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The use of spin labels in looking at subtle conformational changes in blood coagulation proteins

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Abstract - Spin labels have now enjoyed a history of close to twenty-five years in demonstrating the applicability of paramagnetic nitroxides to biochemical problems of structure and function in enzymes, membranes, cells and animals. These have been employed traditionally as reporter groups; that is, the nitroxide spin label serves as a physical probe which reports aspects of its structure and environment at a localized molecular site. The work presented here describes the use of active-site directed fluorosulfonylphenyl nitroxide spin label inhibitors for the blood clotting enzyme, thrombin. The results describe a topographical mapping of the extended active site regions of *coagulant* human α-thrombin, *noncoagulant* human γ-thrombin, and bovine α-thrombin. Subtle differences are revealed between all of these species and forms. Lastly, interactions between the regulatory protein thrombomodulin and spin labeled α-thrombin are described which confirms the propagation of subtle conformational changes to the catalytic site in the thrombin molecule upon complexation.

INTRODUCTION

Spin labels have now enjoyed a history of close to twenty-five years in demonstrating the applicability of paramagnetic nitroxides to biochemical problems of structure and function in enzymes, membranes, cells and animals. These have been employed traditionally as reporter groups; that is, the nitroxide spin label serves as a physical probe which reports aspects of its structure and environment at a localized molecular site. It has been quoted many times that this reporter group must *"report the news"* not *"make the news"*. That is, it is important to insure that the sometimes bulky nitroxide spin label, does not perturb the macromolecular system under study. To this end, several groups, particularly Berliner and colleagues (ref. 1), have employed a *comparative* system of structural investigation to enzyme studies, whereby a spin label is placed at the same homologous site in a family of enzymes whose structures are highly similar. In that way, any perturbations of the molecular structure (if they indeed occur) are identical in all systems tested. Hence, the spin label becomes a valid tool for structural comparisons.

Perhaps one of the most enlightening studies in the use of nitroxide spin labels has been that with the proteolytic blood clotting enzyme, α-thrombin (ref. 2). This is a member of the common serine protease family which, however, displays a much more restricted, unique specificity than the typical digestive enzymes such as α-chymotrypsin or trypsin. Furthermore, to date the crystal structure of this enzyme from either the human or bovine species has yet to be reported. Therefore, a study of the structure and environment of thrombin in solution is even more challenging - results from the x-ray crystal structure may eventually *confirm* results from solution studies (quite the opposite from past history!)

The approach of Berliner and colleagues has been to mimic an inhibitor or substrate analog known to covalently and specifically react with the serine residue 195 at the active site of α-thrombin and other serine proteases (ref. 1). An extensive series of fluorophenylsulfonyl spin labels were synthesized (shown in Fig. 1), all of which had the fluorosulfonylphenyl moiety in common and varied by the position and structure of the nitroxide moiety attached to the aromatic ring (ref. 3). This also satisfied another important requirement for reporter group labeling studies: that is, one must acquire consistent reproducible results from more than one label. It is occasionally possible that structural results derived from the employment of a *single label* may be the reporter group "reporting the news," and not that of the protein structure itself. The spin labels, when organized in the order shown in Fig. 1, may be classified into two general groups: a linear group of labels, mostly *para*-substituted on the phenyl ring; and a bent series of labels mostly those substituted in the *meta* position. Two additional labels, designated as mobile in the Fig. 1, were difficult to classify on the enzyme as their freedom of rotation was so rapid that interactions with the protein structure could not be easily distinguished. These latter two fall in a case of labels which are simply not sensitive enough to the protein structure - not that the spin label method is of little use, but simply again showing the need for following a series of several labels (ref. 4).

