monovalent anions may vary and therefore modulate microvillus membrane proton permeability. Another possibility is that the anion-dependent proton permeability may be a developmental refinement of a transport protein which functioned initially as a Cl^-/OH^- countertransporter.

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Registry No. H_2DIDS, 61481-03-6; DIDS, 53005-05-3; H^+, 12408-02-5; Cl^-, 16887-00-6; Br^-, 24959-67-9; I^-, 20461-54-5; valinomycin, 2001-95-8.

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Properties of Chemically Modified Protein S: Effect of the Conversion of γ-Carboxyglutamic Acid to γ-Methyleneglutamic Acid on Functional Properties†

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ABSTRACT: Protein S, the protein cofactor for activated protein C in the proteolytic inactivation of factor Va, was chemically modified with a mixture of morpholine and formaldehyde. This treatment resulted in the conversion of the γ-carboxyglutamic acid (Gla) residues of this vitamin K dependent protein to γ-methyleneglutamic acid. With a 10000-fold molar excess of morpholine and formaldehyde over protein S it was found that between 10 and 11 Gla residues could be modified. The degree of modification was proportional to the concentration of the modifying reagents used. The modification of as few as two residues resulted in the 70% loss of activity. Calcium inhibited the modification of several residues. In the presence of 3.2 mM calcium ion, a derivative with 2.5 residues modified was prepared that appeared to have full activity. Modification of protein S resulted in the alteration of a number of its properties. The quenching of intrinsic fluorescence by calcium decreased. The quenching effect of terbium ions was also decreased. However, the modified protein and the native protein were equivalent when protein-dependent terbium fluorescence was measured. When modified, protein S would no longer bind to phospholipid vesicles. Finally, the ability of protein S to self-associate was decreased by modification. These findings suggest that the γ-carboxyglutamic acid residues of protein S may play several roles in the maintenance of structure.

Protein S is a vitamin K dependent protein found in blood plasma (DiScipio & Davie, 1979; Stenflo & Jonsson, 1979). It is believed to function as a cofactor for the expression of the anticoagulant activity of activated protein C (Walker, 1980, 1981b). Protein S forms a complex with activated protein C on the surface of membranes that contain negatively charged phospholipids (Walker, 1981a; Suzuki et al., 1983). This complex is thought to be the main form in which activated protein C is active as an anticoagulant that functions through the proteolytic inactivation of coagulation factors V (Walker, 1981a) and VIII (Lawrence, 1985). This is supported by the observation that plasma depleted of protein S is much less

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sensitive to activated protein C than control plasma (Walker, 1980; Comp & Esmon, 1984). Essential to the activity of protein S is its ability to interact with membranes (Walker, (1981a) or platelets (Harris & Esmon, 1985). As a vitamin K dependent protein, protein S is unusual in several respects. First, unlike the other plasma vitamin K dependent proteins, it does not appear to be a zymogen of a serine protease (Dahlback et al., 1985). Second, it has a much higher affinity for membranes that the other proteins of this group (Nelsestuen et al., 1978). The reason for the high affinity for membranes is unclear. One derivative of protein S that has lost its high affinity for membranes is a two-chain version that is the product of proteolysis by thrombin. This version has no cofactor activity, altered calcium binding properties, and a low affinity for membranes (Walker, 1984; Suzuki et al., 1983).

Several groups have used a modifying reaction that converts γ-carboxyglutamic acid (Gla) residues to γ-methyleneglutamic acid to study the role of the Gla amino acids. The modification of the Gla residues in prothrombin fragment 1 resulted in changes in its interaction with calcium (Wright et al., 1984). Modification of the Gla residues in factor X resulted in the inhibition of its activation by a snake venom activator (Sherrill et al., 1984), and conversion of the Gla residues in factor IX resulted in the loss of activity (Straight et al., 1984).

The purpose of this paper is to examine the role of the γ-carboxyglutamic acid residues in the properties of protein S. This paper describes the effects of Gla modification of biological activity as well as the interactions between protein S and ions or membranes.

