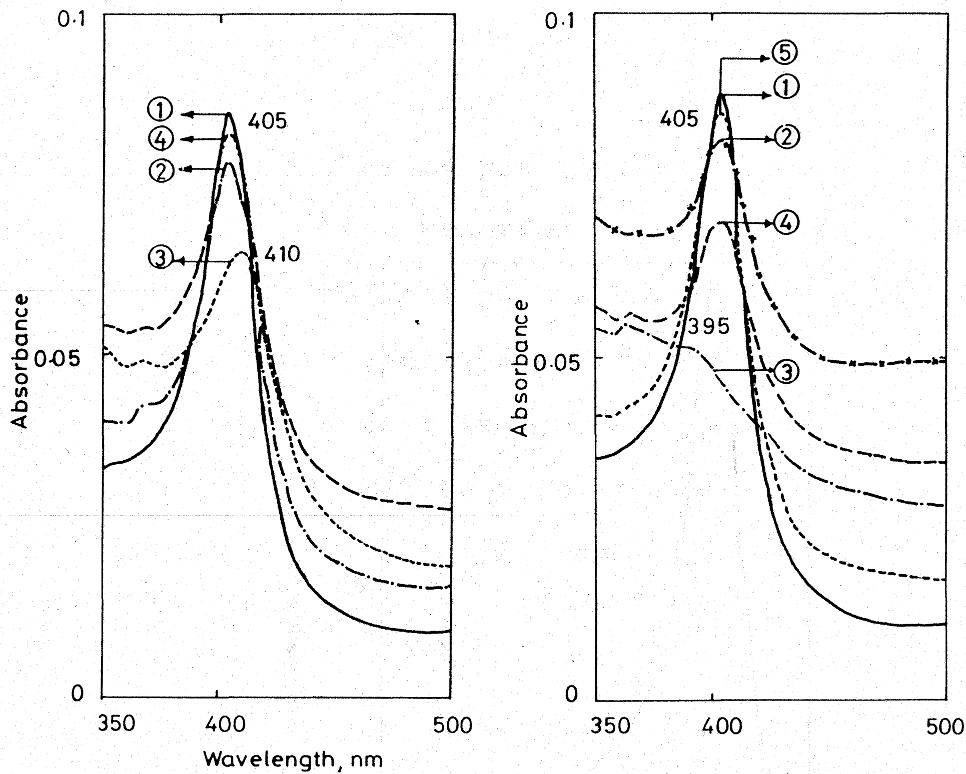


Fig.3.7. Effect of pH on the Soret band absorbance of the hemoprotein. (A) the spectrum of the protein (curve, 1) was recorded at pH 7.22 and the pH was increased by gradual additions of dil. NaOH and spectra recorded at various pH values: curve, 2 at 8.02; 3 at 10.06 and the pH was brought back to the original by adding dil. HCl and the spectrum was recorded at pH 7.2 , (curve, 4). (B) The curve,1 is recording at pH 7.22. Then the pH was reduced with dil. HCl to 6.78, curve, 2; 3.25, curve 3. Then the pH was brought back to normal and curve 4, was recorded at pH 5.78 and curve, 5, at pH 7.1.

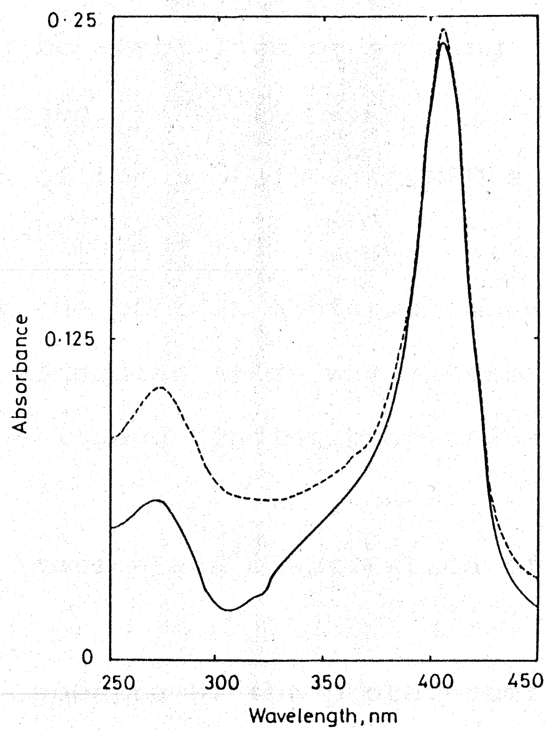


Fig.3.8. Effect of aging on the absorption spectrum of the purified platelet hemoprotein. The spectra of the protein immediately after purification (———) and after 24 h at -20` C (-----) were recorded in quartz cuvettes at 28` C.

The A_{405}: A_{280} ratios of apparently homogeneous preparations of the protein varied between 3 and 4. This ratio of a given preparation reduced markedly upon diluting it with buffer. Storing concentrated solutions at -20` C also resulted in significant changes in spectral properties which reduced $A_{405}$:$A_{280}$ ratios(Fig.8 curve 2) or otherwise increased $A_{280}$. Spectral changes could be retarded or otherwise the protein conformation could be stabilized by storing it at -20`C in an atmosphere of nitrogen.

Interaction of the protein with NEM also stabilized the UV band of the protein(Fig.3.9).

So possibly the protein conformation was stabilized by NEM treatment while molecular size was altered. The effect of NEM modification on the ligand binding properties are described in chapter IV.

III.vii. Charecterization and quantitation of the heme prosthetic group:

Pyrichrome spectra of the protein before reduction showed a band at 395 nm which upon reduction shifted to 418 nm (Fig.3.10).

The 418 nm peak of the reduced pyrichrome is charecteristic of hemin b(19). The unreduced pyrichrome spectrum had also a band at 575 nm and a shoulder at 605 nm (Fig.3.11, solid line) and this is also a charecteristic spectral pattern reported for hemin b (19). Chloroform extracts of the heme showed spectral bands at 635 nm, 534 nm and 379 nm (Fig.3.11, broken line) charecteristic of chloroprotoporphyrin IX (19).

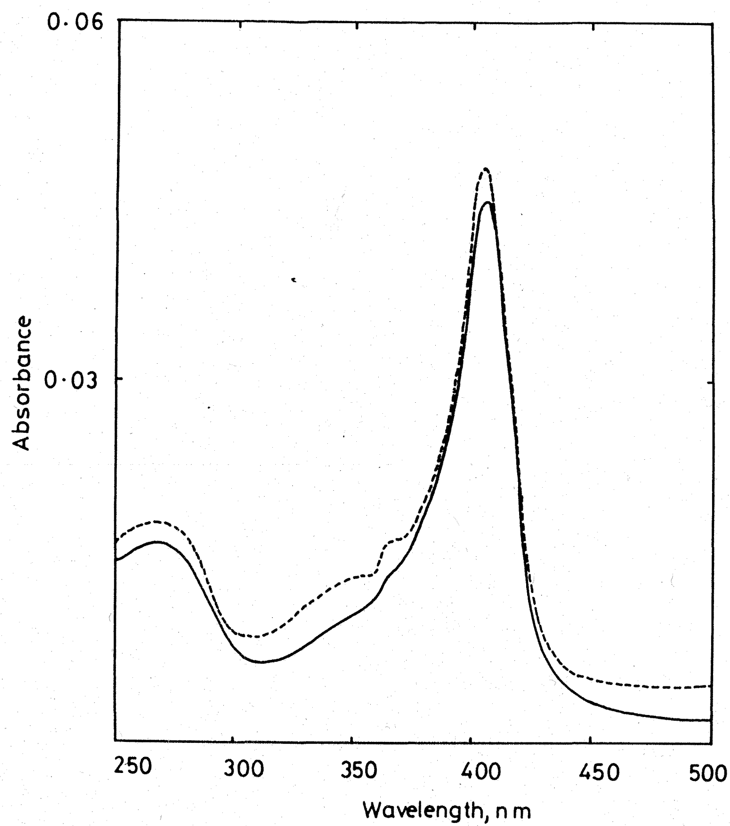


Fig.3.9. Stability of UV absorbance band after NEM modification of the hemoprotein. The spectrum of the NEM- treated protein eluted from a small column (0.9x10 cm) of Sephadex G-200 was recorded immediately (———) and after storing it for 7 days in a 4`C cold room (----).

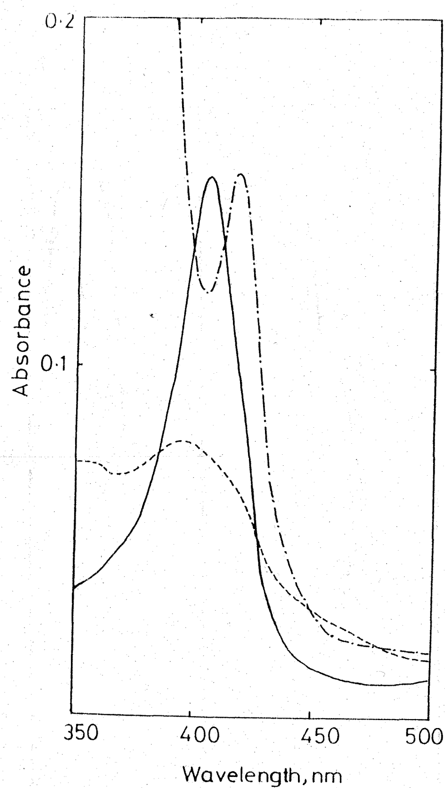


Fig.3. 10. Pyrichrome spectral pattern of the purified platelet protein. The spectrum of the protein (0.8 ml, 0.84 μM) in 0.05M sodium phosphate buffer, pH 7.4 was recorded (———). Then 2 mg of NaOH was added followed by 0.2 ml of pyridine. After recording the spectrum (------) a few grains of $Na_2S_2O_4$ were added and the reduced spectrum was recorded (-.-.-.).

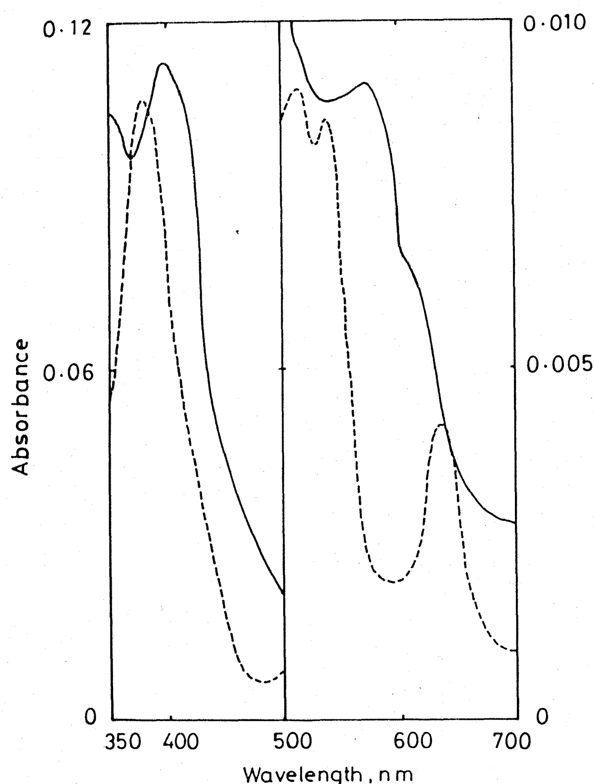


Fig.3.11. The spectrum of the oxidized pyrichrome and that of the prosthetic heme extracted into chloroform. The preparation of pyrichrome was as described in the legend for Fig.8. and the spectrum was recorded from 600-350 nm (———). The protein (1 ml, 1.26 μM) was acidified to pH 3.5 with acetic acid and vortex mixed with 1 ml chloroform. After centrifugation- aided separation of layers , the chloroform layer was drawn out using a Pastuer pipette, dried overnight with anhydrous Na_2SO_4 and the spectrum (-----) was recorded against the solvent.