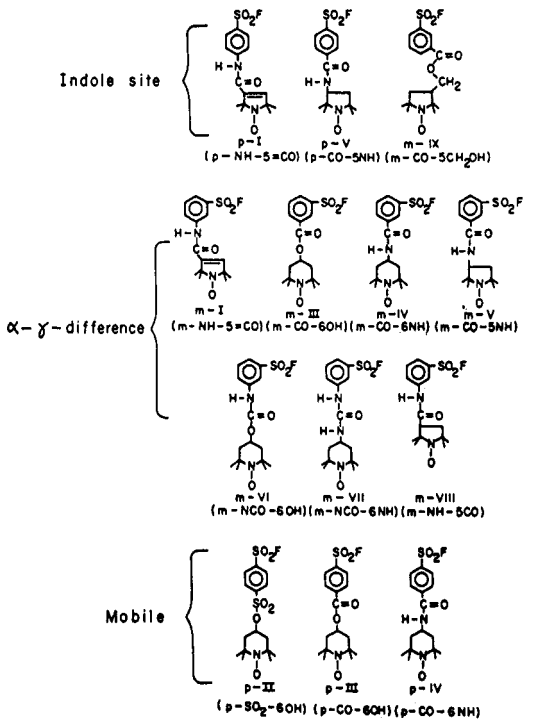

Fig. 1: Structures of spin-labeled sulfonyl fluorides. Compounds which are isomers of the same structure are designated by the same Roman numeral. The three classes of labels, indole site, α-γ difference, and mobile, respectively, are related to the tumbling domains in space of each nitroxide group relative to the sulfonyl substituent on the ring. From ref. 4 with permission.

HUMAN THROMBINS

A schematic of the primary structure of human thrombins is shown in Fig. 2. Note that three species of thrombin are shown, α- β- γ-thrombin. The natural coagulant α-thrombin shown in the top of Fig. 2 consists of two polypeptide chains and an **A** chain which is disulfide bridged to a **B** chain, the latter of which is homologous to the serine protease family. The terminology CHO designates the position of carbohydrate chains attached to the thrombin molecule. Upon standing several hours at room temperature, α-thrombin will autolytically convert to two reduced coagulant forms: β- and γ-thrombin (ref. 5). This conversion is also possible by controlled tryptic treatment (ref. 5). Both of the noncoagulant forms are unique in their noncovalently associated multichain structures. Of particular interest is γ-thrombin which contains

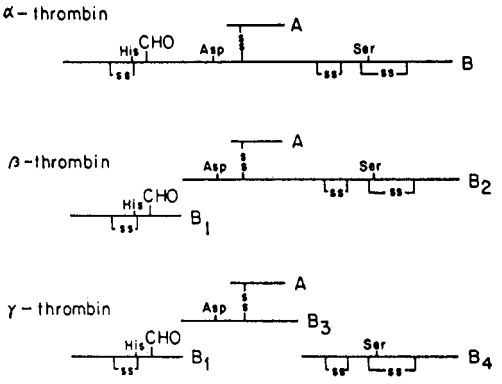

Fig. 2: Schematic polypeptide structures of human α-, β-, and γ-thrombins. While all three forms have estero- and amidolytic activity, only α-thrombin will catalyze fibrinogen cleavage in the clotting event. From ref. 4 with permission.

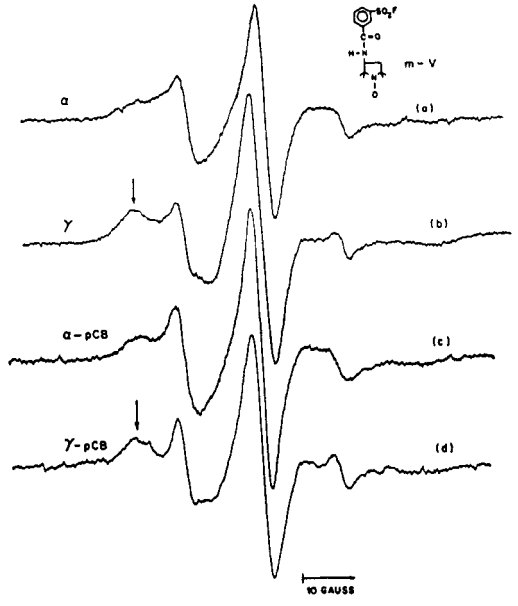

Fig. 3: X-band ESR spectra of human α- and γ-thrombin conjugated at the active serine with ***m*-V** (*m*-CO-5NH) (a) spin-labeled α-thrombin; (b) spin-labeled γ-thrombin; (c) spectrum (a) in the presence of 50 mM *p*-chlorobenzylamine; and (d) spectrum (b) in the presence of 50 mM *p*-chlorobenzylamine. All spectra were measured at pH 6.5, 0.05 M sodium phosphate and 0.75 M NaCl, 26 ± 2°C. Protein concentration was typically 0.07-0.08 mM. From ref. 4 with permission.