Experimental Procedures

Preparation of Proteins. Protein S, protein C, and factor V were prepared from bovine plasma as previously described (Walker, 1981a). Protein C was activated with the factor X activator from Russell's viper venom (Walker et al., 1979) and subsequently purified by ion-exchange chromatography on QAE-Sephadex (Walker et al., 1979). Factor V was activated with thrombin and also purified by ion-exchange chromatography (Esmon, 1979). Bovine thrombin was prepared by activating purified prothrombin with purified factor Xa, factor Va, phospholipids, and calcium as described by Owen and co-workers (Owen et al., 1974). Thrombin was separated from the activation components by chromatography on sulfopropyl-Sephadex (Lundblad, 1971). Thrombin-inactivated protein S was prepared as previously described (Walker, 1984). Purity of the various proteins was ascertained in at least two acrylamide gel electrophoresis systems.

Phospholipid Preparation. Phospholipids dissolved in chloroform were dried under nitrogen onto the wall of a glass tube. Vesicles were prepared by adding buffer to the tube and sonicating for approximately 10 min. The temperature of the tubes during sonication was kept below 37 °C. Following sonication, the lipid preparation was centrifuged for 30 min at 20000*g* to remove large particles and metal fragments generated during the sonication. Phospholipid concentrations were estimated by measuring the organic phosphorus by the method of Chen (1965) and by using a weight conversion factor of 25 (phospholipid/phosphorus). It was assumed that the mole fraction of phospholipids in the vesicles was the same as the mole fraction of phospholipids in the starting material.

Chemical Modification of Protein S. Chemical modifications were carried out exactly as described by Wright and co-workers for the modification of prothrombin fragment 1 (Wright et al., 1984). Protein S samples (0.5 mg/mL) were adjusted to a pH 5.2 with 1% glacial acetic acid prior to the incubation with the formaldehyde morpholine reagent. Samples were separated from the reaction components by exhaustive dialysis against 0.1 M NaCl and 0.02 M Tris-HCl,[1] pH 7.5. The degree of modification was determined by measuring the degree of incorporation of ^{14}C from the (^{14}C)formaldehyde and measurement of protein concentration. The activity of protein S was not affected if the morpholine and formaldehyde were omitted from the reaction mixture.

Fluorescence Measurements of Protein S. The intrinsic fluorescence of protein S was determined on a Perkin-Elmer MPF 66 fluorometer. Fluorescence measurements were made by using an excitation wavelength of 295 nm and integrating the emitted, corrected fluorescence spectrum between 310 and 370 nm. Prior to integration appropriate backgrounds and Raman scattering were subtracted from the spectrum. The areas under the spectrum were calculated by using a base line in which the relative fluorescence at 310 and 370 nm was zero. Calcium quenching experiments were carried out with protein in 0.1 M NaCl and 0.02 M Tris-HCl, pH 7.5. Titration with terbium ions was carried out in 0.1 M NaCl and 0.02 M MES, pH 6.2. the effect of terbium on intrinsic fluorescence was determined in the manner described for calcium except that fluorescence was excited at 285 nm. Terbium fluorescence was measured by integrating the spectrum from 540 to 555 nm. Appropriate backgrounds were subtracted from the corrected spectrum.

Interaction between Protein S and Phospholipid Vesicles. The interaction between protein S and phospholipid vesicles was determined by the method described by Nelsestuen and Lim (1977). Relative 90° light-scattering measurements were made in a Farand Mark I spectrofluorometer at room temperature. All experiments were carried out in a 2.0-mL final volume of 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, and 3.2 μg/mL phospholipid with 0.8 μM protein S. Calcium chloride was added to the indicated concentrations and mixed, and the relative scattering was measured. Scattering was compared to a solution that contained protein and phospholipid. Data were converted to relative molecular weight (M_2/M_1) of the protein–lipid complex by using the method described by Nelsestuen and Lim (1977). In this method the relative light scattering of the phospholipid vesicles (I_{s1}) and protein with lipids (I_{s2}) was determined. The latter was corrected for unbound protein, and the relative molecular weight of the protein–phospholipid complex was determined from the relationship

$$I_{s2}/I_{s1} = [(dn_2/dc_2)/(dn_1/dc_1)]^2(n_2/n_1)^2$$

where dn/dc represents the refractive index increment for each of these species. dn_2/dc_2 was calculated by the iterative method described by Nelsestuen and Lim (1977) using the values of 0.192 for protein and 0.172 for phospholipid that they reported.

Sedimentation Equilibrium. For the determination of the functional apparent molecular weight of protein S, sedimentation equilibrium was carried out in an air-driven ultracentrifuge (Beckman Airfuge) by the method described by Bock and Halvorson (1983). Solutions (0.150 mL) were sedimented at 36 000 rpm for 24–36 h at room temperature. Tubes were then fractionated with a micropipet and then assayed. All samples were sedimented in duplicate tubes. For most runs standards (either prothrombin or factor X) were determined along with the protein S samples. Calculations were performed by using the computer program described by Bock and Hal-

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid.