Quantitation of heme from the spectral data showed the presence of two mol of heme per mol of dimeric protein.

Carbon monoxide was found to bind to the platelet hemoprotein. The binding caused hypochromicity of its Soret band and blue shift of the 574 nm band to 568 nm with a concomittant increase in peak height (Fig.3.12).

Upon reduction, the bands in the visible region merged into one broad band with maximum centered on 540 nm and its Soret band shifted to 420 nm with hypochromicity (Fig.3.12). Difference spectral patterns of CO-reduced protein vs CO-oxidized protein (Fig.3.13) showed peaks at 570 nm, 540 nm and 420 nm. The spectral properties of the protein, its hemochrome and its heme in chloroform suggested it to be a ferric hemoprotein. Although CO is regarded as having little affinity for ferric hemoproteins, CO could bind to this protein. The sixth coordination position of the protein bound heme might be Cl^- a weak ligand . The protein is found to loose its heme easily, even due to mild treatments like dialysis . So the protein - heme bonding may also be weak. Because of the trans-axial ligand effect of the protein Cl^- may be displaced by CO to bind at the sixth coordination position . Ebel et al. (29) showed that CO can interact with the ferric form of cytochrome P-450.

III.viii.Summary

Modifications of the purification procedure introduced improved the yeild of the protein. Storage conditions of the protein was standardized and antibodies were raised against the hemoprotein in rabbits. The only N-terminal amino acid identified was lysine, suggesting the homogeneity of the protein preparation

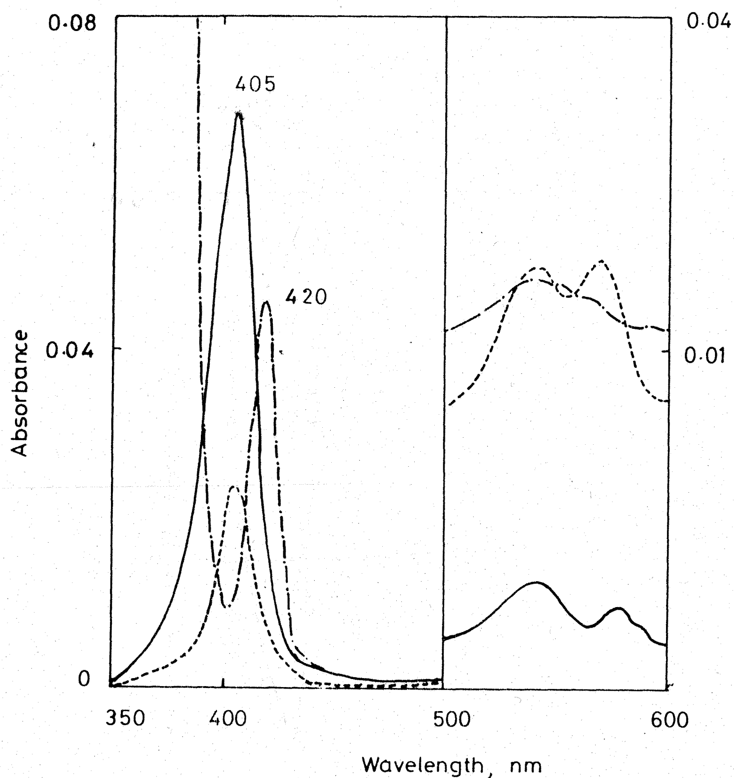


Fig.3.12. Spectral properties of the binding interaction of CO with the purified platelet hemoprotein. The spectrum of a sample of the protein was recorded from 600-350 nm(——). CO prepared by adding HCOOH from a dropping funnel into concentrated H_2SO_4 in a distilling flask, and purified by passing through successive bottles containing KOH solution and distilled water, respectively was bubbled through the protein for 4 min and its spectrum was recorded again (----). A few grains of $Na_2S_2O_4$ were then added to the protein and the reduced spectrum(-.-.-.) was recorded.

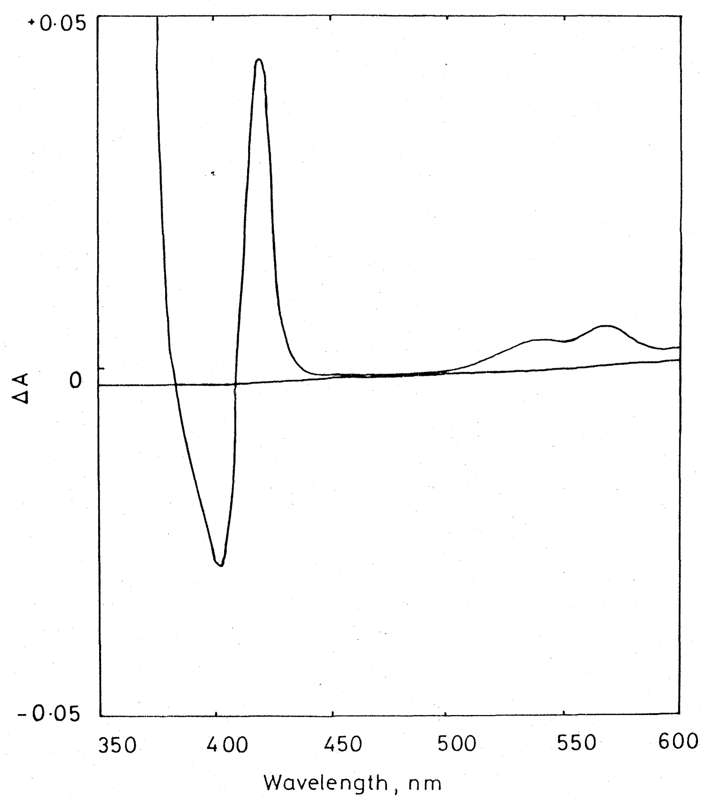


Fig.3.13. Difference spectral pattern of CO-reduced protein vs CO-oxidized protein. CO saturated protein obtained as described in the legend for Fig.9, was taken in both reference and sample cuvettes and the baseline was corrected automatically. a few grains of $Na_2S_2O_4$ were added to the sample cuvette and the spectrum was recorded.

and identity of its subunits. Two mol of cysteine residues were estimated to be present per mol of protein. The heme prosthetic group of the protein was identified as chloroprotoporphyrin IX . Two mol of heme were found per mol of protein. Carbon monoxide could bind to the protein ispite of the iron existing in the ferric state. The protein exhibited association- dissociation equilibria which could be modified by the sulfhydryl modifying agent, NEM. The absorbance ratio of the Soret band to ultraviolet region was found to vary from preparation to preparation. Dilution and storage could also reduce the $A_{405}:A_{280}$ ratios. NEM treatment of the protein could stabilze the UV absorbance.

CHAPTER IV

LIGAND BINDING TO THE PURIFIED HEMOPROTEIN AND ITS CONSEQUENCES

The reported putative receptor of prostaglandin endoperoxide (PGH_2) isolated from calf platelets, could bind arachidonic acid and the PGH_2 analogue U-46619 (67). A receptor is not defined by a single agonist, but rather by a range of chemical structures capable of inducing changes in the state of cellular receptors that interact with it (79,112). The criteria that must be satisfied to identify a receptor include:

(1) affinity, specificity and reversibility of binding and rates of binding should be consistent with those observed for the agonist and the cell.

(2) There should be a functional correlation between binding to the putative receptor and the physiological consequences of receptor activation (82).

So binding of various physiologically relevant ligands, their analogues and antagonists to the purified hemoprotein and its consequences on the protein conformation were charecterized as a prelude to a study of the role of the protein in platelet activation. The results are presented in this chapter.

IV.i. Ligand binding:

As discussed earlier (III.v.) the existence of the protein in association- dissociation equilibria, its temporal conformational changes combined with the ease with which it lost its heme prosthetic group made ligand binding studies by equilibrium dialysis out of the question. Such studies could, however, be performed by difference spectral measurements as

exemplified with PGH_2 (Fig.4.1).

The difference- spectral patterns of the PGH_2 binding showed peaks at 425 nm and troughs at 405 nm (lower inset). Double-reciprocal plots of the data were parabolic and Hill plots linear (upper inset) with h = 2 and $S_{0.5}$ =1-2 μM. Saturation was reached at a ligand : protein ratio of ~2 which is also the value of h suggesting strong positively cooperative interactions.

The binding parameters of a number of ligands determined by difference spectral measurements, employing the same protein preparation (0.7 μg) having A_{405}:A_{280} ratio of ~3.5 are presented in table 1.

A variety of molecules including n-Hexane, a hydrophobic molecule, could interact with the protein. Most of the ligands exhibited positive cooperativity (h>1). The highest affinity in terms of the $S_{0.5}$ (1-2 μM) and the lowest ligand molar excess at saturation(~2) were shown by PGH_2.

PGH_2 was followed in affinity by 5,8,11,14,17-eicosa pentaenoic acid, oleic acid and hydrogen peroxide. Oleic acid exhibited negative cooperativity (h<1) at low concentrations and positive cooperativity (h>1) at higher concentrations. The highest $S_{0.5}$ (>30 mM) was found for n-hexane, which apparently recognised the binding site of the methyl side chain of PGH_2 on the protein.

Spectral properties of the heme prosthetic group of the protein were shown to be identical to those of chloroprotoporphyrin IX (III.vii) indicating that the sixth coordination position of the protein- bound heme might be Cl^-, a weak ligand. This

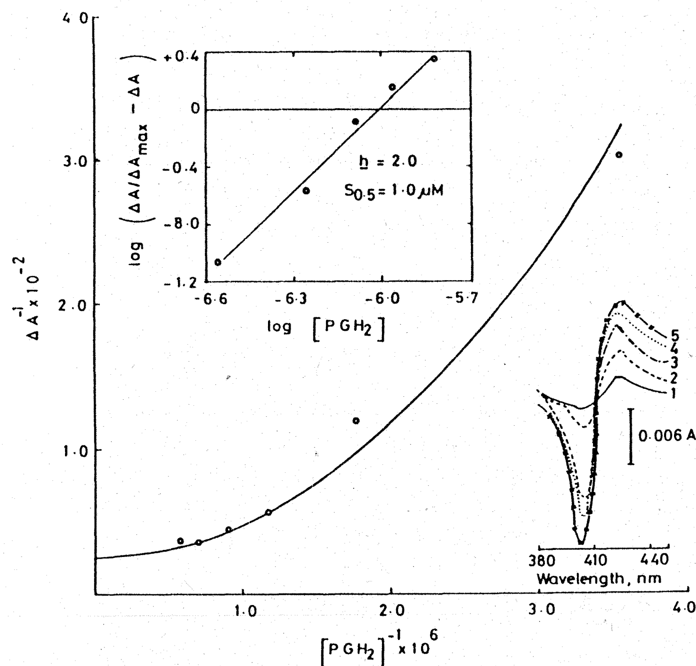


Fig.4.1. Charecteristics of the binding interaction of prostaglandin H_2 with purified platelet hemoprotein. The protein (1 ml, 0.7 μM) was used. PGH_2 (1 μl, at a time, acetone solution) was added to the sample cuvette and acetone to the reference cuvette. Stable difference spectrum obtained after each addition was recorded, at 28`C (right bottom inset). The method of ligand binding study is described in II.5. Spectral patterns shown are those obtained at ligand concentration (μ M) of 0.28 (1), 0.56 (2), 0.84 (3), 1.12 (4), and 1.4 (5). A double- reciprocal plot of the spectral data is shown in the main frame and Hill plot in the top-left inset.

