Phosphorylation/Dephosphorylation and the Regulation of Fibrinogen and Complement Factor C3

Minireview based on the doctoral theses of

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INTRODUCTION

Eucaryotic cells contain a large amount of protein-bound phosphate[1]. The proteins are generally synthesised first and phosphorylated later by protein kinases. Dephosphorylation by phosphoprotein phosphatases removes the covalently-bound phosphate. The biological activity of many proteins has been shown to be affected by such reversible phosphorylation and regulatory phosphorylation has come to be considered as one of the main principles of metabolic regulation.

Regulatory Phosphorylation

The first enzyme which was reported to be regulated by phosphorylation was glycogen phosphorylase in 1955[2]. Since then research has revealed that
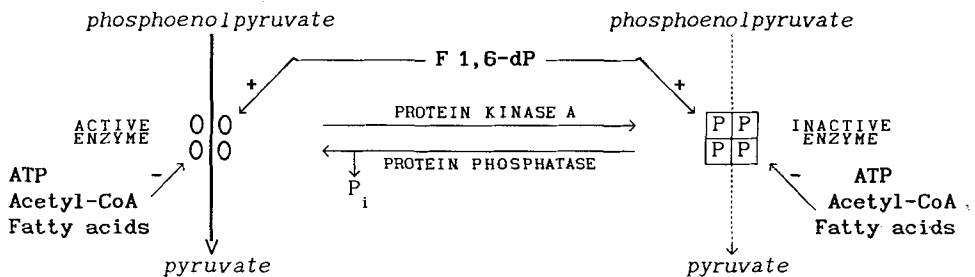

Fig. 1. **The regulation of pyruvate kinase in liver.** The enzyme is a tetramer where each monomer can be phosphorylated (P). The active monomer is denoted by (O). Fructose 1,6-diphosphate (F 1,6-dP) is an allosteric activator of both forms of the enzyme.

protein phosphorylation / dephosphorylation provides a reversible regulatory mechanism that can respond to the needs of the organism faster than the organism can synthesise or degrade proteins and which is a complement to allosteric regulation by small metabolites. Fig. 1 shows the regulation of liver pyruvate kinase which, when phosphorylated, has only 10% of the catalytic activity of the unphosphorylated form[3]. The allosteric effectors 'fine-tune' the activity of the enzyme either in its phosphorylated or unphosphorylated forms.

The protein kinases used in our experiments transfer the γ-phosphate group from magnesium adenosine triphosphate to a serine or threonine residue in the substrate protein. Protein kinases A and C (cyclic AMP-dependent protein kinase and the calcium- and phospholipid-dependent protein kinase, respectively) recognise basic residues around the phosphoacceptor site, while casein kinases I and II (S and TS) prefer acidic residues. The first two kinases are found ubiquitously in mammals with especially high concentrations in platelets[4]. While all four enzymes are expressed intracellularly, there have been reports of casein kinase II activity on the outside of cell membranes[5] and protein kinase A has been reported to be secreted by thrombin-activated platelets[6].

Evidence for extracellular phosphorylation

Some extracellular proteins, such as fibrinogen[7,8], fibronectin[9] and complement S protein (vitronectin)[10] are also known to contain covalently-bound phosphate groups. In these cases, it is not known whether the proteins are completely phosphorylated before secretion or whether the phosphorylation occurs extracellularly, but it is certain that the phosphorylation must have some functional significance. By analogy to the known intracellular regulatory role of protein phosphorylation/ dephos- phorylation it seemed to us possible that regulatory phosphorylation might also occur extracellularly in blood. The coagulation/fibrinolytic and complement activation systems are certainly complicated enough to warrant the extra level of control.

There have been earlier sporadic reports of protein kinase activity and protein phosphorylation in plasma[11-16] while other researchers have established the existance of membrane-bound extracellular protein kinases on various cells[17-20]. Platelets, as well as being rich in protein kinases, store molar quantities of ATP in their dense granules[21] which are released together with the kinases when they are activated by thrombin[6]. The concentration of the released ATP has been measured at 20 μM during haemostasis[22]. Alkaline phosphatase, a broad-specificity phosphoprotein phosphatase, is also found in plasma and extracellularly on the membranes of

endothelial cells. Therefore, there are kinase and phosphatase activities in the blood, and high local concentrations of ATP sufficient to sustain protein phosphorylation - but what of the protein substrates?

Enzymes regulated by phosphorylation/dephosphorylation generally occur at the beginning or the end of a metabolic pathway. We chose to look at fibrinogen and complement factor C3 because they are at the end of the coagulation cascade and the first stage of the complement activation pathway, respectively. If regulatory phosphorylation occurs in these systems, these proteins are likely targets for phosphorylation and the phosphorylation should alter their behaviour.

FIBRINOGEN

Fibrinogen (Mw 340,000) is a dimer, each half consisting of three polypeptide chains. The molecular weight and amino acid sequence have been determined for the different chains namely Aα (64,000, 610 aa), Bβ (57,000, 461 aa) and γ (48,000, 411 aa)[23-30]. The protein is stabilized by 28 internal disulphide bonds and contains 4% carbohydrate, present as neutral hexoses, glucosamine or sialic acid[31,32], for reviews of the structure see[30,33-37]. Most, if not all of the protein is synthesized in hepatocytes[38,39]. Some fibrinogen is found in the α-granules of platelets, but it is probable that fibrinogen is actually taken up by the platelets and stored in these granules[40]. Fibrinopeptides isolated from both platelets and liver show no differences in composition[36].

The plasma concentration is normally between 2.5 to 4 g/l[34,41] and 80% of the total fibrinogen is found in plasma. The plasma concentration is determined by the rate of synthesis.

The half-life of the molecule in plasma is around 4-6 days[42]. Fibrinogen is an "acute-phase reactant" and the highest amount is found 3-4 days after the onset of trauma or infection and the level remains elevated for 3 to 4 weeks. High levels of fibrinogen are also seen with some malignant diseases, especially hypernephroma where the protein is retained in the capillaries due to its molecular weight, while other proteins leak through the glomeruli. Low levels of fibrinogen are mainly found in disseminated intravascular coagulation (DIC)[34].

That fibrinogen from different mammalian species contains phosphate groups was first demonstrated by Blombäck[7] in 1962. It was found that fibrinogen from human and other mammalian species contains around 1-2 moles of covalently bound phosphate per mole fibrinogen. During the clotting process, 30 percent of the total phosphate is split off together with αSer3 in human

fibrinopeptide A. Besides αSer3, αSer345 has also been found to be phosphorylated *in vivo*[43].