three noncovalent fragments, each of which contains one of the critical catalytic residues involved in proteolysis: His 57, Asp 102 and Ser 195. Remarkably, these three chains remain noncovalently associated in the proper conformation, affording catalytic activity to human γ-thrombin, however with the notable lack of fibrinogen clotting activity (ref. 5). Human γ-thrombin catalyzes the hydrolysis of small ester and amide substrates and, most importantly, reacts as efficiently as the α-form with spin labels and other structural probes (refs. 4-6). Human γ-thrombin therefore serves as a perfect analog of thrombin which *does not* clot. An understanding of the structural differences between α- and γ-thrombin helps us to understand the unique molecular interactions involved in thrombin-fibrinogen binding and catalysis in the blood clotting step. Fig. 3 depicts a typical series of X-band ESR spectra for ***m*-VI** labeled human α- and γ-thrombins. Note that with this bent, *meta*-substituted derivative, the γ-thrombin analog is significantly ***more immobilized*** than that of the α-form. Notably, this ***difference*** was found for ***every*** bent fluorosulfonyl phenyl spin label, all of which could be overlapped in structure within a small volume constraint. On the other hand, Fig. 4 shows comparative spectra for a *para*-substituted, linear label, ***p*-I** where we note in spectra **a** and **b** that the lineshapes

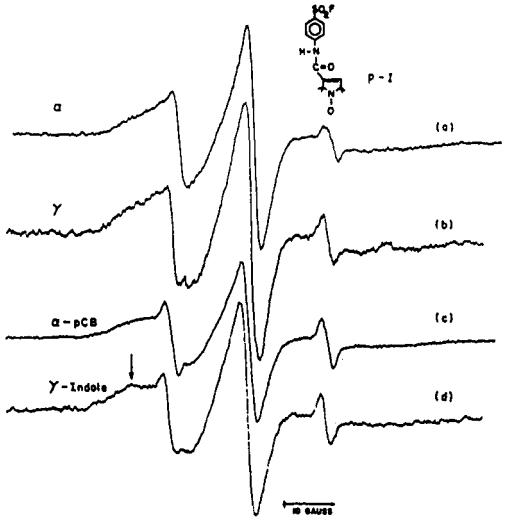

Fig. 4: X-band ESR spectra of human α- and γ-thrombin spin-labeled at the active serine with *p*-I(*p*-NH-5=CO). All conditions were identical with those in Figure 4. (a) ***p*-I-α**; (b) ***p*-I-γ**; (c) ***p*-I-α** plus 50 mM *p*-chlorobenzylamine (***p*-I-γ** under the same conditions gave an identical effect); and (d) ***p*-I-γ** in saturated (~20 mM) indole. From ref. 4 with permission.

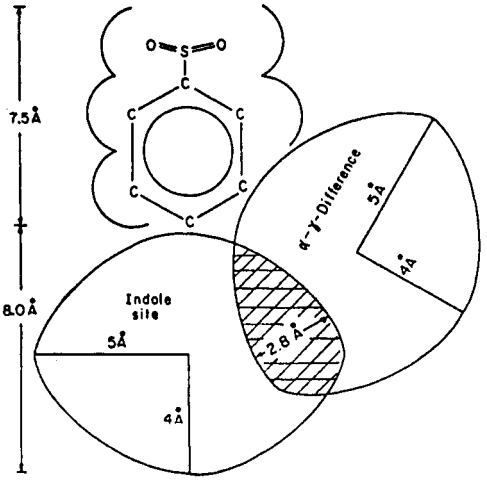

Fig. 5: Two-dimensional representation of human thrombin active site topography as a map constructed from the accessible volume of rotation of each nitroxide moiety with respect to the phenylsulfonyl group. The distances, which include van der Waals radii, were measured from Corey-Pauling-Koltun models. The distance marker at the phenyl ring bisects the covalent bond between the ring and the nitroxide moiety. The oblate ellipsoids of revolution approximate those of the smallest spin label that senses the designated thrombin structural feature. The volume of intersection excludes either the indole or α-γ difference sites. From ref.4 with permission.