TABLE 4.I

Comparison of the parameters of the binding interaction of various ligands with the purified platelet protein.

Ligand	$S_{0.5}$ (M)	Hill coefficient (h)	Molar excess of ligand required for saturation
Prosaglandin H_2	1.5×10^{-6}	2.0	2
11-eicosa monoenoic acid	132×10^{-6}	1.3 &2.8	~200
11,14-eicosa trienoic acid	22×10^{-6}	1.5	~60
8,11,14-eicosa trienoic acid	11×10^{-6}	1.9	~30
5,8,11,14-eicosatetraenoic acid	11×10^{-6}	1.7	~30
5,8,11,14,17-eicosa pentaenoic acid	5×10^{-6}	1.8	~15
Oleic acid	5×10^{-6}	0.7&1.6	~20
Linoleic acid	20×10^{-6}	1.4	~50
Linolenic acid	9×10^{-6}	1.0	~100
Palmitoleic acid	16×10^{-6}	1.8	~20
Imidazole	1.4×10^{-3}	1.4	~2×10^{4}

(contd.)

N^{-3}	5×10^{-6}	1.7	~25
CN^{-}	14×10^{-6}	1.5	~70
n-Hexane	3.5×10^{-6}	1.0	$>10^{3}$

Different batches of protein preparation (0.7 µM) having $A_{405}:A_{280}$ ratio ~3.5 were employed. The binding parameters of H_2O_2 were determined for each preparation and their agreement within 20% of that reported in table 4. 1 was chosen as a test of suitability of the preparation for comparative binding studies. The reported parameters of each of the other ligands are the average of at least two determinations with different preparations. ΔA_{max} values were determined from double- reciprocal plots when they could not be obtained directly (e.g. in the case of n-hexane). Values of $S_{0.5}$ and h were determined from Hill plots of the data.

could be easily displaced by a nucleophilic type of reaction by the peroxo group of PGH_2 and H_2O_2 or by nucleophilic centers of other molecules and ions. Heme may also be a hydrophobic site for ligand interaction. The amino acid composition analysis also showed a preponderence (III.iv) of hydrophobic residues. So hydrophobic domains may be present on the polypeptide chain, which would interact with the hydrophobic site of ligand. Thus n-hexane which cannot be expected to co-ordinate with heme iron, could interact with the protein and produce spectral patterns similar to those produced by PGH_2 and H_2O_2. Binding of many ligands like eicosanoids may also be explained as hydrophobic interaction, because they all possess a common methyl side chain, which could be a common site of their action on the protein. But depending on the nature of other variable groups present, and their stereochemistry, the mode and extent of interaction might differ. Thus although pinane thromboxane A_2 interacts with the protein, it did not produce significant spectral changes, while more spectral effects are shown by U-46619 (67). The interaction of Pinane TxA_2 with the protein was judged by the method of Hummel and Dryer(65).

Two other hemoproteins which interact with PGH_2 and thromboxane A_2 are prostaglandin endoperoxide synthetase and thromboxane synthetase. However, these three proteins show differences in affinity for each ligand. Thus $S_{0.5}$ of CN^- for prostaglandin endoperoxide synthetase is reported to be in mM range (61) while that of the hemoprotein under study was 14 μM (Table 1). The $S_{0.5}$ of imidazole for TxA_2 synthetase is 0.1 mM (28)

while that of this hemoprotein was 1.4 mM (Table 4.1).

IV.ii. Effect of protein modification on ligand binding properties:

The protein exhibited different spectral charecteristics (III.vi) and the molecule existed in association-dissociation equilibria(III.v). So it was investigated whether the conformational state of the protein affected the ligand binding properties.

a) Effect of aging and protein concentration on ligand binding properties:

Both the concentration of the protein sample used and its $A_{405}:A_{280}$ ratio affected the ligand binding parameters (Table 4.II)

Fresh protein preparations with $A_{405}:A_{280}$ ratios of 3-4 at a protein concentration of ~0.5 μM showed $S_{0.5}$ of 10± 2 (mean ± SE, n=5) and h = 1.8± 0.2 with H_2O_2. When the protein concentration was increased to 2.5 μM, the $S_{0.5}$ increased to 30 uM and h decreased to unity. These higher $S_{0.5}$ and lower h values were also found at the same protein concentration (0.5 μM), after the protein had its A_{405}: A_{280} ratio decreased to 0.66 by aging. Thus ligand binding properties of the protein changed not only with its $A_{405}:A_{280}$ ratio but also with the protein concentration at nearly the same $A_{405}:A_{280}$ ratio.

b) Effect of ligand-induced modification on arachidonate binding:

Arachidonic acid binding to the imidazole treated protein was studied (Fig.4.2).

The $S_{0.5}$ of AA binding to the control and imidazole

Table 4.II

Effect of A_{405}:A_{280} ratio and concentration of the protein on the binding parameters of H_2O_2.

protein concentration (μM)	A_{405}:A_{280} ratio	Hill coefficient (*h*)	$S_{0.5}$ (μM)
0.5	4.11	1.8±0.2	10±2
0.05	0.66	1.0	30±2
2.5	3.1	1.0	28±2

Binding studies with 0.5 μM protein having the indicated A_{405}:A_{280} ratios were done at 28° C. Protein sample with a lower ratio of A_{405}:A_{280}, was obtained after storing the protein for 24 h at -20 ° C. Average values ± s.d. of two determinations are shown.

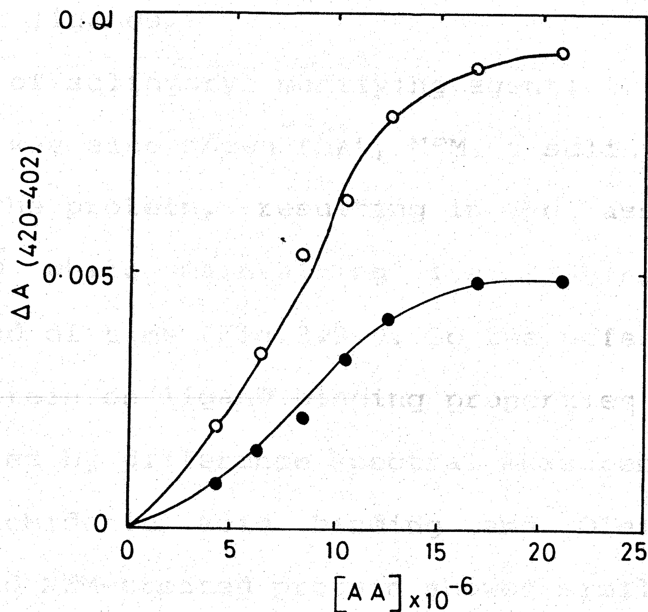


Fig.4.2. Comparison of arachidonic acid binding to the control and imidazole-treated protein samples. 0.4 μM protein was used for each study. Direct plot of $A_{420-408}$ vs concentration of arachidonic acid is given for control (o) and 2mM imidazole -treated protein (●). The binding studies were done by difference- spectral measurements as described (II.5).

treated protein was same (8.5 μM) but the maximum binding (ΔA_{max}) was reduced to half of that of the control protein.

So the modification of protein either by aging or by ligand interaction, resulted in the alteration of its interaction with other ligands.

c) Effect of sulfhydryl modifying agent:

It was also shown that, NEM, a sulfhydryl modifying agent, acted on the protein, resulting in the association of protein (Fig.3.3.) while maintaining its $A_{405}:A_{280}$ ratio stable for a long period of time (Fig.3.9.). So the effect of NEM modification of the protein on ligand binding properties of the molecule were also studied by difference spectral measurements(Table 4.III).

Arachidonic acid binding and Oleic acid binding to the control and NEM-treated protein showed similar h and $S_{0.5}$ values. But the ΔA_{max} or the maximum binding was higher when the protein was modified with NEM. For H_2O_2 binding the h and $S_{0.5}$ were higher while the maximum binding was lower with NEM-treated protein. So it is possible that different molecules recognized different binding sites and/or conformations of the protein.

While reduction in $A_{405}:A_{280}$ ratio by aging reduced the h of H_2O_2 binding, the preservation of this ratio by NEM- treatment increased h. So modification of protein conformation by different treatments was found to alter the ligand binding properties, each in a different manner.

IV.iii. Ligand- induced conformational change of the protein:

a) Gel-exclusion chromatogrphic study;

It was shown (III.v) that the protein existed in

Table 4.III

Effect of NEM treatment of the protein on the ligand binding properties.

Ligand used	Protein	ΔA_{max}	hill coefficient (h)	$S_{0.5}$ (μM)
Arachidonic acid	-NEM	0.06	1.56	12.4
	+NEM	0.065	1.56	14.6
Oleic acid	-NEM	0.052	1.84	2.0
	+NEM	0.059	1.83	1.3
Hydrogen peroxide	-NEM	0.03	2.05	8.3
	+NEM	0.025	3.66	11.5

1 ml of the protein (2.5 μM) treated with 5 mM NEM (1 μl in DMSO) and 1 μl solvent control, were passed through a column of Sephadex G 200 (1x10 cm) and 1 ml hemoprotein fractions collected were pooled and the A_{405} of the test and control were adjusted to the same (0.11A) and used .

association-dissociation equilibria detected by large-zone gel-exclusion chromatography (Fig.3.3). Interaction of the protein with various ligands were found to modify the elution pattern and area under the elution peak. The ligands used included physiologically relevant platelet agonists their analogues, and platelet antagonists. A schematic representation of the results is presented in Fig.4.3.

Out of the ligands used for this study, hydrogen peroxide and the prostglandin endoeroxide analogue U-46619 are platelet agonists and arachidonic acid which can also induce platelet aggregation independently of its metabolic conversion to PGH_2 and thromboxane A_2 also has been shown to interact with the protein and modify the gel-exclusion pattern (67). Other ligands which were able to modify the large-zone gel-exclusion chromatographic pattern were pinane thromboxane A_2, ajoene, N-ethylmaleimide and dithiothreitol. Though all these compounds are able to modify the protein, no distinction was observed between the modifying effects of agonists and antagonists.

b) **Immunodiffusion experiments:**

This was chosen as a valuable technique to detect conformational change of the protein because a relationship between protein conformation and antigenicity was recognized and it was shown that a subtle change in conformation was invariably associated with a reduction in immunogenicity and an alteration in antigenicity (31,75).