As already mentioned, the rate of synthesis increases under acute phase conditions and this is accompanied by an increase in the phosphate content of Ser3 of the Aα-chain. Human foetal fibrinogen has been found to contain twice as much phosphate as fibrinogen from adult blood[44], here both αSer3 and αSer345 have higher amounts of phosphate. The foetal fibrinogen has a higher affinity for thrombin and the fibrin gels are more transparent than those from adult fibrinogen, indicating a smaller fibrin fibre thickness[45]. The protein kinase(s) responsible for the phosphorylation *in vivo* are not known at present.

Engström et al[46] found that human fibrinogen is a substrate for protein kinase A *in vitro* and, later, a Ca^{2+}-stimulated protein kinase activity in liver cell sap, and casein kinase TS were both found to phosphorylate fibrinogen[47,48]. Recently the probable sites for protein kinase C and casein kinase TS phosphorylation in fibrinogen have been determined[49,50].

Fibrinogen is the substrate of two serine proteases, thrombin and plasmin. Activation of the coagulation system leads to the convertion of inactive prothrombin into thrombin which cleaves off the A and B fibrinopeptides from the amino-terminals of the α- and β-chains. This limited proteolysis converts soluble fibrinogen into insoluble fibrin, which polymerises to form the fibrin clot. Dissolution of the fibrin mesh is catalysed by plasmin which degrades fibrin (or fibrinogen) into small peptides known as fibrin degradation products. Partially plasmin-degraded fibrinogen is found *in vivo*. An overview of the coagulation and fibrinolytic pathways is given in Fig. 2.

Changes in Fibrinogen's Behaviour after Phosphorylation or Dephosphorylation

Fibrinogen phosphorylated by protein kinase C was reacted with thrombin and the gelation was followed spectrophotomerically. The resulting gels were consistantly found to be more translucent than gels from untreated fibrinogen. The optical difference is partially due to inhibited lateral growth of the fibre bundles in the phosphorylated sample[51,52], that is, the extra phosphate groups added by the protein kinase interfere with the side-to-side polymerisation of fibrin fibres into fibrin bundles, but not with the end-to-end polymerisation of fibrin monomers into fibrin fibres.

Both protein kinase C-phosphorylated and untreated fibrinogen were dephosphorylated with alkaline phosphatase from calf intestine, and then reacted with thrombin. Both samples gave an increase in optical density during gelation after the phosphatase treatment, confirming that the phosphate groups play a role in determining the fibre thickness in the fibrin clot, and that the effect of the phosphorylation of fibrinogen on gelation could be reversed
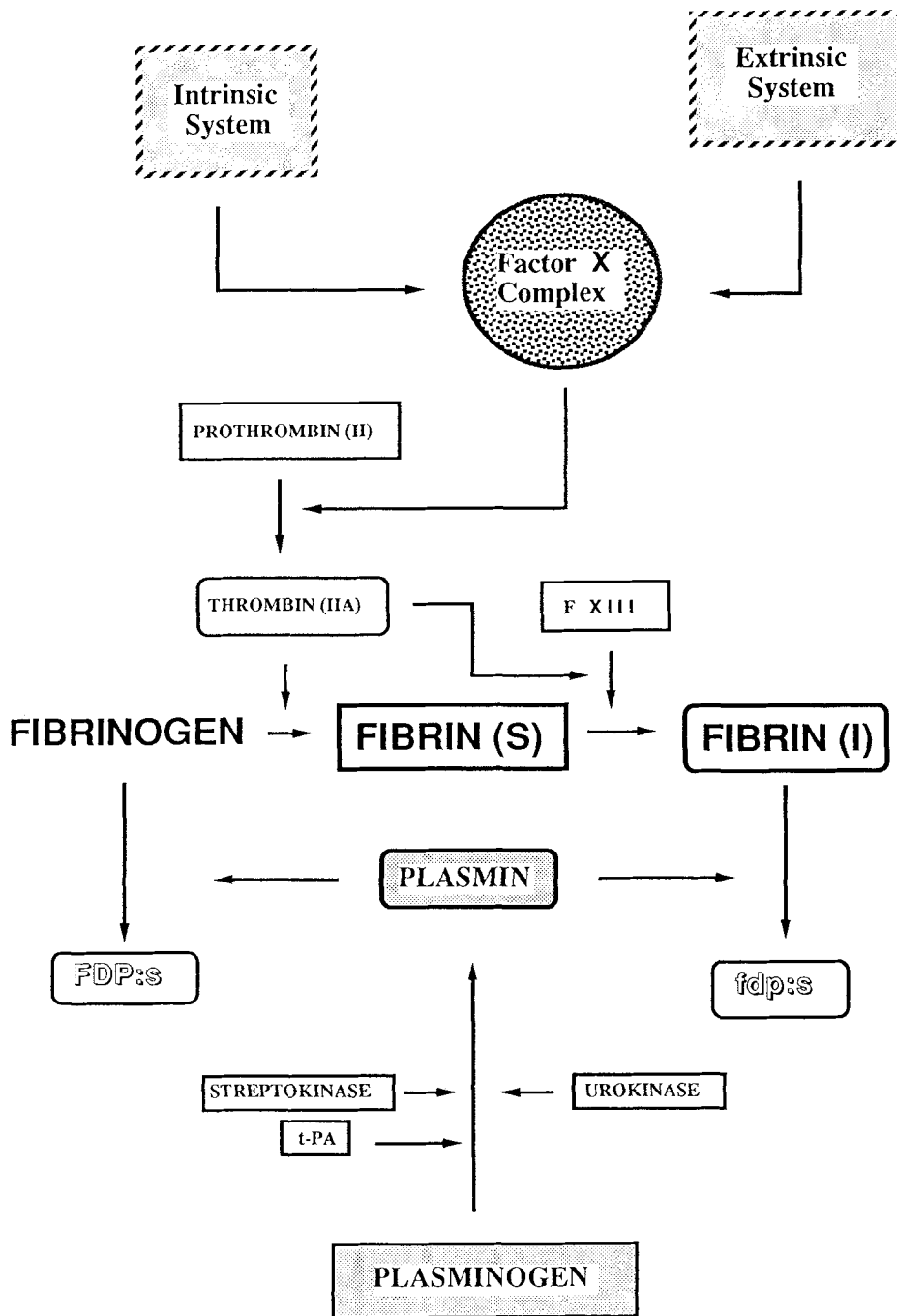
Fig. 2. The coagulation and fibrinolytic pathways. Coagulation can be
initiated through either intrinsic or extrinsic routes. The extrinsic
activation depends on factors from the vessel wall or surounding cells while
in the intrinsic system all factors exists in unactivated states in plasma.
Fibrinolysis starts with an activation of plasminogen by different activator
proteins.

by phosphatase treatment, both in the case of *in vitro* (^{32}P)-phosphorylated fibrinogen and endogenously phosphorylated fibrinogen[53]. Table 1 gives the mass-length ratios of the fibrin fibre bundles resulting from the various treatments of fibrinogen.