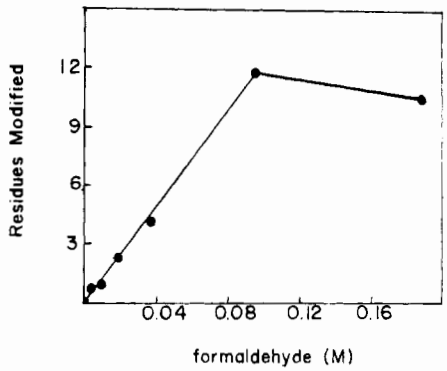

FIGURE 1: Effect of the concentration of the modifying reagent on the degree of modification of Gla residues. Protein S was incubated for 24 h with the indicated concentrations of formaldehyde and an equal concentration of morpholine as described under Experimental Procedures. Following modification, the samples were dialyzed against 8000 volumes of buffer (0.1 M NaCl and 0.02 M Tris-HCl, pH 7.4) for 36 h. The degree of modification was determined by measuring the incorporation of (^{14}C)formaldehyde into the protein.

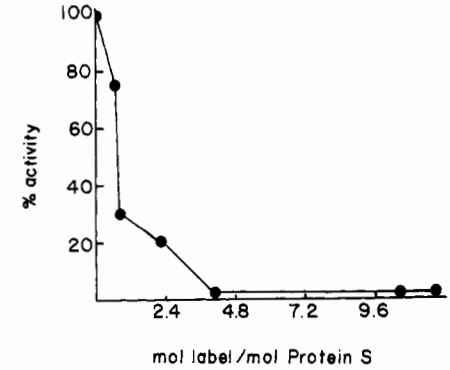

FIGURE 2: Relation between degree of modification and activity. Modified protein S samples were assayed in the one-stage clotting assay for protein S. No effect of incubation on the control samples was observed.

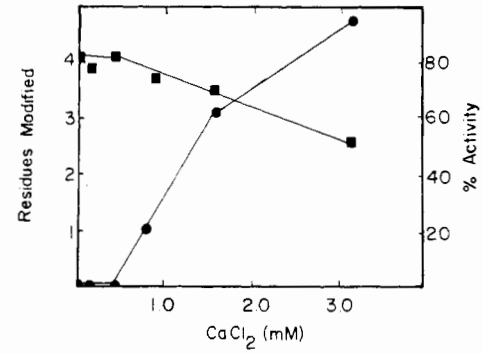

FIGURE 3: Effect of calcium ions on modification. Protein S samples were incubated with 0.04 M morpholine/formaldehyde reagent as described in Figure 1 except that the indicated concentrations of calcium chloride were included during the first 24-h incubation. The number of residues modified (squares) and activity (circles) were determined as described under Experimental Procedures.

vorson (1983). Molecular weight determinations for the standards were within 10% of the literature values.

Apparent molecular weights were also determined on purified samples of protein S in the analytical ultracentrifuge by using the method of miniscus depletion as described by Yphantis (1964). Prior to centrifugation in the analytical ultracentrifuge all samples were centrifuged for 5 min in a microfuge to remove any large particles that might be present. A 1.5 mg/mL solution (0.12 mL) of protein S in 0.1 M NaCl and 0.02 M Tris-HCl was loaded into a 12-mm double-sector cell with interference windows and sapphire windows that contained 0.02 mL of FC-43 fluorocarbon oil. Samples were centrifuged for 24 h at 18 000 rpm. At this time interference fringe photos were taken every 2–6 h until equilibrium was achieved. For the calculation of protein concentrations from fringe displacements a protein refractive increment of 0.19 mL/g (at 514.5 nm) (Sober, 1970) was used. Measurements of the fringe displacements and calculations of the molecular weights and association constants were carried out as described by Yphantis (1964).

Assay of Protein S. Protein S was assayed by a modification of previously reported methods. Samples to be assayed for protein S were added to protein S deficient plasma. Clotting was initiated by the addition of phospholipid (0.11 mg/mL), calcium (6 mM), activated protein C (0.01 mg/mL), and factor Xa (the factor Xa concentration was adjusted to give a blank time of 18 s). The total volume of the assay was 0.4 mL. There was a linear relation between prolongation of clotting time and protein S concentration. Standard curves were prepared by diluting citrated plasma into the deficient plasma. Protein S deficient plasma was prepared as described previously (Walker, 1980).