For determining the change in antigenicity of the hemoprotein, antibodies raised against purified protein was

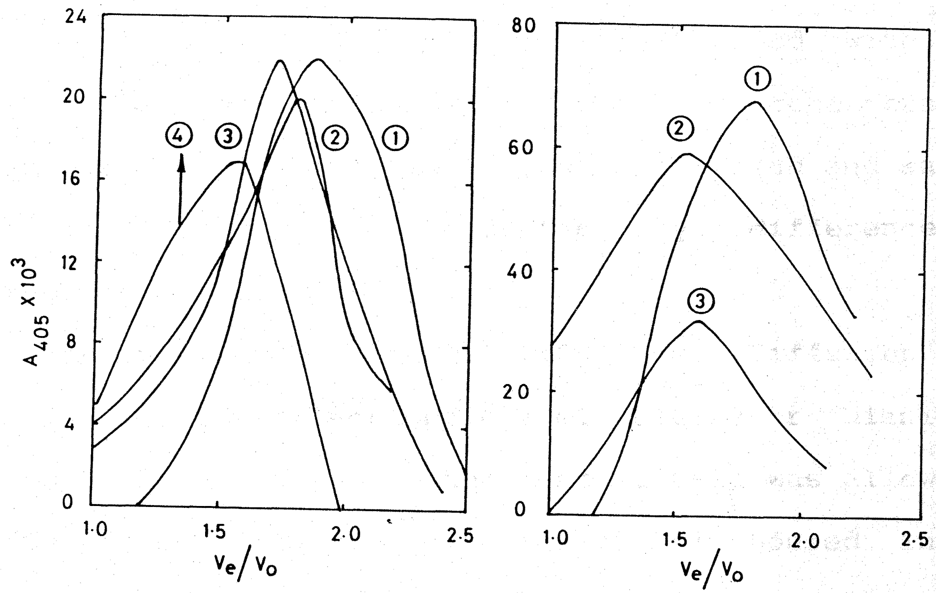


Fig.4.3. Modification of the large-zone gel-exclusion chromatographic pattern of the platelet hemoprotein by ligand interaction. The protein (1 ml) treated with reagent diluent or various ligands was applied to a column of Sephadex G-200 (1x10 cm) equilibrated with 0.05 M sodium phosphate buffer, pH 7.4, at 4` C. The column was eluted at a flow rate of 10 ml h^{-1} and fractions (0.5 ml) were collected and their absorbance values at 405 nm were determined. The A_{405} of each fraction was plotted as a function of its elution volume (Ve) relative to the elution volume of blue dextran (VO). Experimental points were omitted for the sake of clarity. The left panel represents elution patterns of the protein (0.5 µM) (1) ad the same amount of protein treated with ligands as follows: 30 µM H_2O_2 (2), 3 µM pinane thromboxane A_2 (3), and 12 µM U46619 (4). The right panel represents elution patterns of the protein (0.8 µM) (1), and the same concentration of protein treated with 144 µM ajoene (2), and 5 mM N-ethylmaleimide (3).

allowed to cross-react with protein treated with saturating concentrations of various ligands. Same concentration of the protein was used for treatment with each ligand and saturation of ligand interaction was detected by difference spectral measurements (Fig.4.4).

For the Ouchterlony's (100) immunodiffusion experiment, which can demonstrate antigenic similarities or dissimilarities, the undiluted antiserum in the central well was allowed to react with the ligand- treated and native protein poured in alternate peripheral wells . The ligands used included physiologically relevant as well as common heme ligands and the results are illustrated in (Fig.4.5).

The results showed that when arachidonate, or 5,8,11-eicosa trienoic acid, or H_2O_2 was bound, only faint precipitin lines were formed which contrasted with those formed with authentic protein in shape and position. In the case of PGH_2 only a slight shift in position of the precipitin line towards the peripheral well occured (Fig.4.5.B-2). The protein was, apparently, not saturated with PGH_2 as may be judged from Fig.4.4B because of the limitations of the volume of the solvant (acetone) that could be added to the protein and instability of the compound.

The precipitin lines formed in the presence of saturating concentrations of CN^- and imidazole seemed indistinguishable from those of the authentic protein (Fig.4.5B).

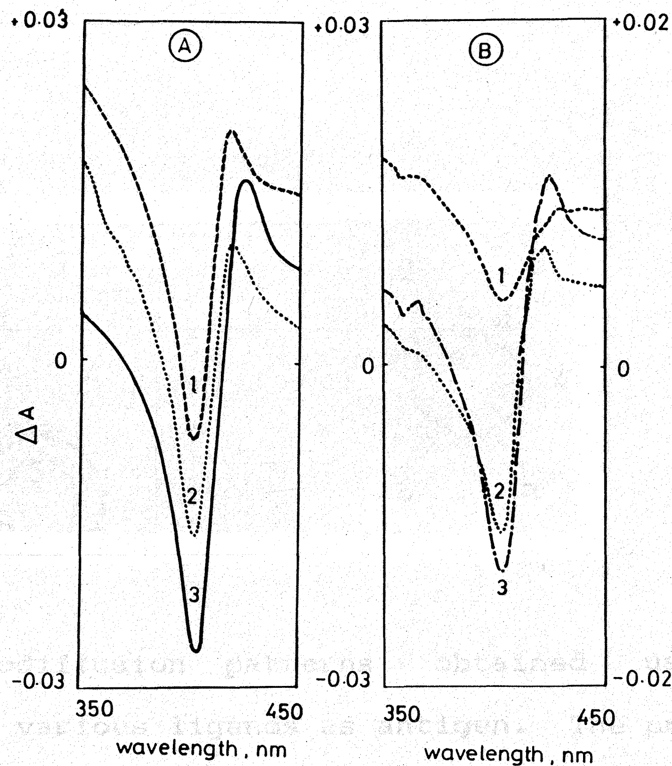


Fig.4.4. **Difference-spectral patterns obtained on saturating the calf platelet hemoprotein with various ligands.** The purified protein sample (1 ml,45 µg, A_{452}=0.088) was taken for each ligand and difference spectra was recorded two minutes after each addition and mixing, until no further spectral change was detected. The spectra shown in A are those obtained with 33.8 µM 5,8,11- eicosa trienoic acid (1), 39 µM arachidonic acid (2), added as their ethanolic solutions, and 25 µM NaCN (aqueous solution adjusted to pH 7.4 with dilute HCl) (3). Spectra in B are those obtained with 6 µl of an acetone solution of PGH_2 (~0.5 µM) (1), 8.5 mM imidazole (aqueous solution adjusted to pH 7.4) (2), and 56 µM H_2O_2 (3).

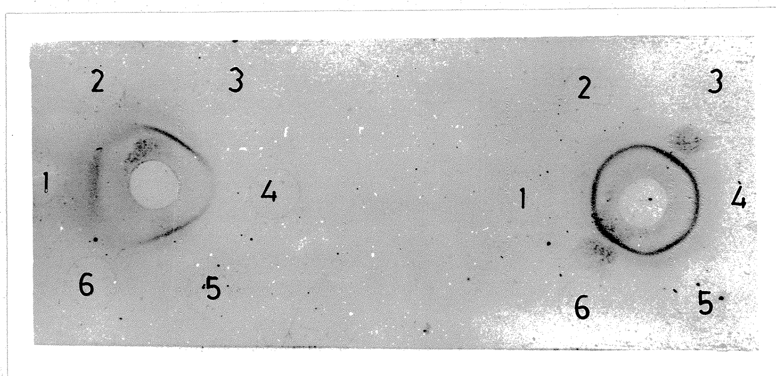


Fig.4.5. Immunodiffusion patterns obtained using protein saturated with various ligands as antigen. The protein samples (30 µl) from the sample cuvettes of experiments described in the legend for Fig.4. were taken in peripheral wells alternated with the same volume of the contents of their respective reference cuvettes. Wells 2,4 and 6 contained protein saturated with H_2O_2, arachidonic acid and 5,8,11- eicosa trienoic acid, respectively in A and PGH_2, imidazole and CN^- respectively in B. Wells 1, 3 and 5 contained 30 µl of protein from the reference cuvettes.

IV.iv. Ligand -induced alterations of charge charecteristics of the protein:

a) ion-exchange chromatographic experiments:

Charge charecteristics of the purified protein were altered to a greater extent by treating it with specific ligands. The saturation of the protein with ligands were detected as described in the legend for Fig.4.4. The recovery of the control and ligand saturated protein from the DEAE Sephadex A-50 column were compared (Table 4.IV).

The protein treated with physiologically relevant ligands had also greatly decreased $A_{405}:A_{280}$ ratios compared to the protein treated with heme ligands (Fig.4.6). The 280 nm absorbance of the ligand- treated proteins could not be reliably measured directly because the fatty acid ligands and the H_2O_2 also absorb in the UV region. So the 280 nm absorbance values of the ligand bound protein samples were compared after removing the unreacted ligands by passing through a column of Sephadex G-25. The Soret band of the gelfiltered protein returned to 405 nm showing that bound ligand was removed . No fatty acid could be recovered from the gelfiltered protein by solvant extraction and HPLC analysis. These results attested to the reversibility of the interaction.

The binding of arachidonic acid which decreased the $A_{405}:A_{280}$ ratio to 0.25 was totally retained by the ion-exchange column, which could be eluted with 0.2 M NaCl. The eluted protein had lost its heme prosthetic group completely and showed only high 280 nm absorbance and no Soret band absorbance.

Table 4.IV

Modification of the net charge charecteristics of the purified platelet hemoprotein.

Ligand employed	A_{405} after ligand treatment	% recovery of A_{405} after Sephadex G-25 gelfiltration	$A_{405}:A_{280}$ ratio	% recovery DEAE Sephadex 50 column chromatography
None	0.065	81	2.94	72
CN^- (30 μM)	0.059	77	2.14	56
Imidazole (10 mM)	0.058	74	2.86	53
H_2O_2 (30 μM)	0.042	69	0.41	41
5,8,11-eicosa tri-enoic acid (120 μM)	0.053	83	0.65	27
Arachido-nic acid (34 μM)	0.044	52	0.25	0

(contd.)

The protein(1 ml, 0.44 μM, A_{405}:A_{280} ratio 4) was treated with a given ligand (final concentration in brackets) at 30° C. After measuring its stable A_{405} value, the treated protein was gelfiltered in a column of Sephadex G-25 (1x10 cm) at 4° C to remove the unreacted ligand. Fractions (1 ml) were collected and their A_{405} and A_{280} values determined. The respective values were summed up to calculate the per cent recovery (third column) and the A_{405}:A_{280} ratio (fourth column). The fractions were then loaded on a DEAE Sephdex A-50 column (1x10 cm) in the order in which they emerged from the Sephadex G-25 column. Fractions (1ml) were collected and the total A_{405} recovered was determined to calculate the per cent recovery of A_{405} loaded (last column).

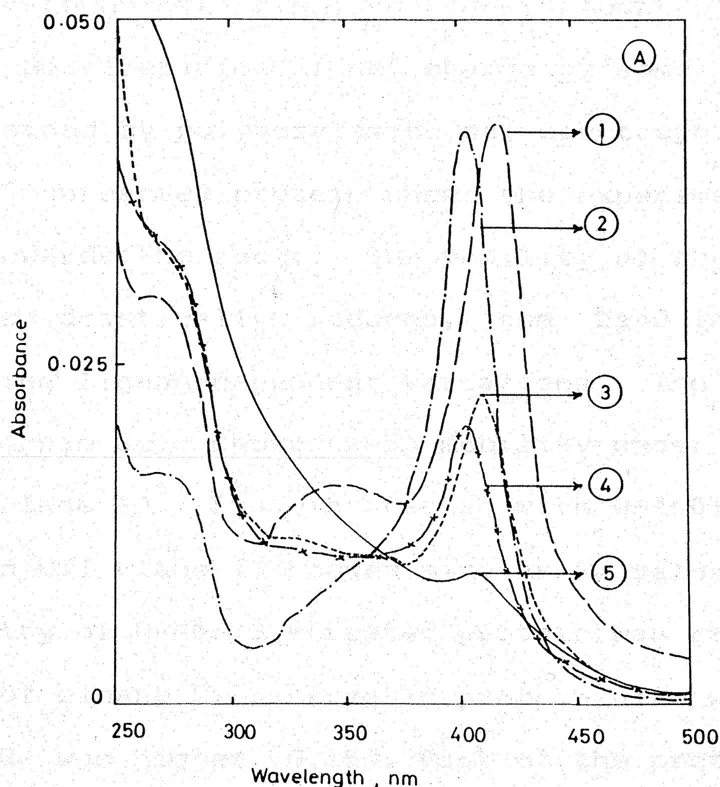


Fig.4.6. Comparison of the $A_{408}:A_{280}$ ratio of the ligand-treated, gelfiltered protein samples. 1 ml protein (45 µg, A_{408}=0.088) treated with various ligands were passed through a column of Sephadex G-25 (1x10 cm) and 1 ml fractions were collected. The spectra given are those of the peak fractions of the CN^- (30 µM)treated- (1); imidazole (10 mM) treated- (2); 5,8,11- eicosa trienoic acid (120 µM) treated (3); hydrogenperoxide (30 µM) treated- (4); and arachidonic acid (34 µM) treated-(5) samples eluted from the column.

b) Polyacrylamide gel electrophoretic analysis:

The effect of all the ligands on the net charge of the protein could not be tested by ion-exchange chromatography because this technique required higher amount of protein and was laborious. So alteration of net charge by some of the ligands were ascertained by polyacrylamide gel electrophoresis (Fig.4.7).