Table 1.   Mass-length Ratios for Fibrin Fibre Bundles from various Fibrinogens.

	Control Fibrinogen		Phosphorylated Fibrinogen	
Alkaline phosphatase treatment	−	+	−	+
Mass-length ratio (x 10^{-12} dalton/cm.)	10.4	16.9	8.3	11.0

Footnote: The mass-length ratios were calculated from the data given in[53] and (unpublished) using the formulae given in[52].

Experiments were then carried out to investigate whether there is any protein phosphatase activity in the blood. Whole blood anticoagulated with heparin, EDTA or citrate was incubated at 37°C with trace amounts (less than 0.5% of the total fibrinogen) of *in vitro* phosphorylated fibrinogen for different times. Aliquots were then taken and the (^{32}P)orthophosphate released was determined. Up to 30% of the ^{32}P-labelled phosphate in fibrinogen was released after four hours with heparinised blood. With EDTA, around 15% of the radioactivity was liberated while incubation with citrated blood only released 5 % of the phosphate from (^{32}P)fibrinogen. The differing amounts of ^{32}P released might be explained by the chelating action of citrate and EDTA, as magnesium and ions are essential for full phosphatase activity. These results proved that there was a dephosphorylating activity in whole blood, as if only serum or plasma was used no dephosphorylation was obtained[53].

Fibrinogen is one of the main targets of plasmin and contains loosely-bound plasminogen. The degradation of fibrinogen *in vivo* is partly due to plasmin activation. Therefore, we were interested to see if phosphorylation of fibrinogen affected its susceptibility to plasmin proteolysis. Fig. 3 shows the major plasmin digestion fragments from fibrinogen.

Fibrinogen was first phosphorylated with protein kinase C and then either dephosphorylated with alkaline phosphatase or treated with the phosphatase dialysis buffer. Thereafter the degradation of the protein with plasmin was studied. Aliquots from reaction mixtures of fibrinogens degraded with plasminogen activated by streptokinase, urokinase, or tissue plasminogen.
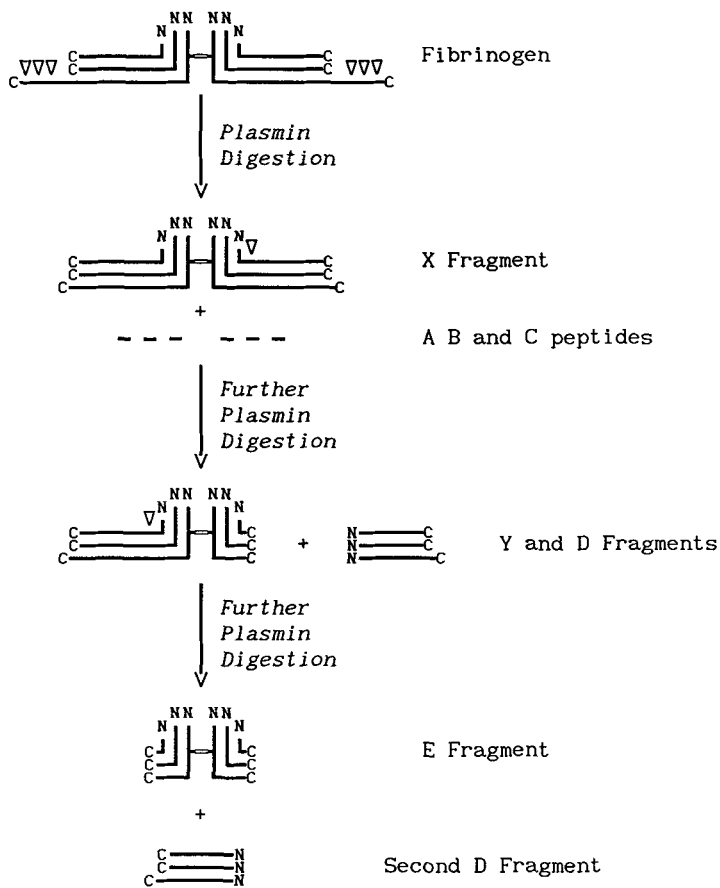
Fig. 3. Plasmin digestion of fibrinogen. Plasmin cleavage is shown ($\frac{\nabla}{-}$) beginning from the carboxy-terminal of the α-chain, where three small peptides are released and the X fragment (mw ≈ 250,000) is formed. Next, the X fragment is split into the first of two D fragments (mw ≈ 100,000) and the Y fragment. Finally, plasmin removes the second D fragment and the E fragment (mw ≈ 45,000) which is left consists of the tightly cross-linked amino-terminal regions of the three peptide chains.

activator were separated on non-reducing polyacrylamide gels (7.5%) under denaturing conditions, stained and densitometrically scanned. The amounts of fibrinogen fragments X, Y and D were expressed as a percentage of the total stained protein in each lane, Fig.4.

It was found that the rate of degradation of fibrinogen with plasmin was halved, if fibrinogen was phosphorylated prior to the plasmin degradation.
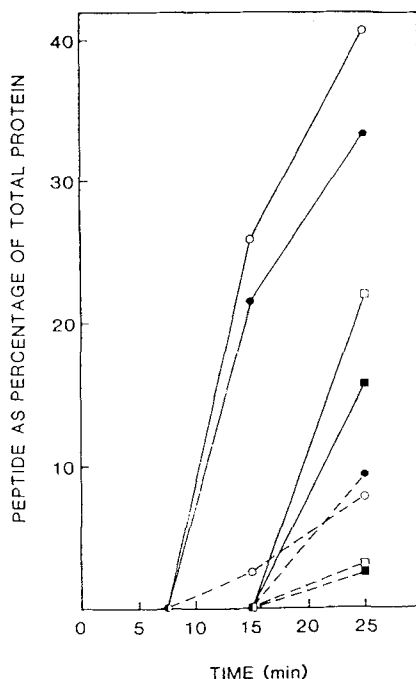
Fig. 4. Plasmin digesttion of human fibrinogen (phosphorylated and control) after dephosphorylation by alkaline phosphatase. Control fibrinogen by (O), phosphorylated sample by ( ), dephosphorylated control by (O) and dephosphorylated sample by ( ). Solid lines represent the X fragment and dashed lines represent the D fragment.