RESULTS

Degree of Modification. A linear relationship between the number of residues modified and the concentration of the formaldehyde–morpholine reagent used in the modification was observed (Figure 1). Maximum modification was observed when the mole ratio of formaldehyde to protein S was approximately 10 000. The cofactor activity of protein S was found to be sensitive to the degree of modification. Modification of one residue resulted in a 50% loss of activity. The modification of four residues resulted in a derivative with no cofactor activity (Figure 2).

Modification in the Presence of Calcium. In order to determine if the modification pattern might be sensitive to calcium, tests were carried out in the presence of various concentrations of calcium ions. The concentration of morpholine/formaldehyde used resulted in the modification of between four and five residues. Below 0.5 mM, calcium ions had no effect on either the loss of protein S activity or the number of residues modified (Figure 3). In the presence of 3.2 mM calcium between two and three residues of Gla were modified. In addition to the inhibition of the chemical reaction, calcium also appeared to prevent the loss of activity. This result suggests that some of the modified residues may not be essential for the activity of protein S.

Effect of Modification on Intrinsic Fluorescence. Modification of the protein altered some of the fluorescence properties of protein S. In some samples that were fully modified a slight red shift in the emission maxima was observed. In addition, there was a small decrease in the maximum effect of calcium on the quenching of intrinsic fluorescence (Figure 4). The effect was maximally observed in the samples that had 10–11 residues modified. There was an increase in the apparent dissociation constant for calcium from 0.87 to 2.08 mM between the native and fully modified samples. Samples with only two to three residues modified were intermediate between the native protein and the completely modified protein. Hill plots of the data gave slopes that were not significantly different ranging from 1.25 to 1.62. The results obtained from all of the fluorescence experiments were representative examples obtained from six independent protein preparations.

The intrinsic fluorescence of protein S and modified protein S was examined in the presence of terbium ions. Like calcium,

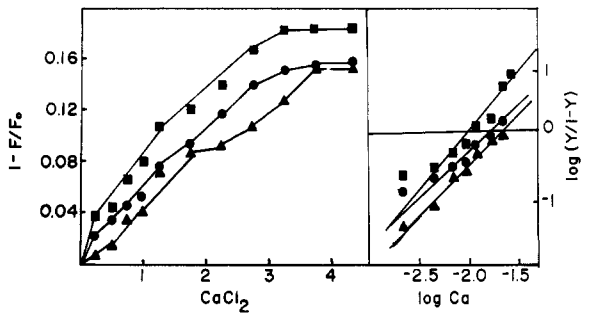

FIGURE 4: Fluorescence quenching of native and modified protein S. (Left panel) Intrinsic fluorescence of native protein S (squares) (0.4 μM), 10-Gla-modified protein S (triangles) (0.4 μM), and 2-Gla-modified protein S (circles) (0.4 μM) was measured in the presence of increasing concentrations of calcium as described under Experimental Procedures. 2-Gla-modified protein S was prepared by modification of the protein in the presence of 3.2 mM $CaCl_2$. (Right panel) Hill plot of fluorescent data. Y represents the fraction of saturation. The slopes and dissociation constants from the plots are 1.62, 0.87 mM (squares); 1.25, 1.55 mM (circles); and 1.54, 2.08 (triangles).

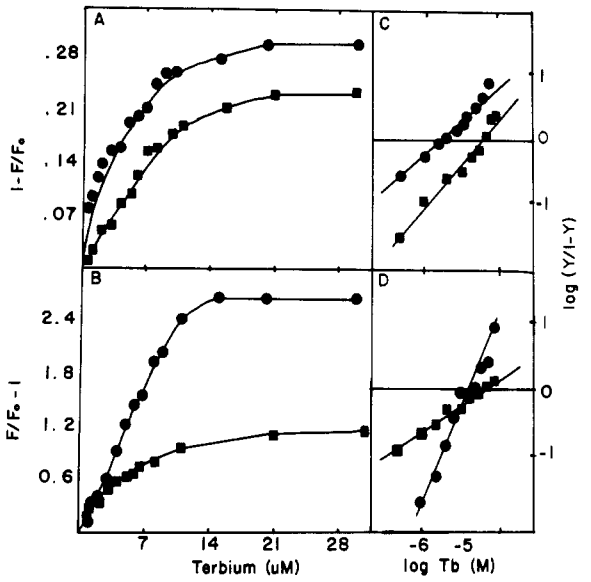

FIGURE 5: Fluorescence determination of effect of terbium ions on protein S. Intrinsic protein fluorescence (A) and terbium fluorescence (B) were determined as described under Experimental Procedures for the native protein (circles) (0.45 μM) and samples with 10 Gla residues modified (squares) (0.45 μM). The data in panels A and B have been replotted by using the Hill equation in panels C and D, respectively. Y represents the fraction saturation of protein S.