The unreacted protein under the experimental conditions, moved far towards the anode. The mobility of the ligand treated protein was drastically reduced, the band intensities and R_f values showing ligand-dependent variations. The protein treated with arachidonic acid showed zero mobility under the experimental conditions (lane 3). Protein treated with U-46619 (lane 2), NEM (lane 6) and DTT (lane 7) showed similar R_f values (0.11) but the band intensity of U-46619 -treated protein was reduced. The band intensity of pinane TxA_2 -treated protein also was reduced (lane 5) but the R_f was higher (0.15). Part of the protein treated with U-46619 and pinane Tx A_2 seemed to have its charge reversed. Another possibility was that the affinity of the protein for the dye was altered by these ligands.

IV.v.Summary

The purified protein could bind various ligands including unsaturated fatty acids, prostaglandin endoperoxide (PGH_2), its analogue U-46619 , pinane thromboxane A_2, common heme ligands like azide, cyanide and hydrogen peroxide, and a hydrophobic molecule like n-hexane; and all of them showed positive cooperativity. Among all the ligands studied, prostaglandin endoperoxide, PGH_2 showed maximum affinity. Modifications of the

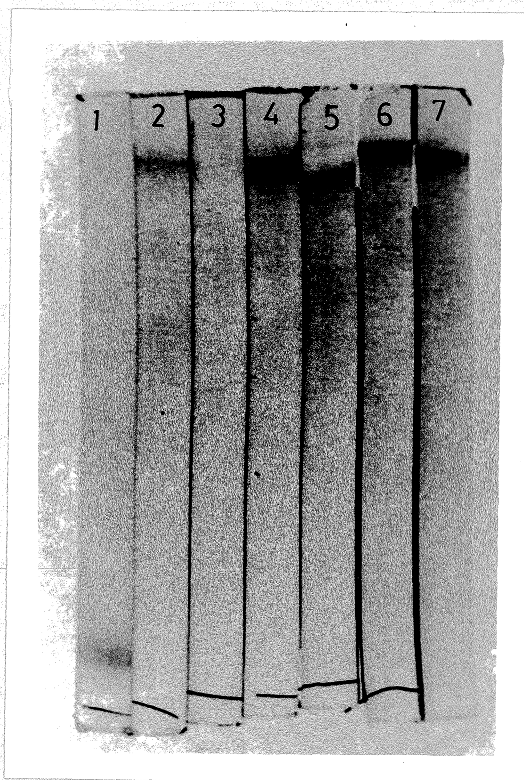


Fig.4.7. Results of polyacrylamide gel electrophoresis of native and ligand-treated proteins. Equal amount of protein (50 µg) was loaded in each tube. Protein used in lanes 1-7 were as follows : 1, control; 2, U-46619 (12.3 µM) treated-; 3, arachidonic acid (30 µM) treated-; 4, ajoene (144 µM) treated-; 5, pinane Tx A_2 (3 µM) treated-; 6, NEM (5 mM) treated-; and DTT (4 mM) treated. Experimental details are given in II.7.ii.

protein by aging , ligand interaction or by variations of protein concentration altered its ligand binding properties. The treatment of the protein with NEM, a sulfhydryl modifying agent, also affected the ligand binding properties. Ligand -induced conformational change of the protein was detected by large-zone gel- exclusion chromatography and immunodiffusion. While the latter technique could distiguish the modifying effects of physiologically relevant ligands, no distinction could be made between the modifying effects of agonists and antagonists using the large-zone gel exclusion chromatographic technuque. Ligand-induced alteration of the net charge of the protein was detected by ion-exchange chromatography where physiologically relevant ligands specifically modified the protein charge. Polyacrylamide gel electrophoresis also detected charge variations of ligand-treated protein in terms of their mobility and band intensity.

CHAPTER V

CONFORMATIONAL MODIFICATIONS OF THE HEMOPROTEIN DURING AGONIST-INDUCED ACTIVATION OF GELFILTERED CALF PLATELETS

The ultimate goal of measurement of ligand binding to receptor proteins is to provide a rational basis for understanding, the mechanism of their action. And the quantitative relationship between ligand binding and the consequent response become an important aspect of receptor identification. Ligand-receptor complex formation plays an important role in the sequence of biochemiocal events leading to biological action(3,128). Ligand interaction of the purified platelet hemoprotein resulted in the alteration of its conformation and charge chatecteristics(IV.iii&iv). Experiments were designed to test whether analogous modifications of the protein took place on cell stimulation. Experiments were also conducted to asess the accessibility and localization of the protein in platelet sub fractions.

The criteria for receptor identification are various, but a very strong evidence for receptor identity can be gained if antibodies against the purified protein selectively block a receptor system (60,103,132). So the effect of anti- IgG , raised against the purified protein, on the aggregation kinetics of various ligands were also investigated. The results of these investgations are presented in this chapter.

V.i. Spectral effects:

Binding of most of the ligands to the protein resulted in a red-shift of the Soret band from 405 nm to 410 nm or 415 nm depending on the nature of the ligand(67). In the crude extracts

or the sonicated , clarified, platelet supernatents, a batch to batch variation of the Soret band maximum was observed. This was suspected to be due to varying degrees of activation of the platelets in vivo or during handling and the consequent binding of low Mr ligands, that are released or generated in the platelet on cell stimulation, to the protein. To test this possibility, GFP, of which Soret band maximum of crude extracts had previously been determined to occur at 405 nm, were activated with ADP (10 μM) and A23187 (5 nM), as being representative of stimuli acting through cell- surface receptor-dependent and -independent mechanisms. These agents by themselves did not affect the Soret bands of the purified protein. The Soret bands of crude extracts of the activated platelets were found to be red-shifted with hypochromicity compared to those of the control platelets (Fig.5.1)

V.ii. Change in conformation, detected by immunodiffusion:

a. Activation with different ligands; Conformational change of the protein with ligand interaction was demonstrated using immunodiffusion (IV.ii.b.) experiments. Immunologicl methods were also employed to test whether similar conformational changes could be induced in platelet during activation. Thus when Triton X-100 extracts of the activated platelets were used in Ouchterlony`s immunodiffusion experiments, double precipitin lines were observed whereas only one line could be detected in extracts of control platelets (Fig.5.2).

So it appeared that when the platelets are stimulated ligands may be liberated or released at specific sites or compartments where they could interact and modify the protein.

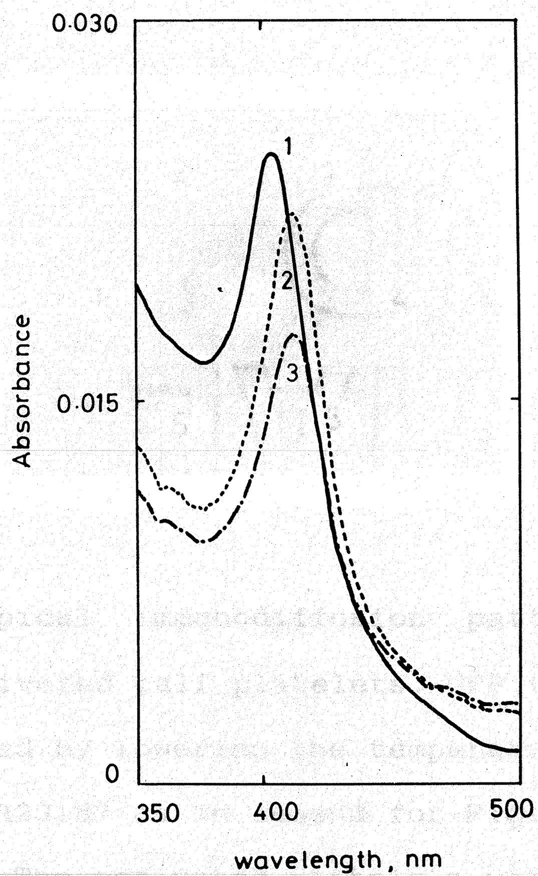


Fig.5.1. Spectral properties of crude extracts of gel-filtered calf platelets before and after activation. GFP (2 ml, 6x10^{8} platelets / ml) were activated with ADP (10 µM) or A23187 (5 nM) for 10 min at 30` C. Crude extracts of the platelets were prepared as described (II.2.4.) and their absorption spectra recorded at 25` C as in (II.4.5.). The spectra shown are those of the crude extracts of control platelets (1), ADP- activated platelets (2), and A23187- activated platelets(3).

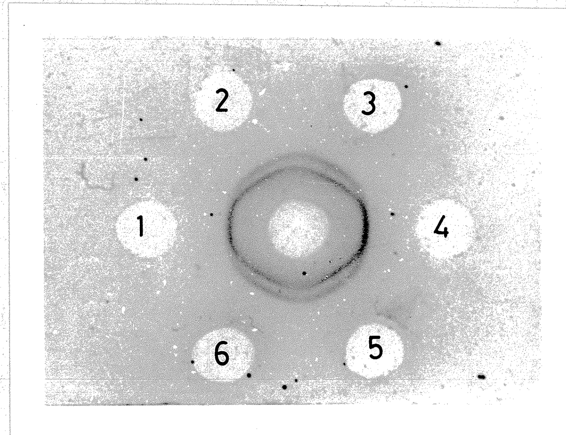


Fig.5.2. Typical immunodiffusion patterns of Triton X-100 extracts of activated calf platelets. GFP (1 ml, 6x10⁸ platelets) were activated by lowering the temperature (1 h in melting ice), with ADP and A23187 as in legend for Fig.5.1. and with 0.5 units of thrombin. The activated platelets were treated with Triton X-100 added to a final concentration of 1% (v/v) and mixed. The Triton X-100 exracts (30 µl) were then taken in the peripheral wells, 2, 3, 5 and 6, respectively. Similarly prepared Triton X-100 extracts of the control platelets and the purified protein were taken in wells 1 and 4, respectively. The undiluted antiserum was in the central well. Other details were as described in (II.6.ii).

The protein which is inaccessible for the formed molecules may remain in their native state. Thus double precipitin lines formed in immunodiffusion could correspond to : 1, native protein and 2, the modified protein.

The implied role of this protein in cold -induced activation of platelets is intriguing. Enzymatic reactions undoubtedly occur by cold activation as evidenced by the phosphorylation of myosin light chain (12,98). Arachidonate liberation could also occur since phospholipase A_2 might act at low temperatures (104). But metabolic conversion of arachidonate did not occur as we could not detect malondialdehyde formation by the thiobarbituric acid reaction(14,129). So arachidonate which might be released during cold activation could bind directly to the protein triggering the activation cascade.

b. Time-course of protein conformational change in platelets activated with A23187: Platelet activation by the calcium ionophore A23187 is known to be through arachidonate release and metabolism (73). This agonist was used to measure the effect of time on the antigenic variation of the hemoprotein, using Ouchterlony`s immunodiffusion technique. The intensity and the position of the precipitin lines were altered in the course of platelet activation by A23187 (Fig.5.3).