This change was, in contrast to the result from the thrombin gelation assay, unaffected by the dephosphorylation of fibrinogen with alkaline phosphatase, even though the dephosphorylation always exceeded 75% of the (^{32}P)phosphate incorporated. If unphosphorylated fibrinogen was dephosphorylated no change in the rate of degradation by plasmin was seen. The same result was obtained irrespective of the plasminogen activators used (streptokinase, urokinase or t-PA), or of the supplier of the fibrinogen (Kabi or Sigma)[54]. These results suggested that phosphorylation caused a conformational change in the fibrinogen molecule making it less susceptible to plasmin. This conformational change must remain even after the dephosphorylation of the fibrinogen, as the plasmin degradation rate does not increase after the dephosphorylation. To test this hypothesis, the near-ultraviolet circular dichroism spectra of control and phosphorylated fibrinogen were compared before and after alkaline phosphatase treatment and it was seen that phosphorylation with protein kinase C led to an increase in ordered secondary structure which remained after dephosphorylation[55]. As plasmin cleaves regions of disordered structure, the increase in ordered structure may explain the reduction in the plasmin degradation rate.

82

COMPLEMENT

The complement system is responsible for the cytotoxic part of humoral immunity, as well as opsonisation of bacteria and viruses, anaphylaxis and stimulation of the immune system. Like the extrinsic and intrinsic activation pathways in coagulation, complement activation can occur in two separate ways, the classical (antibody-dependent) pathway or the alternative pathway, which is activated by foreign carbohydrates on the surfaces of viruses and bacteria. Fig. 4 shows the proteins and reactions involved in the complement pathway. Antigen-antibody complexes activate C1s, which activates C4 and C2; these two factors form the classical pathway C3 convertase when bound to a unusual thiol-ester bond which is ruptured by proteolytic activation[56] resulting in a reactive glutamyl group that rapidly binds to either the bacterial surface or to water. The alternative pathway is less well understood, but involves activation by foreign carbohydrates. Factor $\bar{D}$ (believed to exist solely as the active form) proteolytically converts factor B to Ba and Bb in the presence of activated C3. Complement factor C3 is a glycoprotein of molecular weight 185,000 present in plasma at around 1-2 mg/ml ($\approx$ 8 μM).

It was first isolated in Uppsala by Müller-Eberhard et al. in 1960[57] and consists of two dissimilar polypeptide chains with molecular weights of 110,000 (α) and 75,000 (β) joined by disulphide bonds. As mentioned above, C3 (shown in bold type in the diagram) contains an unusual modification in that the side chains of Cys 988 and Glu 990 form a thiolester bond in the native α-chain. This ester bond is susceptible to hydrolysis in plasma and a small percentage of the C3 molecules are believed to circulate as C3i molecules which express the factor B binding site necessary for forming the C3iB complex and the priming of the alternative pathway C3 convertase. Otherwise, for 50 μs after the conversion of C3 to C3a and C3b, the activated carboxy-group in C3b can bind to -OH and -NH$_2$ groups on the bacterial or viral surface. Like C4, this concentrates the complement response onto the target surface. The C3b generated is deposited next to the convertase, and alters the specificity of the convertase by binding C5 and making the C5 susceptible to the C3 convertase (now called the C5 convertase). This process is the same for both C3 convertases and here complement activation enters the final common pathway. C5b binds reversibly to the surface and C6, C7, C8 and up to 18 molecules of C9 bind to the C5b, forming the membrane attack complex. Control over the complement activation pathway is exercised by a variety of specific proteases and binding proteins including vitronectin (S protein).

Two of the small peptides released during complement activation (C3a and C4a) are anaphylatoxins, they cause vasodilatation and stimulate mast cells to release their granular contents; while C5a is a chemoattractant for neutrophils. (For a review of the complement system see[58,59]).

Phosphorylation of C3 in vivo

Complement factor C3 being at the centre of the complement activation pathway, was considered to be a prime candidate for regulatory phosphorylation if this control mechanism existed extracellularly. Therefore the phosphate content in C3 was determined and found to be around 0.15 moles/ mole protein. This showed that C3 was a phosphoprotein like fibrinogen and although the degree of phosphorylation was low it is comparible to fibrinogen which has about 1 mole of phosphate divided among possibly six phosphorylation sites. We assume that the phosphate in C3 is added during synthesis and secretion but, because of the reports of plasma protein kinase activity, phosphorylation experiments were carried out which demonstrated that the α-chain of C3 became phosphorylated when whole blood and trace amounts of AT^{32}P were incubated together at 37°C. Further, it was possible to localise the phosphorylation site to a 47,000 dalton fragment which contains the 10,000 dalton C3a anaphylatoxin[60]. Finally, the plasma ATP concentration was measured and found to be around 2μM.

Changes in the behaviour of C3 after phosphorylation

In vitro experiments with purified C3 and protein kinases A and C showed that both kinases were able to stoichiometrically phosphorylate the α-chain of C3 and that the phosphorylation site appeared to be the same for both kinases. Protein kinase C also phosphorylated a site in the β-chain, but the function, of this phosphorylation was not determined. The phosphorylation site on the α-chain was located in the C3a portion, the peptide which is first removed in the activation of C3. This led us to investigate whether phosphorylation might affect the behaviour of C3 in complement activation tests.