terbium quenches the intrinsic fluorescence of protein S (Figure 5A). Quenching of intrinsic fluorescence was only slightly reduced in the modified protein. There was a slight difference between the apparent dissociation constant for terbium in the native (1.4 μM) and the modified protein (5.5 μM). The maximum quenching was slightly reduced for the modified protein (Figure 5A). The slopes of the Hill plots were also not significantly different (1.2 vs. 1.4), indicating that the binding that caused quenching was probably not cooperative. Terbium ions also exhibit fluorescence when they bind to proteins adjacent to aromatic amino acids. The excitation spectrum suggested that it was due to energy transfer from tryptophan residues (data not shown). The addition of calcium reduced this fluorescence, suggesting that the terbium could bind to calcium binding sites (data not shown). Terbium fluorescence was also observed with modified protein S (Figure 5B). There was no significant difference between the dissociation constant for the native (3.4 μM) and modified protein

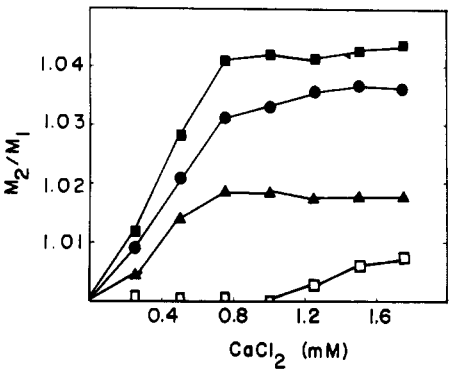

FIGURE 6: Effect of modification on lipid-binding properties. Protein S binding to phospholipid vesicles was determined by the light-scattering technique described under Experimental Procedures. The protein concentration used in these experiments was 0.8 μM. Binding was measured for the native protein (filled squares), 1-Gla-modified protein S (circles), 2-Gla-modifed protein S (triangles), and 10-Gla-modified protein S (open squares). Scattering measurements were converted to relative molecular weights by the method described under Experimental Procedures.

(3.5 μM) (Figure 5D). Total fluorescence at saturating terbium was reduced in the modified samples, and there was a significant difference in the Hill plots (2.8, native; 0.9, modified) (Figure 5D). This result suggests that the Gla residues can bind terbium ions and that the binding in the native protein is cooperative.

Effect of Modification on Lipid Binding. Protein S is thought to act by stimulating the formation of a factor Va inactivation complex consisting of itself, activated protein C, calcium ions, and phospholipids (Walker, 1981a). The binding of protein S to phospholipid membranes is essential for the expression of its cofactor activity. Most models of the interaction between the vitamin K dependent proteins and phospholipids have been that the Gla residues are essential for this interaction. To test this hypothesis the binding of modified protein S to phospholipid vesicles was measured. As the amount of modification increased, the degree of binding to lipid vesicles decreased (Figure 6). This finding confirms the importance of Gla residues in the maintenance of the lipid-binding properties of this protein.

Effect of Modification on Self-Association. Self-association has been observed with prothrombin. This interaction appears to depend on the presence of calcium and is mediated through the Gla residues in the fragment 1 region of the protein. To determine if protein S has a similar property its solution properties were examined in the ultracentrifuge. The molecular weight of protein S was measured by using three initial concentrations and several rotor speeds. The molecular weight appeared to be heterogenous under all conditions chosen. This was apparent both from curvature of log concentration vs. r^2 plots as well as from point-averaged molecular weight vs. concentration plots of the data, representative examples of which are shown in Figure 7. The apparent molecular weight of the monomer appeared to be between 62 000 and 64 000. The apparent molecular weight of the higher molecular weight form appeared to be between 120 000 and 130 000. Under the assumption that protein S could exist as either a monomer or a dimer, association constants were calculated for a number of conditions. In the absence of added metal ion the association constant appeared to be approximately 1.8×10^5 M^{-1}. There was no significant change in the molecular weight distribution when measured in the presence of 2.5 mM calcium chloride. Two-chain protein S derived from thrombin cleavage was also analyzed. Thrombin-cleaved protein S has been observed to have no cofactor activity (Walker, 1984; Suzuki et al., 1983).