for α- and γ-thrombin, respectively, are identical. That is, the structural environment sensed by the nitroxide in this case is ***identical*** in both enzyme forms. Thus, here we have an example of nitroxide spin labels detecting subtle conformational differences between human α- and γ-thrombin where in one case we are detecting those regions which are ***different***, while in the second case those regions that are ***identical***. Upon binding specific reversible basic inhibitors, such as benzamidine or *p*-chlorobenzylamine, to any of the spin-labeled thrombin forms, we note (e.g., Figs. 3c,d and 4c) an increased immobilization of the nitroxide. That is, upon binding the ligand at the active site specificity pocket, the phenylsulfonyl moiety is effected similarly in <u>all</u> cases, presumably causing the phenylsulfonyl nitroxide spin label to adopt an alternate orientation in order to accommodate benzamidine binding simultaneously. In most cases this results in a ***more immobilized*** spectrum.

Fig. 4d depicts thrombin labeled with the linear nitroxide spin label ***p*-I**, after exposure of either α- or γ-thrombin to the hydrophobic, apolar ligand indole. Here we note an ***increased*** immobilization; that is, upon binding this ligand to some specific apolar site on the thrombin molecule, a change in the structure or environment of the nitroxide is detected with this particular spin label, resulting in increased immobilization. This phenomenon occurred for **all** of the linear *para-*

substituted nitroxides while the effect was totally absent in all of the bent (α- γ-thrombin difference) labels (ref. 4). Here again is another example of the detection of subtle differences in the thrombin active site structure, here where the phenomenon exists in both α- and γ-thrombin at some site **different** from that where the two structures differ significantly. By model building, a topographical map, shown in Fig. 5, was constructed based on the rotational space available to this series of labels.

BOVINE THROMBINS

The primary structure of bovine α-thrombin is highly homologous to that of its human counterpart. In fact, from a quick perusal of their minor amino acid differences, one would never suspect any major differences in their catalytic and/or specificity properties. Yet, while the noncoagulant γ-form of human α-thrombin has essentially zero clotting activity, the corresponding bovine form (β-thrombin) retains 10% clotting activity: K_m is unchanged and k_{cat} is reduced 90% of that of the α–form (ref. 7). Figs. 6 and 7 depict spin label spectra comparing human and bovine α-thrombins with bent, *meta*- and linear *para*-substituted labels. It is important to note that in **all** cases the bovine analogs are more immobilized than that in the case of the human forms (Fig. 6). That is, it is clear that in the extended active site structure of bovine thrombin (i.e., the region at 10 - 12Å from serine 195) distinct differences exist in the structure and environment of the species (ref. 4). When examining aspects such as indole binding (Fig. 7), we note that the indole site also exists in bovine thrombin as well. However, with the label ***m*-VII**, these indoleeffects were noted in the case of bovine, but *not* human α-thrombin. On the other hand, the three other indole sensitive labels shown in Fig. 1 were common to both species in terms of eliciting an effect. That is, the bovine α-thrombin structure differs subtly in its apolar (indole binding) region from that of the human form, as detected by spin labels (particularly in the case of the nitroxide ***m*-VII**).