Fig. 6 shows how haemolysis due to the classical activation pathway declines as the phosphate content of C3 increases. The slope of the line indicates that at a phosphorylation of 1 mole phosphate / mole C3, the classical activation pathway would be inhibited by 80%. This inhibition was found to be of the same order for each of the two protein kinases. When phosphorylated C3 was tested in alternative pathway activation tests, an inhibition amounting to 60% at 1 mole phosphate / mole C3 was seen with both kinases[61]. These results indicate that the phosphorylation of C3 in the C3a portion of the α-chain causes an almost stoichiometric inhibition of both complement activation pathways.
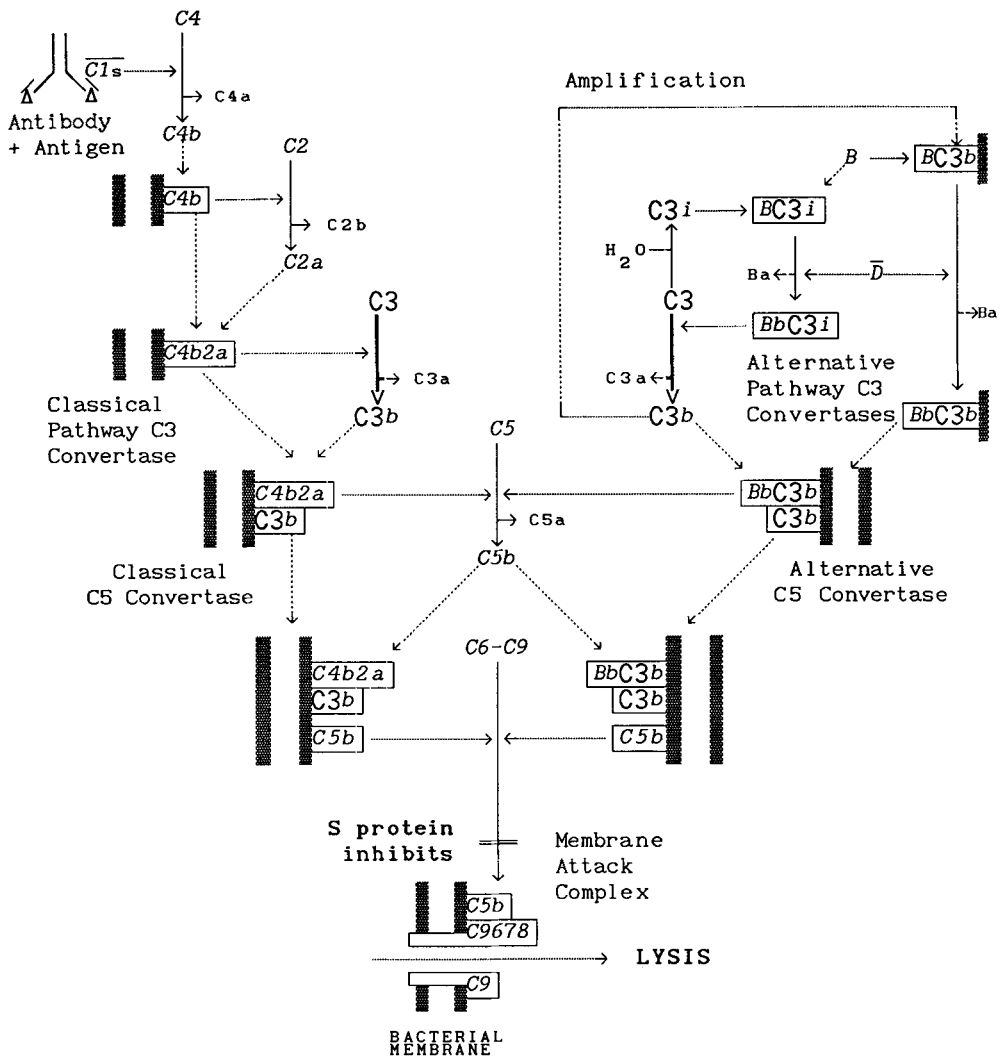
84

Figure 5. The Complement Activation Pathway. The classical pathway is activated by the binding of C1 to antigen-antibody complexes. C1s cleaves C4 and C2 which bind to the target molecule to form the C3 convertase. The alternative pathway is activated by carbohydrates and the convertase is formed by C3iBb or C3bBb in analogy to the classical pathway. The C3b formed binds to the convertase and alters its specificity to favour C5. The C5b formed binds widely over the target surface and forms the nucleus of the membrane attack complex (C5-9) which penetrates the membrane to cause lysis. The central role of C3 is highlighted in this diagram.

As yet the effects of dephosphorylation of the phosphorylated C3 on its
behaviour have not been tested, but in preliminary experiments it has been
possible to remove 30% of the (^{32}P)phosphate with calf intestinal alkaline
phosphatase (Forsberg, P.-O. & Martin, S. C. unpublished results)
demonstrating that the *in vitro* phosphorylation is at least partially
reversible.

GENERAL DISCUSSION

The research team to which we belong has shown that human fibrinogen and
complement factor C3 can be phosphorylated *in vitro* by various protein
kinases. Other investigators have recently shown that plasma proteins such as
albumin[62,63], fibronectin[9], thrombin[64], complement S protein[10,65] and
complement factor C9[66] can also be phosphorylated *in vitro*. Taken together
with the reports of protein kinase[11-20] and protein phosphatase
activity[53] and the detection of μM quantities of ATP in plasma[22], these
results strongly suggest that extracellular regulatory phosphorylation of
plasma proteins occurs.

In the 1970's, Krebs[67] and Nimmo & Cohen[68] formulated a series of
criteria to establish whether a protein phosphorylation reaction was of a
regulatory nature or not. The four criteria are:-

   1.   A protein substrate for a protein kinase should be phosphorylated in
its native state at a rate fast enough to be realistic *in vivo*.

   2.   The activity of the protein should be altered by phosphorylation and
the effect reversed by dephosphorylation *in vitro*.

   3.   A reversible change in the function of the protein should occur *in
vivo* under conditions which stimulate the protein kinase.

   4.   Phosphorylation should occur *in vivo* at the same sites as are
phosphorylated *in vitro*.

   These criteria are only a guide to indicate which proteins are likely to
be regulated by phosphorylation, and few investigated enzymes have fulfilled
them all.

   If our results for fibrinogen and C3 are tested against the four criteria
we find that both fibrinogen and C3 are phosphorylated at a realistic rate by
protein kinase C with K_m's of 0.2 μM and 2 μM, respectively. These values
are in the range reported for other substrates of this kinase[69-73]. The
behaviour of both proteins is affected by the phosphorylation and, for
fibrinogen, partially reversed by dephosphorylation *in vitro*. The third
criterion has not been studied other than confirming that both proteins can

be phosphorylated by protein kinases present in anticoagulated whole blood. As for the phosphorylation sites being the same *in vitro* as *in vivo*, protein kinase C phosphorylates αSer 345 *in vitro*[49] which is also phosphorylated *in vivo* and the α-chain phosphorylation sites in C3 have been localised to a 47,000 dalton area both *in vivo* and *in vitro*. In summary, both fibrinogen and C3 partially fulfill the criteria for regulatory phosphorylation and their behaviour may therefore be considered to be regulated by protein phosphorylation / dephosphorylation just like the many intracellular proteins described in the litterature.