Conformational change of the protein ocurred at 7 s , the earliest time interval of manual sampling, after agonist addition and mixing. Spectrophotometric and microscopic examination under the conditions showed shape change of platelets but no aggregation (69,127).

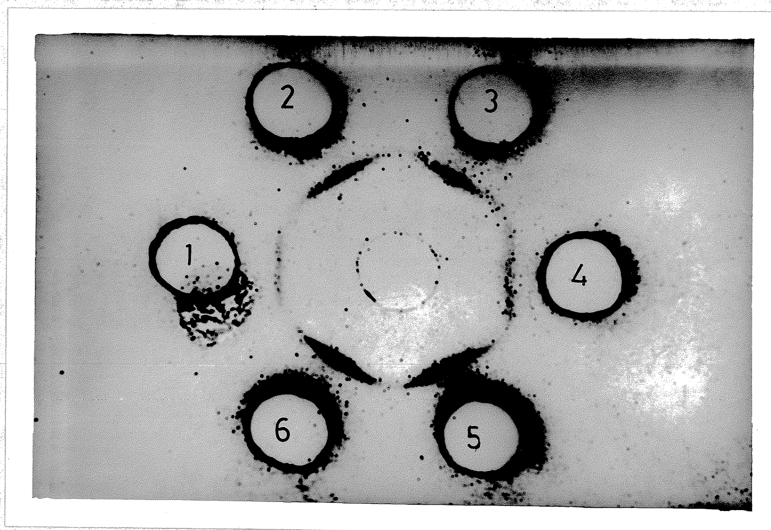


Fig.5.3. Time course variations of immunoprecipitin lines obtained with lysates of A23187 - activated platelets. GFP (1 ml, 3x10[illegible] platelets) were activated with 10 nM- A23187. 90 μl of the activated platelets were transferred into tubes containing 10 ul of 1 % Triton X-100 at 7 s, 15 s, 30 s, 60 s, and 120 s after stimulation. The lysates were poured into the immunodiffusion wells nos 2, 3, 4, 5, and 6, respectively. Well no.1 contained 1 % Triton X-100 lysate of the normal platelets as control.

V.iii. Comparison of molecular size and spectral properties of the protein from native and activated platelets:

In chapter IV.iii it was shown that ligand interaction resulted in the modification of association- dissociation equilibria of the purified protein. So it was tested, whether similar changes in the properties of the protein could occur when native platelets, whose crude extrcts showed Soret band at ~405 nm, were activated. For this study the elution patterns from the Sephadex G-200 gel-exclusion column, of the $(NH_4)_2SO_4$ fractions of the hemoprotein, from activated platelets and native platelets were compared (Fig.5.4).

Whereas the protein from unactivated platelets emerged from the column as one narrow peak (curve,1) with Ve/V0 value between 2.1 and 2.2, that from activated plaelets emerged in a broad peak having Ve/V0 values greater than 2.2 with a shoulder at Ve/V0 less than 2 (curve 2).

The spectral recording of each of the hemoprotein fraction, originated from normal and ADP- activated platelets, after the Sephadex G-200 column chromatography was analysed. The Soret band of the protein in the ascending limb of the elution peak appeared at or near 410 nm, reducing gradually as the elution progressed, reaching 405 nm before the peak was reached when the protein was from apparently unactivated platelets (Fig.5.5A).

When the protein was from platelets activated by ADP , the spectral difference between such fractions decreased in proportion to the concentration of ADP used to activate platelets (Fig.5.5 B&C). Their difference spectral patterns reported in Fig.5.5 D showed that when the fractions were from normal

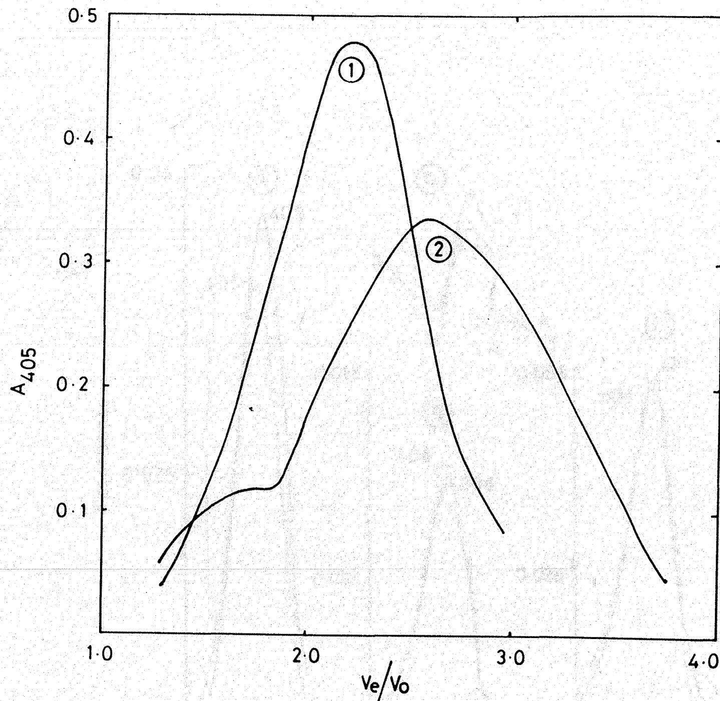


Fig.5.4. Schematic representation of the elution pattern of the hemoprotein isolated from normal and ADP-activated platelets. 100 ml each of normal and 5 μM ADP-activated platelets (GFP) were used for protein isolation. The protein precipitating between 50-90% ammonium sulphate was dissolved in 1 ml of 0.05 M phosphate buffer pH 7.4 and loaded on a column of Sephadex G200 (0.7x30 cm), equilibrated with the same buffer. 405 nm absorbance of the 1 ml fractions collected were plotted against Ve/V0 (Ve, elution volume of each fraction; V0, void volume). Curve 1 and 2 represent elution patterns of hemoprotein from normal and ADP- activated platelets respectively.

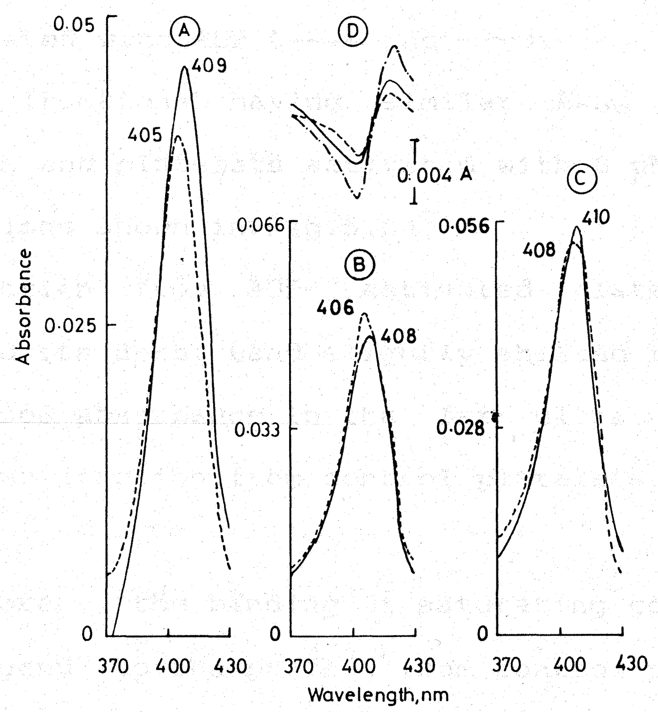


Fig.5.5. Heterogeneity of Soret band maximum of the protein after gel exclusion chromatography. The experiment was done as described in the legend for Fig.5.3. Spectra of a hemoprotein fraction from normal platelets eluted in the ascending limb (——) and that from the descending limb (---) but with similar 405 nm absobance are compared in (A). In B and C similar curves compare similar protein fractions from 2 µM and 8 µM ADP- activated platelets, respectively. D gives difference spectra of the two fractions represented in A(-.-.) ; B,(———); C, (----).

platelets the difference was large with a peak at ~425 nm and a trough at 402 nm (Fig.4.D,-.-.) . The magnitude of the spectral difference decreased and the peak and trough appeared at ~420 nm and ~405 nm, respectively. When the fractions were derived from platelets activated with ADP (---- and ——).

Protein fractions having similar A_{405} obtained from normal platelets and platelets activated with 8 μM ADP showed the spectral variations shown in(Fig.5.6).

The fraction from ADP- activated platelets (5.3 μg protein/ml) had its Soret band slightly shifted to the red, and markedly increased absorbance in the far ultra violet region, compared to the fraction from control platelets (6.7 μg protein per ml).

Furthermore , the binding of saturating concentrations of H_2O_2 as a ligand to the protein from control platelets showed greater magnitude of spectral change compared to that produced by its binding to the protein from activated platelets (Fig.5.6, inset) .There was no change in $S_{0.5}$ (12 μM) or h (1.6).

Comparison of the elution patterns of the soluble protein from apparently normal and ADP- activated platelets (Fig.5.4) showed that it had undergone both association and dissociation reactions during platelet activation (curve 2, compared to curve 1). The associated form of the protein, eluting earlier from the column, from both normal and activated platelets had Soret band at 410 nm. The dissociated form eluting later from the column, from control platelets had Soret band at 405 nm but this form of the protein from activated platelets had Soret band at >405 nm. In addition the protein from activated platelets had greater

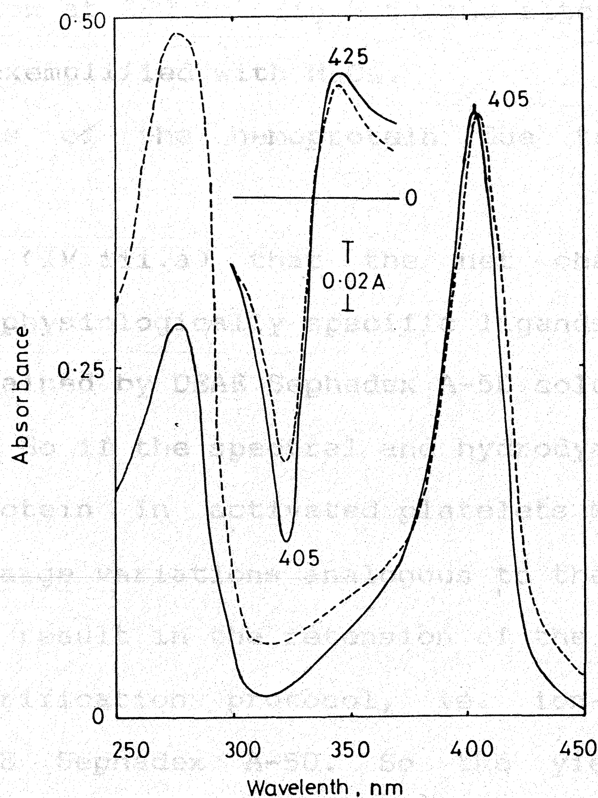


Fig.5.6. Comparison of the spectral properties of the hemoprotein from normal and ADP- activated platelets having similar elution volume and A_{405}. From the experimental system explained in the legend for Fig.5.4, one fraction each were chosen from control and test chromatograms, with similar elution position and 405 nm absorbance. Spectra of these fractions were recorded from 500-250 nm . Solid line represents the protein from normal platelets and broken line that from ADP- activated platelets. Both these fractions were then used for hydrogen peroxide binding as described (II.5.). The difference spectral patterns obtained at the saturating concentration of the ligand (82.5 µM) to the normal platelet protein(———) and ADP- activated platelet protein(----) are given in the inset.

intensity of absorption at 280 nm (Fig.5.6) and altered ligand-binding properties as exemplified with H_2O_2.