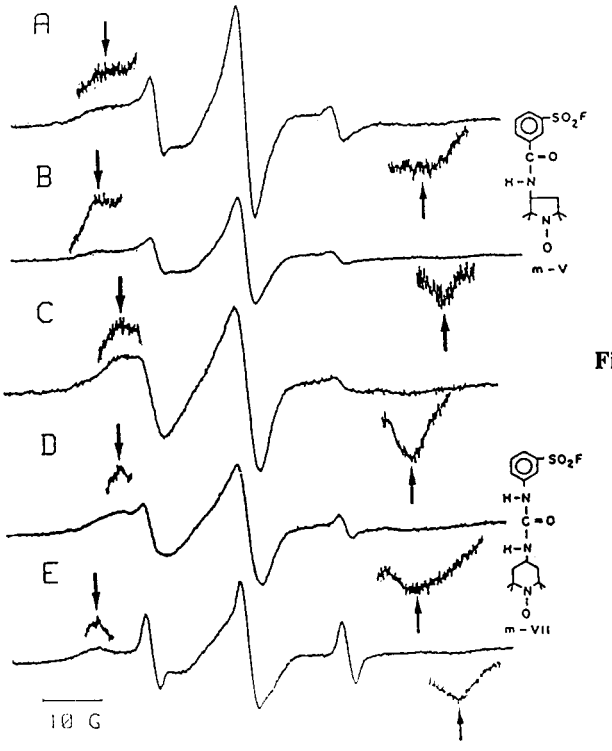

Fig. 6: X-band ESR spectra of active site spin-labeled human and bovine α-thrombins (a) **m-V** labeled human α-thrombin; (b) **m-V** bovine α-thrombin; (c) **m-VII** human α-thrombin; (d) **m-VII** bovine α-thrombin; and (e) spectrum (d) in 100 mM benzamidine hydrochloride. Conditions were pH 6.5, 0.05 M Tris-HCl, 0.75 M NaCl, 20°C. Protein concentrations were typically 0.07-0.08 mM. Arrows (↓) indicate points used in determining the maximum hyperfine splitting (2T//). From ref.7 with permission.

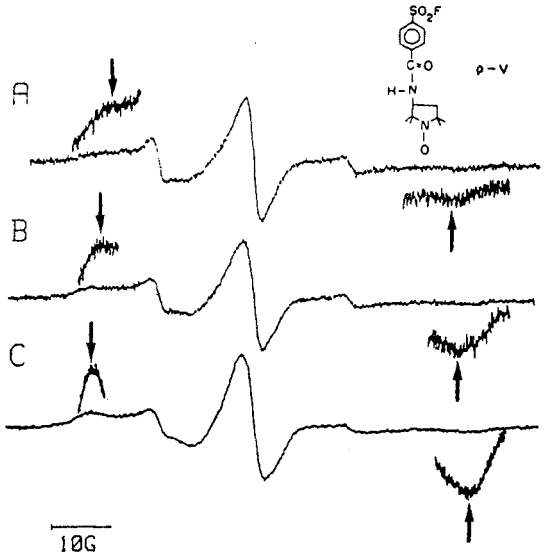

Fig. 7: X-band ESR spectra of p-V (*p*-CO-5NH) spin-labeled human and bovine α-thrombin in the presence of saturated sucrose (a) human; (b) bovine; (c) bovine in the presence of 10 mM indole in saturated sucrose. All other conditions were identical to those in Fig. 2. From ref.7 with permission.