What function does this regulation serve? Here we can as yet only speculate, but many of the factors involved in coagulation / fibrinolysis or complement activation can act in the other system, e.g., kallikrein, plasmin, S protein (vitronectin) and C1 inhibitor, therefore activation of one system could directly effect the other. When there is a vascular injury, platelets form the primary haemostatic plug and their phospholipid membranes provide the focus for the developing coagulation cascade. During this process, the platelets are activated (e.g., by thrombin, or ADP) and they release their kinases and stored ATP. As mentioned earlier, the local concentration of ATP can rise to levels sufficient to maintain protein phosphorylation, and one of protein phosphorylation is ADP which is itself a platelet activator. From our results, phosphorylation of fibrinogen under these circumstances by protein kinase C (or protein kinase A, unpublished results) would lead to thinner fibrin fibre bundles which were twice as resistant to plasmin cleavage as normal fibrin (assuming that our results for fibrinogen reflect the degradation of fibrin by plasmin.

We feel that this is a reasonable assumption based on the similar degradation rates for fibrinogen and fibrin seen by Šonka et al.[20]). A fibrin mesh that was more resistant to plasmin would make sense during acute vascular injury where the priority is haemostasis, resolution of the clot would only be delayed not totally inhibited. The kinases and ATP released by the platelets might also reasonably be expected to phosphorylate other plasma proteins besides fibrinogen under these conditions, S protein, and complement factor C3 for example. While any effect of the phosphorylation on the behaviour of S protein has not been reported, it is known that the phosphorylation site lies in the heparin-binding region of the protein[10,40] . Phosphorylation of C3, however, with protein kinases A or C would result in the partial inhibition of the complement activation cascade, which could be physiologically relevant as the activation of proteolytic enzymes such as kallikrein and plasmin by coagulation / fibrinolysis might result in inadvertant complement activation.

Thus the phosphorylation of C3 and its consequent inactivation might protect the body from accidental complement activation and tissue damage under circumstances (acute vascular injury) where the coagulation / fibrinolytic system is active.

Recently, interest has grown into fibirnogen as a factor in atherosclerosis and cardiovascular disease. evident. Fibrinogen levels seem to correlate with survival after cardial infarctions[74,75] but what happens to the phosphorylation state of the fibrinogen is not known. Our work suggests that measurement of the phosphate content of the fibrinogen as well as measuring total fibrinogen might be valuable as a predictive indicator.

We have been unable to pinpoint where phosphorylation takes place *in vivo*, but we have demonstrated the possibility of extracellular phosphorylation. Evidence for such phosphorylations is growing with new reports from research groups all over the world.

Here, we will continue our effort to identify these phosphorylations by investigating fibrinogen from patients with inflammatory responses as well as the thromboembolic diseases and altered platelet function. The future will hopfully give us the clues to extracellular phosphorylation in general and to the importance of fibrinogen and C3 as phosphoproteins. We feel this is the beginning of an new field in the area of protein phosphorylation.

REFERENCES

1. Forsberg, H., Zetterqvist, Ö. & Engström, L.: Protein-bound phosphorylserine in different tissues and organisms. Biochim. Biophys. Acta **181**: 171-174, 1969.

2. Sutherland, E. W., Jr. & Wosilait, W. D.: Inactivation and activation of liver phosphorylase. Nature **175**: 169-170, 1955.

3. Titanji, V. P. K., Zetterqvist, Ö. & Engström, L.: Regulation *in vitro* of rat liver pyruvate kinase by phosphorylation-dephosphorylation reactions, catalyzed by cyclic AMP-dependent protein kinases and a histone phosphatase. Biochim Biophys Acta **422**: 98-108, 1976.

4. Kawahara, Y., Takai, Y., Minakuchi, R., Sano, K. & Nishizuka, Y.: Phospholipid turnover as a possible transmembrane signal for protein phosphorylation during human platelet activation by thrombin. Biochem Biophys Res Comm **97**: 309-317, 1980.

5. Pyerin, W., Burow, E., Michealy, K., Kübler, D. & Kinzel, V.: Catalytic and molecular properties of highly purified phosvitin/casein kinase type II from human epithelial cells in culture (HeLa) and relation to ecto protein kinase. Biol. Chem. Hoppe-Seyler's **368**: 215-227, 1987.

6. Korc-Grodzicki, B., Tauber-Finkelstein, M. and Shaltiel, S.: Platelet stimulation releases a cAMP-dependent protein kinase that specifically phosphorylates a plasma protein. Proc Nat Acad Sci USA **85**: 7541-7545, 1988.

7. Blombäck, B., Blombäck, M., Edman, P. & Hessel, B.: Amino-acid sequence and the occurence of phosphorus in human fibrinopeptides. Nature **193**: 883-884, 1962.

8. Blombäck, B., Blombäck, M. & Searle, J.: On the occurence of phosphorus in fibrinogen. Biochim. Biophys. Acta **74**: 148-151, 1963.

9. Imada, S. Sugiyama Y. & Imada, M.: Fibronectin phosphorylation by Ecto-protein Kinase. Exp. Cell Res. **179**: 554-564, 1988.

10. McGuire, E. A. Peacock, M. E. Inhorn, R. C. Siegel, N. R. & Tollefsen, D. M.: Phosphorylation of vitronectin by a protein kinase in human plasma. J. Biol. Chem. **263**: 1942-1945, 1988.

11. Galabru, J. Kurst, B. & Hovanessian, A. G.: A protein kinase system from platelet rich plasma. Biochem. Biophys. Res. Commun. **113**: 370-376, 1983.

12. Kurst, B. Galabru, J. & Hovanessian, A. G.: Phosphorylation of the α-chain of fibrinogen by a platelet kinase activity enhanced by interferon. Biochem. Biophys. Res. Commun. **117**: 350-357, 1983.

13. Chiang, T. M. & Kang, A. H.: Identification, purification, and partial characterisation of plasma protein kinases. Arch. Biochem. Biophys. **243**: 530-541, 1985.