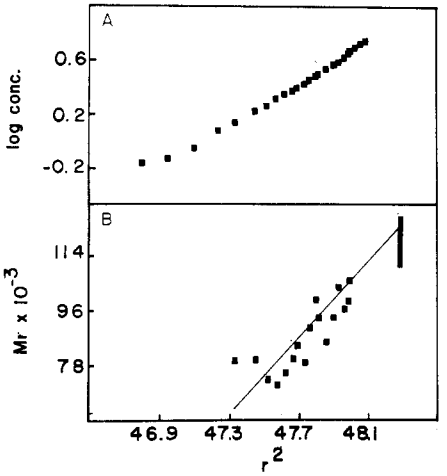

FIGURE 7: Sedimentation equilibrium of protein S. Protein S (1.5 mg/mL, initial concentration) was centrifuged at 18 000 rpm for 24 h in the analytical ultracentrifuge. (A) log concentration vs. radius squared. (B) Molecular weight vs. radius squared.

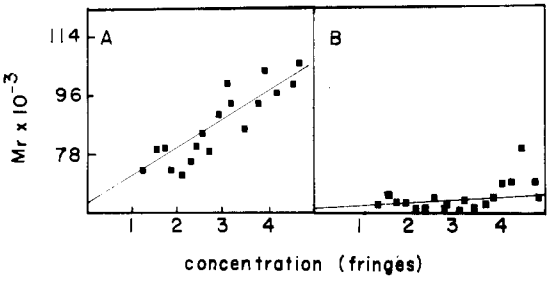

FIGURE 8: Sedimentation equilibrium of protein S (initial concentration 1.5 mg/mL) (A) and two-chain protein S (initial concentration 1.0 mg/mL) (B). Plots of apparent molecular weight vs. concentration in fringes. Molecular weights were calculated by taking the first derivative of the log concentration vs. r^2 plots.

This appears to be related to its altered calcium and lipid binding properties. Two-chain protein S appeared to exist entirely as a monomer with a molecular weight of 62 000 (Figure 8).

Though there did not appear to be a calcium dependence to self-association, the effect of Gla modification was examined. Molecular weights of native protein S and protein S modified to the degree of 2 residues in the presence of calcium were determined in the air-driven ultracentrifuge. This was carried out so that aggregation or denaturation of the sample would not be a problem. Native protein S had a molecular weight of approximately 120 000, and the modified protein S had a molecular weight of 62 000 (Figure 9). The initial protein concentration in these experiments was 1.0 mg/mL. Molecular weight determinations of completely modified protein S were also carried out in the analytical ultracentrifuge. In these experiments (data not shown), it was determined that the association constant for the modified protein was less than 5×10^3 M^{-1}.

Discussion

The γ-carboxyglutamic acid residues of protein S were modified to γ-methyleneglutamic acid by using a method described by Wright and co-workers (1984). Using this method it appears that protein S contains 10 or 11 residues of Gla. This corresponds well to the 10 residues of Gla found in protein S by DiScipio and co-workers (1977). The results suggest that not all of the Gla residues are equal with respect to cofactor function. While some of the Gla residues appear to be essential for the expression of the cofactor activity, others

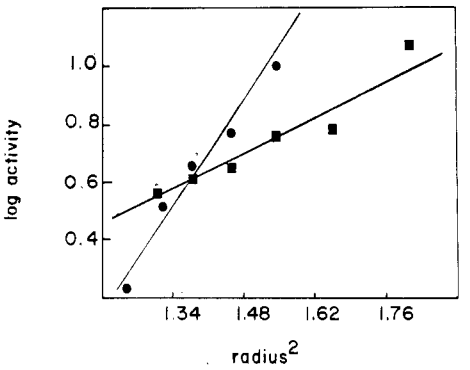

FIGURE 9: Effect of modification on self-association. Protein S (circles) and 2-Lal-modified protein S (squares) were centrifuged for 30 h in the air-driven ultracentrifuge. 2-Gla-modified protein S was prepared by modification in the presence of 3.2 mM $CaCl_2$. The initial concentration of each protein was 1.0 mg/mL. The total volume of sample was 0.150 mL.

appear to be involved in the maintenance of other structural properties of the protein. When the protein is treated with the modifying reagents in the absence of added divalent cations, the most reactive residues are those closely related to the expression of the cofactor activity. Between two and four of these Gla residues must be intact for the expression of cofactor activity. When the protein is modified in the presence of calcium, a different pattern is observed. In the presence of 3.2 mM calcium ions the more reactive residues are not essential for cofactor activity. These residues, however, are important for the maintenance of the self-association properties of the protein.