V.iv. Charge variations of the hemoprotein due to platelet activation:

It was shown (IV.iii.a) that the net charge of the protein was altered by physiologically specific ligands and the altered protein was retained by DEAE Sephadex A-50 column in 0.05 M phosphate at pH 7.4. So if the spectral and hydrodyanamic size variations of the protein in activated platelets were due to ligand interaction, charge variations analogous to that in the purified system might result in the retension of the protein at the last step of the purification protocol, ie. ion- exchange chromatography in DEAE Sephadex A-50. So the yield of the hemoprotein from ion-exchange chromatography, isolated from platelets before and after actvation with various concentrations of ADP was compared. Data reported in Table 5.1 showed that whereas ~60% of A_{405} of the previous step was recovered after gelfiltration irrespective of the starting A_{405} and ADP concentration used, the recovery of pure protein after the DEAE Sephadex A-50 column decreased sharply and in proportion to ADP concentration. Clearly, the protein assumed , progressively stronger anionic charecter after activation of platelets with increasing concentrations of ADP.

The protein from the activated platelets showed greater retension in the DEAE Sephadex A-50 column in proportion to the dose of ADP used to stimulate the cell.

The foregoing results, thus, showed that the protein from activated platelets had its spectral properties, charge and

Table 5.1

Loss of hemoprotein from activated platelet(absorbance at 405 nm, A_{405}) at the DEAE Sephadex A-50 column chromatographic step.

Source of protein	Total A_{405} after $(NH_4)_2SO_4$ precipitation	% A_{405} recovery after chromatography in	
		Sephadex G200	DEAE Sephadex A-50
Normal platelets	1.52	60	38
Pltelets activated			
with 2 μM ADP	2.99	58	14
5 μM ADP	1.05	61	11
8 μM ADP	0.906	59	6

Different concentrations of ADP, were employed to activate platelets prior to isolation of protein. Experimental protocol is described in (II.2.). The A_{405} of the chromatographic fractions was summed up to calculate the per cent recovery.

hydrodynamic size charecteristics as well as ligand binding properties altered.

V.v.Localization of the protein:

Immunofluorescence experiments showed that anti-IgG but not preimmune-IgG reacted with intact platelets (Fig.5.7).

Furthermore, anti-IgG formed precipitin lines with Triton X-100 extracts of isolated membranes of both control and activated platelets(Fig.5.8).

Though the hemoprotein under study was purified from the soluble fraction of platelets, its anti- IgG formed precipitin lines with Triton extracts of membrane and granule fractions (Fig.5.8) and fluorescent anti-IgG interacted with intact platelets (Fig.5.7). Thus the protein was present both in the membrane and in the soluble fractions. The membrane protein also changed conformation during platelet activation(Fig.5.8) and so the membrane protein was both accessible and functional.

V.vi. Effect of anti- IgG on the kinetics of agonist-induced aggregation:

Rates of agonist-induced aggregation of GFP were found to be modified by anti- IgG compared to preimmune-IgG. In the case of A23187, maximum rate R, was reduced without affecting $S_{0.5}$ or h (Fig.5.9).

The effect of anti-IgG on the ADP- and H_2O_2- induced aggregation kinetics were also investigated (Table 5.II).

With ADP as the agonist 20-40 % inhibition of rates was found at low ADP concentrations (2 μM) but the inhibition was overcome by increasing the ADP concentration.

With H_2O_2 as the agonist, inhibition occured at lower

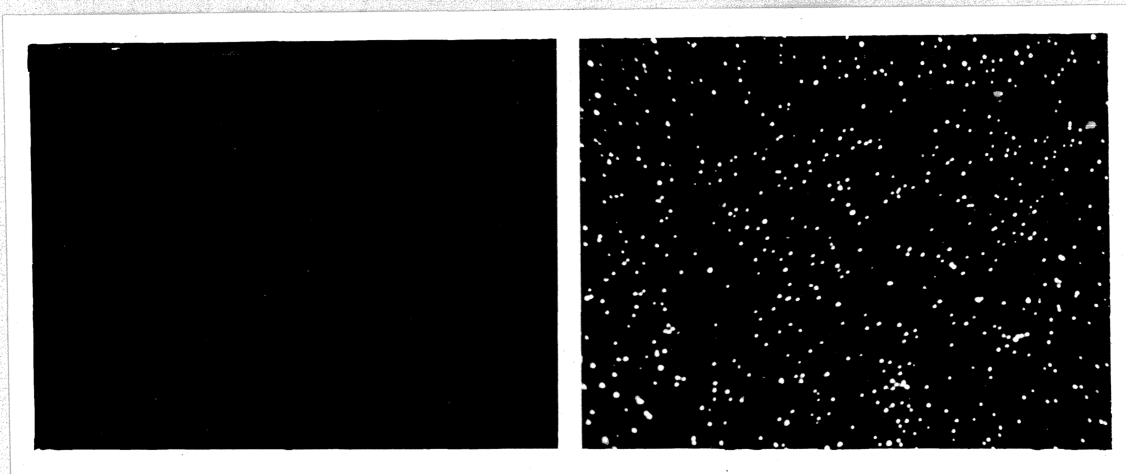


Fig.5.7. Results of immunofluorescence experiments employing gelfiltered whole platelets. FITC- conjugated preimmune-IgG was employed to label the platelets in the left hand-side frame and FITC-conjugated anti-IgG was employed in the right handside frame . Experimental details are given in II.9.

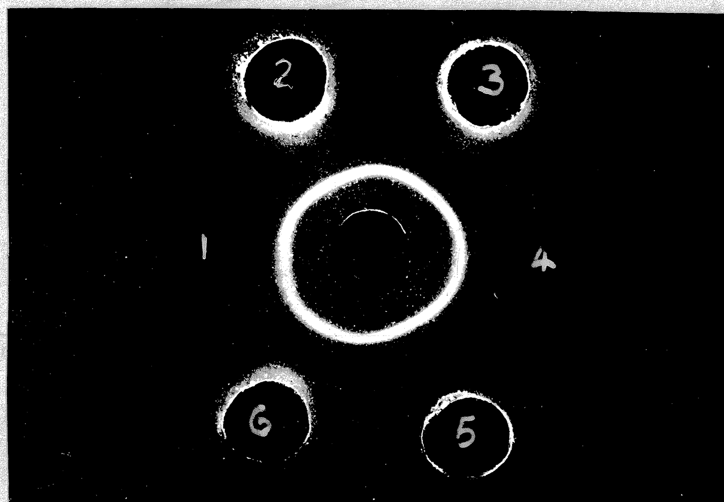


Fig.5.8. Results of the immunodiffusion experiments with subcellular fractions of platelets, as antigen. Undiluted antiserum was poured in the central well. Peripheral wells, 1 and 4 contained Triton X-100 lysates of normal platelets as control. Wells 2, 3, 5 and 6 contained, normal membrane lysate, ADP- activated membrane lysate, normal granule lysate, and ADP- activated granule lysate, respectively. Experimental details were as described in II.10.

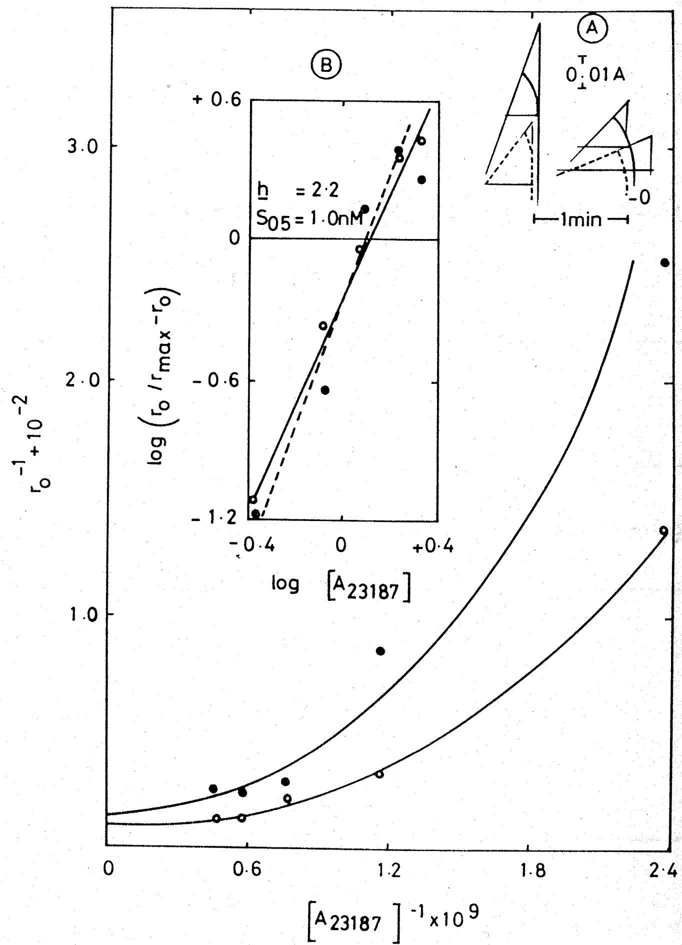


Fig.5.9. Effect of anti-IgG on the kinetics of A23187-induced aggregation of GFP. Isolated platelets (~0.6 A at 540 nm), preincubated with 62.4 µg of anti-IgG or an equal amount of normal IgG per ml of GFP was used. A typical pen-response pattern obtained with 0.42 nM (right) and 2.11 nM (left) are given at the right hand-side inset. The solid line represents the normal IgG treated- and broken line the anti-IgG- treated platelet aggregation. In the double reciprocal plot of rate of aggregation vs agonist concentration and Hill plot (inset), the open circles represents the normal IgG- treated, and closed circles represents the anti- IgG - treated platelet aggregation kinetics. Other experimental details were described in II.12.

Table 5.II

Effect of anti- IgG on rates of H_2O_2- and ADP- induced aggregation of gelfiltered calf platelets.

Agonist (M)		rate of aggregation		percentage inhibition
		with normal IgG	with anti-IgG	
ADP	1.08×10^{-6}	0.0446	0.035	21
	2.16×10^{-6}	0.056	0.043	23
	10.8×10^{-6}	0.086	0.082	5
H_2O_2	5.5×10^{-3}	0.026	0.018	30
	7.34×10^{-3}	0.036	0.026	28
	9.17×10^{-3}	0.038	0.074	-95

The GFP in the sample and reference cuvettes were incubated for 1 min with preimmune IgG or anti-IgG.

agonist concentrations but activation at higher agonist concentrations.

Thus the effect of anti- IgG showed agonist-specific variations. Depending on the dose of agonist used, the influence of anti- IgG on the aggregation rate also varied. Rates of aggregation induced by A23187 were inhibited at all agonist concentrations without changing $S_{0.5}$ or h (Fig.5.9). Rates of ADP- induced aggregation were inhibited only at lower agonist concentrations and rate accelaration at higher agonist concentrations. Since anti- IgG cannot be expected to penetrate the cell membrane, its effect must be exerted on the protein at the cell membrane which was found to be accessible in intact platelets. Different agonists are known to operate different pathways, releasing components from different granules. The released components may act at different compartments and the protein is shown to be present in granules, unavailable for anti- IgG. However, the response elicited by various agonists may ultimately operate through a common effector pathway leading to aggregation. These points may explain the diversity of anti-IgG effect with different agonists.

V.vii.Summmary

Analogous to the modifications observed in the purified platelet hemoprotein with ligand interaction, alterations of the spectral, size and charge charecteristics of the protein were detected in whole platelets due to its activation, using similar techniques. The protein was found to be accessible for exogeneous ligands. Though the protein was purified from the soluble fraction, it was also detected in the granule and plasma membrane

fractions by immunodiffusion. The membrane protein also changed its antigenicity on ADP activation of the cell. Anti- IgG raised against the protein influenced the aggregation kinetics of platelets stimulated by various agonists, like the calcium ionophore A23187, ADP and hydrogen peroxide.