14. Kubler, D., Fehst, M., Garcon, T., Pyerin, W., Burow, E. & Kinzel, V.: Substrates for the cell surface protein kinase in blood. A claf serum protein and it's precursor in plasma. Biochem. Int. **15**: 349-357, 1987.

15. Lin, M. F., Lee, P. L. & Clinton, G. M.: Characterization of Tyrosyl kinase activity in human serum. J. Biol. Chem. **260**: 1582-1587, 1985.

16. Haas, A., Heller, I. & Presek, P.: Detection of a tyrosine kinase in human sera and blood cells by pp60^{src} antiserum. Biochim. Biophys. Res. Commun. **135**: 426-434, 1986.

17. Kleine, L. P., Whitfield, J. F. & Boynton, A. L.: Ca^{2+}-Dependent cell surface protein phosphorylation may be involved in the initiation of DNA synthesis. J. Cell. Physiol. **129**: 303-309, 1986.

18. Kleine, L. P. & Whitfield, J. F.: Serum activated T51B rat liver cells transiently accumulate cyclic AMP-Dependent protein kinases on there surfaces during the G1 phase. J. Cell. Physiol. **132**: 354-358, 1987.

19. Dusenbery, K. E., Mendiola, J. R. & Skubitz, K. M.: Evidence for ecto-protein kinase activity on the surfaces of human neutrophils. Biochem. Biophys. Res. Commun. **153**: 7-13, 1988.

20. Šonka, J., Kübler, D. & Kinzel, V.: Phosphorylation by cell surface protein kinase of bovine and human fibrinogen and fibrin. Biochim. Biophys. Acta **997**: 268-277, 1989.

21. Ugurbil, K. & Holsen, H.:Nucleotide compartmentation: Radioisotopic and nuclear resonance studies. In "Platelets and pathology-2" (ed Gordon) Elsevier/North Holland Biomedical press. pp 147-177, 1981

22. Born, G. V. R. & Kratzer, M. A. A.: Source and concentration of extracellular adenosin triphosphate during haemostasis in rats, rabbits and man. J. Physiol. **354**: 419-429, 1984.

23. Watt, K. W. K., Takagi, T. & Doolittle, R. F.: Amino acid seqence of the β-chain of human fibrinogen. Biochemistry **18**: 68-76. 1979.

24. Watt, K. W. K., Cottrell, B.A., Strong, D. D. & Doolittle, R. F.: Amino acid sequence studies on the chain of human fibrinogen. Overlapping sequences providing the complete sequence. Biochemistry **18**: 5410-5416, 1979.

25. Doolittle, R. F., Bouma, H., Cottrell, B. A., Strong, D. & Watt, K. W. K. In "The Chemistry and Physiology of the Human Plasma Proteins" (DH Bing, ed.), pp 77-95. 1979. Pergamon, Oxford.

26. Doolittle, R. F., Watt, K. W. K., Cottrell, B. A., Strong, D. D. & Riley, M.: The amino acid sequence of the α-chain of human fibrinogen. Nature **280**: 464-468, 1979.

27. Henschen, A., Lottspeich, F., Southan, C. & Töpfer-Petersen, E. Protides Biol. Fluids **28**: 51-56, 1980.

28 Henschen A, Graeff H & Lottspeich F eds, In "Fibrinogen: Recent Biochemical and Medical Aspects", de Gruyter, Berlin, 1982.

29. Hoeprich, P. D. Jr, & Doolittle, R. F.: Dimeric half-molecules of human fibrinogen are joined through disulfide bonds in an antiparallel orientation. Biochemistry, **22**: 2049-2055, 1983.

30. Putnam, F. In "The plasma proteins IV", (ed Putnam, F.) Academic press 2nd ed. New York, 1984.

31. Blombäck, B.: On the proterties of fibrinogen and fibrin. Arkiv för Kemi **12**: 99-113, 1958.

32. Henschen, A.: Number and reactivity of disulfide bonds in fibrinogen and fibrin. Arkiv för Kemi, **22**: 355-373, 1964.

33. Doolittle, R. F. In "The plasma proteins II", (ed Putnam, F.) Academic press, 2nd ed. New York, pp 109-161, 1975.

34. Blombäck, B. In "Plasmaproteiner", (ed Blombäck, B. & Hansson, L. Å.), AB KABI AWE/GEBERS, pp 231-258, 1976.

35. Henschen, A.: On the structure of functional sites in fibrinogen. Tromb. Res. **Suppl V**: 27-39, 1983.

36. Doolittle, R. F.: Fibrinogen and fibrin. Ann. Rev. Biochem. **53**: 195-229, 1984.

37. Furie, B. & Furie, B. C.: The molecular basis of blood coagulation. Cell, **53**: 505-518, 1988.

38. Barnhart, M. I. & Forman, W. B.: The cellular localization of fibrinogen as revealed by the fluorescent antibody technique. Vox Sang. **8**: 461-473, 1963.

39. Kudryk, B., Okada, M., Redman, C.M. and Blombäck, B. Biosynthesis of dog fibrinogen. Characterization of nascent fibrinogen in rough endoplasmic reticulum. Eur. J. Biochem., **125**, 673-682, 1982

40. Harrison, P., Wilbourn, B., Debili, N., Vainchenker, W., Breton-Gorius, J., Lawrie, A. S., Masse, J.-M., Savidge, G. F. & Cramer, E. M.: Uptake of Plasma Fibrinogen into the Alpha Granules of Human Megakaryocytes and Platelets. J. Clin. Invest. **84**: 1320-1324, 1989.

41. Fuller, G. M., Bunzel, R. J. & Nesbitt. J. E.: Fibrinogen. Meth. Enzymol. **163**: 474-485, 1988.

42. Duckert, F.: In "Clinical Biochemistry: Principles Methods", pp 1515-1530, 1974.

43. Seydewitz, H. H., Kaiser, C., Rothweiler, H. & Witt, I.: The location of a second in vivo phosphorylation site in the Aα-chain of human fibrinogen. Thromb. Res. **33**: 487-498, 1984.

44. Witt, I. & Müller, H.: Phosphorus and hexose content of human foetal fibrinogen. Biochim. Biophys. Acta **221**: 402-404, 1970.

45. Müller, M., Burchard, W. & Witt, I.: Fibrinogen - fibrin transformation. Part III. Particularities for foetal fibrinogen. Thromb. Res. **24**: 339-46, 1981.