It has been proposed that protein S acts by forming a lipid-bound complex with activated protein C (Walker, 1981a). This complex is the main inhibitor of activated factor V. Several studies have demonstrated a relationship between lipid binding and the cofactor activity of protein S. Studies of the binding of activated protein C to phospholipid vesicles in the presence and absence of protein S indicated that protein S can enhance the binding of activated protein C to phospholipid vesicles. Thrombin-treated protein S, a two-chain protein, has lost both cofactor activity and its lipid-binding properties (Walker, 1984; Suzuki et al., 1983). None of the Gla-modified proteins have been found to inhibit the function of activated protein C (unpublished observation), suggesting that lipid binding is important for the formation of the activated protein C binding site on protein S. This also appeared to be the case with two-chain protein S (Walker, 1984).

The observation that vitamin K promotes the carboxylation of glutamic acid residues in the vitamin K dependent proteins (Stenflo et al., 1974; Nelsestuen et al., 1974) has led to a number of studies to determine the role of the Gla residues in calcium binding, the effect of calcium binding on protein structure, and the role of bound calcium ions in the mediation of the interaction between the proteins and membranes. Both Gla-dependent and Gla-independent calcium binding has been observed for the vitamin K dependent proteins. Using the quenching of intrinsic protein fluorescence as a measure of the interaction between calcium and the protein, it has been possible to characterize each type of interaction. In the case of prothrombin, it has been observed that calcium, magnesium, manganese, and terbium can quench the intrinsic fluorescence of prothrombin fragment 1 (Prendergast & Mann, 1977). Modification of the Gla residues in fragment 1 lead to the complete loss of calcium quenching of the intrinsic fluorescence, suggesting that the Gla residues are involved in this property (Wright et al., 1984). Calcium also quenches the

intrinsic fluorescence of factor X (Skogen et al., 1983; Sugo et al., 1984). For this protein, it also appears that the Gla residues are involved, as a proteolytic derivative that lacks these amino acids cannot be quenched. On the other hand, several proteins including factor IX (Morita et al., 1984) and protein C (Johnson et al., 1983) appear to have non-Gla binding sites for calcium that lead to fluorescent quenching. In this respect protein S resembles the latter two proteins since modification of the Gla residues has only a small effect on the ability of calcium to quench the fluorescence.

It has been suggested that the quenching of intrinsic fluorescence is associated with a structural transition. For prothrombin it has been observed that the transition precedes prothrombin–phospholipid interactions (Nelsestuen, 1976). In addition, a number of investigators have isolated antibodies that are specific for the calcium-induced conformer of prothrombin (Madar et al., 1982; Tai et al., 1980, 1984). Others have suggested that the observed quenching might be due to calcium-induced self-association of the protein (Jackson et al., 1979). Though most of the studies have involved prothrombin, studies with protein C also suggest that calcium quenching is associated with a conformational change, as its ability to act as a substrate for thrombin is associated with this change (Esmon et al., 1982), though it appears that this transition is not associated with the Gla residues. The quenching of intrinsic fluorescence in protein S did not appear to be strongly related to Gla-dependent binding of calcium since modification had only a modest effect on calcium quenching. There appeared to be a greater effect on terbium binding following Gla modification as the total terbium effect was reduced and the cooperativity of its interaction was lost. Though protein S has been observed to self-associate, the process did not appear to be calcium dependent (data not shown). The role of the calcium-induced conformational change in protein S is unclear since it did not appear to be associated with changes in function.

Two hypotheses have been developed to explain the role of the Gla residues in the interaction between membranes and the vitamin K dependent proteins. The first hypothesis suggests that calcium forms an ion bridge between the protein and the phospholipid (Suttie & Jackson, 1977). The second is that calcium binding induces a conformational change that exposes a membrane-binding surface (Rhee et al., 1982; Madar et al., 1982). Gla residues have been shown by a number of investigators to be associated with calcium-mediated protein binding to membranes (Nelsestuen & Suttie, 1972a, 1972b; Stenflo & Ganrot, 1972; Stenflo, 1972; Esmon et al., 1975). When Gla residues have been removed, lipid binding is always lost (Bajaj et al., 1982; Tuhy et al., 1979). This was also found to be true for protein S, as modification of the Gla residues resulted in the loss of lipid binding. Since the structural transition could still be observed, as monitored by changes in fluorescence, it appears that, at least in this case, the observed transition is not involved in membrane binding.