CONCLUSIONS

CHAPTER VI

CONCLUSIONS AND PROJECTIONS FOR THE FUTURE

Experiments for this dissertation were designed with the aim of delineating the role of a newly reported hemoprotein in platelet function and to define the biochemical mechanism of its action.

Minor modifications of the original protein purification procedure reported by us (67) ennabled me to get stable and homogeneous preparations of the protein in adequate yield for the purposes.

Antibodies were raised against the purified protein in rabbits. The antibodies proved to be powerful tools to obtain several major results of this thesis.

Charecterization of the prosthetic group of the hemoprotein was given prime importance. Spectral properties of its pyrichrome prepared in alkaline pyridine and those of the heme extracted into chloroform suggested it to be chloroprotoporphyrin IX or hemin chloride. Other than forming coordination compounds with suitable ligands, the heme could also be a hydrophobic site for ligand interaction. Inspite of the iron existing in the ferric state, carbon monoxide, which is a weak ligand of ferric heme, was found to interact readily with this protein. This is an interesting observation.

The detection of L- lysine as the sole N-terminal residue supported two of the earlier findings (67) : 1) the protein preparation was homogeneous as suggested employing PAGE; and 2) the subunits of the protein may be identical as inferred from subunit molecular weight determination employing SDS-PAGE.

Amino acid composition analysis as their dansyl derivatives could identify 2 mol cysteine per mol of protein. Out of the total identified aminoacids, 54% were nonpolar and 31 % were charged. One half of the nonpolar residues was phenylalanine.

The spectral properties of the protein also supported ferric hemoprotein nature with a well defined Soret band at 405 nm. Storage of the protein under atmospheric oxygen resulted in a sharp increase of its UV absorbance, which is otherwise a low intensity band in fresh preparations. The detection of cysteine residues raised the possibility of -SH group oxidation, to increase UV absorbance. Both storage of the protein under N_2 atmosphere at -20°C and treatment with NEM, a sulfhydryl modifying agent could stabilize the UV absorbance.

Interaction of this protein with common heme ligands resulted in a red-shift of its Soret band . This charecteristic property enabled me to study the binding of a variety of ligands including platelet agonists their analogues and antagonists gently and rapidly without using time-consuming conventional methods employing radioactive isotopes, or equilibrium dialysis. These methods are also unsuitable for applying to unstable proteins. Out of the ligands tested prostaglandin endoperoxide (PGH_2) showed maximum affinity and lower molar excess of ligand at saturating concentration of the ligand.

Binding of many of the physiologically relevant ligands increased the UV absorbance, even after removing the ligand by gelfiltration. This effect may be due to 1) altered stereochemistry of the heme; 2) exposure of phenylalanine residue as a result of the conformational alteration.

Irrespective of whether it is due to storage or ligand interaction, the modified protein showed altered ligand binding properties. However, the -SH group may not be directly involved in the ligand binding, because even after modifying the cystiene residues with NEM, the $S_{0.5}$ of the ligand interaction was comparable to that of the control protein. The Hill coefficient or maximum binding was altered. So the -SH group may play an important role to maintain the conformation of the protein for ligand interaction and in modulating ligand- induced conformational changes.

The possible conformational change evidenced from the spectral variations of the ligand-bound protein was confirmed employing immunodiffusion and large-zone gel-exclusion chromatographic techniques. Immunodiffusion was found to be more informative because it could distinguish the effects of common heme ligands and physiologically rlevant ligands.

Large-zone gel exclusion chromatography detected conformational changes(changes in hydrodynamic size) even when no considerable spectral changes were observed, e.g. pinane thromboxane A_2. Since the protein was found to exist in association- dissociation equilibria, we expected that possibly agonists may convert it to one form, for e.g. associated form while the antagonists may modify it to the dissociated form. But no such distinction could be detected using large-zone gel-exclusion chromatography.

Charge variations of the protein induced by ligands were detected using two techniques: ion-exchange chromatography and PAGE. In the ion- exchange chromatography, specific charge

variations induced by physiologically relevant ligands were distinguished from those induced by common heme ligands. Poly acrylamide gel electrophoresis also showed decrease in the mobility of ligand-bound protein samples compared to the native protein. The only ligand which was used in both ion-exchange chromatography and poly acrylamide gel electrophoresis was arachidonic acid and the results obtained with both techniques were comparable. Arachidonic acid treated protein was completely retained by the DEAE Sephadex A-50, while similarly treated protein showed zero mobility in PAGE.

Analogous variations in the spectral, antigenic and charge properties of the protein were detected in whole platelets due to activation. The Soret bands appearing at ~410 nm in crude extracts of agonist activated platelets, compared to the appearence of the band at 405 nm in apparently unactivated platelets may indicate interaction of the protein with ligands liberated in platelets upon activation.

Evidence for the conformational change include observations like altered antigenic property and gelfiltration chromatographic patters of the hemoprotein from activated platelets.

Charge variations of the protein due to activation was evidenced by the retension of a considerable amount of the hemoprotein by the DEAE Sephadex A-50 column. The modification of the protein isolated from ADP- activated platelets detected by its increased UV absorbance, compared to that isolated from unactivated platelets was reflected in its altered ligand binding properties. Thus a parallelism was found between the behavior of

the purified protein during ligand binding and its behavior in intact platelets during platelet activation. This established an important role for this hemoprotein in platelet activation.

Before employing anti-IgG to detect the active participation of this protein in platelet aggregation and kinetics, the accessibility of the protein for the membrane impenetrable anti-IgG was assessed by labelling platelets with FITC-conjugated anti-IgG. The resultant fluorescing intact platelets observed under the fluorescence microscope established its accessibility to other exogeneous ligands on intact platelets.

When anti-IgG was found to block the response induced by different agonists variously , the presence of the protein at sites inaccessible to anti- IgG were looked for. Employing immunodiffusion, the protein was localized in platelet granules and integral membranes. This subcellular protein was also affected by ADP activation. Thus a definite role for this hemoprotein is established.

Using immunodiffusion technique it was shown that the antigenicity of the protein was altered even at 7 s after stimulating platelets with calcium ionophore, A23187. This non-physiological agonist is known to function through arachidonate liberation and metabolism. Seven seconds was too early a time to detect aggregation induced by any ligand(69,127). So the conformational change may precede the signal transmittance rather than occur subsequent to response. So the ligand- induced hydrodynamic size and charge alteration of the protein, is proposed as a biochemical mechanism of signal transduction in

platelets. While Rink (113) speculated that ligand-induced conformational change in the cytoplasmic part of a receptor molecule could be an as-yet unidentified transduction pathway, we presented experimental evidence to prove that such a mechanism exists. Further it was established that it was a hemoprotein that underwent ligand-induced conformational changes.

Since PGH_2 showed maximum affinity for this protein, our earlier notion (67) that the protein may be a putative receptor for prostglandin endoperoxide is further strengthened.

PROJECTIONS FOR FUTURE RESEARCH

The topology of the ligand-binding sites on the protein and the amino acid residues involved in the recognition and discrimination of various ligands remain undetermined. The mechanism by which protein conformational change induced platelet shape-change and aggregation is also obscure. Calcium is an important modulator of platelet activation. Does it modulate conformational changes of the protein induced by other ligands? These are some of the problems for future research. Solutions of these problems by appropriately designed experiments should throw much light on the larger problem of mechanisms and modulations of platelet activation and pave the way for designing more rational platelet supressive therapies.

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APPENDIX A

i) Acid Citrate Dextrose (ACD):

Trisodium citrate.2 H_2O	=2.2 g
Citric Acid	=0.8 g
Dextrose	=2.5 g

Made up to 100 ml with distilled water (pH = 5-5.1)

ii) Tris- HCl buffer:

Dextrose	=1.0 g
Calcium Chloride	=5.5 mg
Magnesium Chloride	=0.199 g
Potassium Chloride	=0.402 g
Sodium Chloride	=8.12 g
Trizma base	=1.756 g

Dissolved in 900 ml distilled water, the pH was adjusted to 7.4 with dil HCl and made up to 1 l with distilled water

iii) Phosphate buffer:

Disodium hydrogen phosphate.2H_2O	=7.14 g
Sodium dihydrogen phosphate.2H_2O	=1.56 g

Made up to 1 l with distilled water

iv) Gradient programme for HPLC:

Solvant A : 0.6% acetic acid-0.008% Triethylamine made in distilled water

Solvant B : Methanol, the percentage of which was varied as given below

Time	%B	Time	%B
0	30	40	52
1	32	45	56
3	34	50	60
5	36	55	64
10	39	60	67
20	42	70	75
30	45	90	75
35	48	100	80

v) DTNB reagent:

Urea = 4.8 g

Trizma base = 0.0605g

DTNB = 0.004 g

Made in 10 ml distilled water

vi) Borate Buffered Saline:

Boric acid	= 6.184 g
Sodium tetra borate.10H_2O	= 9.536 g
Sodium Chloride	= 4.384 g

Made up to 1 l in distilled water and the pH was adjusted to 7.6

vii) Reagents for polyacrylamide gel electophoresis:

0.5 M phosphate buffer pH 7.2 (stock)

$Na_2HPO_4.2H_2O$	=13.54 g
$NaH_2PO_4.2H_2O$	=7.83 g

Made up to 250 ml with distilled water

Gel formula:

Acrylamide- bis acrylamide (30:0.8)	=10 ml
0.5 M phosphate buffer	=6 ml
1.5% ammonium persulphate	=1.5 ml
Distilled water	=12.2 ml
TEMED	=0.015 ml

Mixed well and poured into glass (4 x70 mm) tubes

Running buffer: 1:5 diluted stock buffer

Stain:

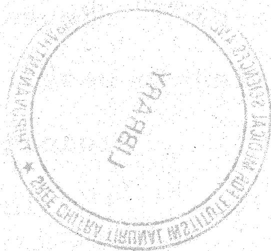
Coomassie brilliant blue R-250 =1 g

dissolved in methanol, 45 ml; glacial acetic acid,10 ml;

and distilled water,45 ml and filtered.

Destain: Acetic acid, 70 ml; methanol, 200 ml; and

water 730 ml.



APPENDIX B

Abbreviations used are:

ACD	- Acid citrate dextrose
ADP	- Adenosine di phosphate
cAMP	- Adenosine 3`:5`-cyclic monophosphate
AA	- Arachidonic acid
Dansyl	- 1-dimethyl amino naphthalene-5-sulfonyl
DEAE	- Diethyl amino ethyl
DTNB	- 5,5` dithio bis-(2-nitro benzoic acid
DTT	- Dithio threitol
F(n)	- Blood coagulation factor
FITC	- Fluorescein isothio cyanate
GFP	- Gelfiltered platelets
GP	- Glycoprotein
cGMP	- Guanosine 3`:5`-cyclic monophosphate
h	- Hill coefficient
HPLC	- High performance liquid chromatography
IgG	- Immunoglobulin G
IP_3	- Inositol 1,4,5 -triphosphate
mA	- milli amperes
NEM	- N- ethyl maleimide
PAF	- Platelet activating factor
PAGE	- Poly acrylamide gel electrophoresis
PG	- Prostaglandin
PIP_2	- Inositol 4,5- biphosphate
PLA_2	- Phospholipase A_2
PRP	- Platelet rich plasma

$S_{0.5}$	- half maximal saturation concentration
TEMED	- N,N,N`,N`- Tetra methyl-ethylene diamine
Tris	- Tris (hydroxy methyl) amino methane
Tx	- Thromboxane
Ve	- Elution volume
VO	- Void volume