46. Engström, L., Edlund, B., Ragnarsson, U., Dahlqvist-Edberg, U. & Humble, E.: Phosphorylation of human fibrinogen *in vitro* with cyclic 3',5'-AMP stimulated protein kinase and (^{32}P)ATP. Biochem. Biophys. Res. Commun. **96**: 1503-1507, 1980.

47. Papanikolaou, P., Humble, E. & Engström, L.: Phosphorylation of human fibrinogen *in vitro* with calcium-activated, phospholipid-dependent protein kinase and (^{32}P)ATP. FEBS lett. **143**: 199-204, 1982.

48. Itarte, E., Plana, M., Guasch, M., D. & Martos, C.: Phosphorylation of fibrinogen by casein kinase 1. Biochem. Biophys. Res. Commun. **117**: 631-36, 1983.

49. Heldin, P. & Humble, E.: Phosphorylation of human fibrinogen *in vitro* with protein kinase C: characterization of the phosphorylated sites. Arch. Biochem. Biophys. **256**: 49-59, 1987.

50. Heldin, P.: Phosphorylation *in vitro* of human fibrinogen with casein kinase TS and characterization of phosphorylated sites. Arch. Biochem. Biophys. **257**: 269-275, 1987.

51. Okada, M., Blombäck, B., Chang, M.-D. & Horowitz, B.: Fibronectin and fibrin gel structure. J. Biol. Chem. **260**: 1811-1820, 1985.

52. Carr, Jr., M.E. & Hermans, J.: Size and density of fibrin fibers from turbidity. Macromolecules **11**: 46-50, 1978.

53. Forsberg, P.-O.: Dephosphorylation of human fibrinogen, previously phosphorylated *in vitro* by protein kinase C, by whole blood or intestinal alkaline phosphatase. Thromb. Res. **53**: 1-9, 1989.

54. Forsberg, P.-O., & Martin, S.C.: Plasmin digestion of human fibrinogen previously phosphorylated by protein kinase C or dephosphorylated by alkaline phosphatase *in vitro*. Thromb. Res. **58**: 119-127, 1990.

55. Martin, S.C. & Björk, I.: Conformational Changes in human fibrinogen after *in vitro* phosphorylation and their relation to fibrinogen behaviour. FEBS. Lett. **272**: 103-105, 1990.

56. Janatova, J., Tack, B. F., & Prahl, J. W.: Third component of human complement: Structural requirements for its function. Biochemistry **19**: 4479-4485, 1980.

57. Müller-Eberhard, H. J., Nilsson, U. R. & Aronsson, T.: J.: Isolation and characterization of two β₁-Glycoproteins of human serum. Exp. Med. **111**: 201-215, 1960.

58 Reid, K. B. M.: Activation and control of the complement system. Essays in Biochem. **22**: 27-68, 1986.

59. Müller-Eberhard, H. J.: Molecular organization and function of the complement system. Ann. Rev. Biochem. **57**: 321-347, 1988.

60. Martin, S.C.: Phosphorylation of complement factor C3 *in vivo*. Biochem. J. **261**: 1051-1054, 1989.

61. Forsberg, P.-O., Martin, S.C., Nilsson, B., Ekman, P., Nilsson, U.R. & Engström, L.: *In vitro* Phosphorylation of Human Complement Factor C3 by Protein kinase A and Protein Kinase C: Effects on the Classical and Alternative Pathways. J. Biol. Chem. **265**: 2941-2946, 1990.

62. Martin, S. C. & Ekman, P.: *In vitro* Phosphorylation of serum albumin by two protein kinases: A potential pitfall in protein phosphorylation reactions. Anal. Biochem. **265**: 2941-2946, 1986.

63. Chen, S.-L. & Kim, K.-H.: Effect on sulfhydryl-disulfide state on protein phosphorylation: Phosphorylation of bovine serum albumin. Arch. Biochem. Biophys. **239**: 163-171, 1985

64. Chanderkar, L. P. & Nadkarni, G. B.: Phosphorylation of thrombin during thrombokinase-mediated activation of prothrombin. Indian J. Biochem. Biophys. **16**: 196-199, 1979

65. Korc-Grodzicki, B., Tauber-Finkelstein, M., Chain, D. & Shaltiel, S.: Vitronectin is phosphorylated by a cAMP-dependent protein kinase released by activation of human platelets with thrombin. Biochem. Biophys. Res. Comm. **157**: 1131-1138, 1988.

66. Fishelson, Z., Kopf, E., Paas. Y., Ross, L. & Reiter, Y.: Protein phosphorylation as a mechanism of resistance against complement damage. Prog. Immunol. **7**: 1989.

67. Krebs, E. G. In: Endocrinology, Proc. 4th. Int. Congr., Scow, R.O. ed., pp 17-29, Exerpta Medica, Amsterdam. 1973.

68. Nimmo, H. G. & Cohen, P.: Hormonal control of protein phosphorylation. In: Advances in Cyclic Nucleotide Research, Vol. 8, pp 145-266, Raven, New York. 1977.

69. Woodgett, J. R., Gould, K. L. & Hunter, T.: Substrate specificity of protein kinase C: Use of syntetic peptides corresponding to physiological sites as probes for substrates recognition requirements. Eur. J. Biochem. **161**: 177-184, 1986.

70. Schatzmann, R. C., Raynor, R. L., Fritz, R. B. & Kuo, J. F.: Purification to homogeneity, characterization and monoclonal antibodies of phospolipid sensitive Ca^{2+}-dependent protein kinase from spleen. Biochem. J. **209**: 435-443, 1983.

71. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. & Nishizuka, Y.: Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. J. Biol. Chem. **254**: 3692-3695, 1979.

72. Nishikawa, M., Sellers. J. R., Adelstein, R. S. & Hidaka, H.: Protein kinase C modulates in vitro phosphorylation of the smooth muscle heavy meromyosin by myosin light chain kinase. J. Biol. Chem. **259**: 8808-8814, 1984.

73. Katoh, N., Wise, B. C. & Kuo, J. F.: Phosphorylation of cardiac troponin inhibitory subunit (Troponin I) and tropomyocin binding subunit (Troponin T) by cardiac phospholipid sensitive and Ca^{2+}dependent protein kinase. Biochem J **209**: 189-195, 1983.

74. Ernst, E.: Plasma fibrinogen – an independent cardiovascular risk factor. J Int Med **227**: 365-372, 1990

75. Cooper, J. & Douglas, A. S.: Fibrinogen levels as a predictor of mortality in survivors of myocardial infarction. Fibrinolysis **5**:105-108, 1991

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