The trend from the literature is that two types of calcium-induced protein transitions can be observed. One, that associated with the calcium ion–Gla interaction, is important for membrane binding. The other, associated with the non-Gla calcium binding sites, is important in the mediation of other functions of the protein. Calcium quenching of protein S appears to be predominantly of the second type with a minor transition associated with the Gla residues. Those Gla residues that were most reactive to modification were found to be essential for membrane binding and cofactor activity. The residues of lesser reactivity did not appear to be as important in the maintenance of function.

Registry No. Ca, 7440-70-2; blood coagulation factor XIVa, 42617-41-4; γ-carboxyglutamic acid, 53445-96-8.

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Chemical Modification of Rhodopsin and Its Effect on Regeneration and G Protein Activation†

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ABSTRACT: The studies reported are concerned with the functional consequences of the chemical modifications of the lysines and carboxyl-containing amino acids of bovine rhodopsin. The 10 non-active-site lysine residues of rhodopsin can be completely dimethylated and partially acetimidated (8–9 residues) with no loss in the ability of the proteins to activate the G protein when photolyzed or to regenerate with 11-*cis*-retinal. These modifications do not alter the net charge on the protein. Surprisingly, heavy acetylation of these lysines (eight to nine residues) with acetic anhydride, which neutralizes the positive charges of the lysine residues, yields a modified rhodopsin fully capable of activating the G protein and being regenerated. It is concluded that the non-active-site lysine residues of rhodopsin are not importantly and directly involved in interactions with the G protein during photolysis. However, this is not to say that they are unimportant in maintaining the tertiary structure of the protein because heavy modification of these residues by succinylation and trinitrophenylation produces proteins incapable of G protein activation, although the succinylated protein still regenerated. The active-site lysine of rhodopsin was readily modified and prevented from regenerating with 11-*cis*-retinal and with *o*-salicylaldehyde and *o*-phthalaldehyde/mercaptoethanol, two sterically similar aromatic aldehyde containing reagents which react by entirely different mechanisms. It is suggested that rhodopsin contains an aromatic binding site within its active-site region. Monoethylation, but not monomethylation, of the active-site lysine also prevented regeneration. Finally, the functional consequences of the chemical modification of the aspartate and glutamate residues were studied. These residues were modified by their carbodiimide-mediated coupling to (1) taurine, (2) glycine methyl ester, and (3) ethylenediamine, reagents which (1) do not alter the negative carboxyl group charge, (2) neutralize it, and (3) convert it into a positive charge, respectively. These modifications did not alter the abilities of the modified rhodopsin derivatives to activate the G protein after photolysis, but they did prevent it from regenerating with 11-*cis*-retinal after bleaching.

Rhodopsin is an integral membrane protein found in the disks of rod outer segments. This protein, of molecular weight 39K, contains a chromophore, 11-*cis*-retinal, covalently bound to lysine-296 by means of a protonated Schiff base (Hargrave et al., 1983). The absorption of a photon of light by rhodopsin results in the cis to trans isomerization of the chromophore (Hubbard & Kropf, 1958). This configurational change about the double bond is translated into a series of conformational changes in the protein (Wald, 1968). One of the spectroscopically defined conformers, metarhodopsin II (Parkes et al., 1979; Calhoon et al., 1981), interacts with and catalyzes a GTP for GDP exchange in a G protein, resulting in the activation of the latter (Fung & Stryer, 1980). This interaction with the G protein is the only known biochemical process directly affected by rhodopsin and serves as a probe for the activated form of the pigment (R*).[1] Activated G protein, in turn, releases a cGMP-specific phosphodiesterase from inhibition which results in the lowering of the free cGMP

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[1] Abbreviations: OPA, *o*-phthalaldehyde/mercaptoethanol; PIPES, 1,4-piperazinediethanesulfonic acid; PM-Rh, non-active-site lysine permethylated rhodopsin; R*, photochemically activated rhodopsin; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; Tris, tris(hydroxymethyl)aminomethane.

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