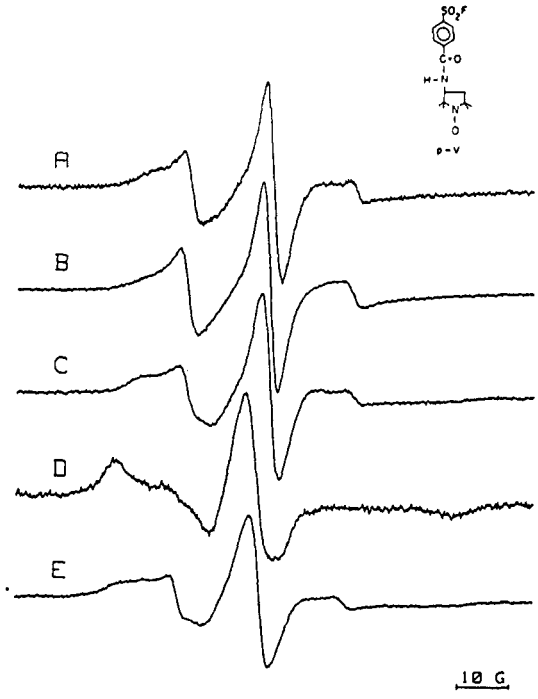

Fig. 8: X-band ESR spectra of **p-V** spin-labeled α-thrombin-TM complexes: (a) **p-V** spin-labeled α-thrombin-ε-TM; (b) *isokylindric* control of **p-V**-labeled thrombin in 24% (w/v) sucrose; (c) **p-V**-labeled thrombin-TM; (d) as in (c) but in saturated sucrose; and (e) **p-V**-labeled thrombin in saturated sucrose. Experimental conditions: 0.16 M NaCl; 33 μM labeled thrombin and 35 μM ε-TM (a); 200 μM labeled thormbin (b); 66 μM labeled thrombin and 98 μM TM (c,d); 66 μM labeled thrombin (e). From ref. 10 with permission.

THROMBIN INTERACTIONS WITH OTHER COAGULATION RELATED MACROMOLECULES

Thrombin, besides being responsible for the catalysis of the hydrolysis of fibrinogen to fibrin (and eventually the blood clot), interacts with several other factors on hemostasis. In fact, it is the central enzyme in the control of hemostasis. Of particular interest is a protein called thrombomodulin, which has the properties of activating thrombin efficiency in a unique anticoagulant pathway; specifically, the protease, protein C, is involved in a negative feedback control process which inactivates enzymes in the blood clotting cascade which normally turns on thrombin (ref. 8). That is, there is a chain reaction for the onset of coagulation and a chain reaction for the shutdown of coagulation. Once thrombin levels are high enough, it is necessary not only to both inactivate thrombin but those enzymes which catalyze thrombin propagation. Protein C (PC) is one such anticoagulant enzyme, which is turned on by thrombin. In a unique regulatory system, the protein thrombomodulin (TM), activates thrombin **activation** of protein C from an inactivated noncatalytic form (PC) to the activated form (APC). The activation factor may be from 1,000 to 20,000, depending upon the species (refs. 8,9). It was already known that thrombomodulin binds to some part of the thrombin surface which is not at the active site but somewhat removed, but the interaction causes conformational changes at the active site. Musci, Berliner, and Esmon examined the interactions between phenylsulfonyl spin-labeled human α-thrombin and a 50,000 MW active fragment (ε–) of thrombomodulin (ref. 10). Fig. 8 shows an example of one of these studies. Fig. 8a is that for ***p*-V** labeled human α-thrombin in aqueous solution. Fig. 8c is the spectrum obtained for the ε-thrombomodulin-thrombin complex. Since the resultant molecular weight is now ca. 86,600, we would expect that macromolecular tumbling contributions should effect the overall nitroxide lineshape. Therefore, we examined isolated spin-labeled human α-thrombin in both 24% (w/v) and saturated sucrose (Figs. 8b,e) and compared this with the analogous thrombin-thrombomodulin complex in both the presence (Fig. 8d) and absence of saturated sucrose (Figs. 8a,c). Comparison of both samples in saturated sucrose is important since the resultant lineshape reflects not only a reduced tumbling rate of the thrombin monomer (to near the time sensitivity limit of the conventional X-band ESR experiment), but also reflects microviscosity contributions the nitroxide moiety and the solution environment. As noted in the comparison between Figs. 8cand 8d for the thrombin-thrombomodulin complex, there is additional line broadening in the presence of saturated sucrose, even though the overall macromolecular complex has slowed motion well below that of the ESR time sensitivity. By comparing the results of Figs. 8a and 8c, we may conclude that upon complexation with thrombomodulin, a conformational change is elicited in the active center around the region where the nitroxide moiety tumbles.

CONCLUSIONS

These results show the ability of spin labels to detect subtle conformational changes in a thrombin macromolecular complex, which will not be observable by X-ray crystallographic methods in the very near future.

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