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Active-Site Topography of Human Coagulant (α) and Noncoagulant (γ) Thrombins†

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ABSTRACT: Human α- and γ-thrombins (clotting vs. nonclotting activities, respectively) were conjugated at their active-site serine with a series of 13 phenylsulfonyl fluoride spin-labeling reagents. One class (*indole-site reagents*), predominantly para-substituted phenylsulfonyl fluorides with an overall linear conformation, was sensitive to indole binding at an apolar site common to both α- and γ-thrombins. A second class (α–γ *difference reagents*), all meta substituted, which had an overall bent conformation, exhibited more immobilization when conjugated to γ- vs. α-thrombin, attributable to either a steric obstruction or increased hydrophobicity where the nitroxide moiety binds. A third class exhibited relatively *mobile* spin-label spectra, indicative of little or no interaction with either the indole site or α–γ difference site mentioned above. When spin-labeled bovine trypsin and α-thrombin were compared, several similarities were found with most of the meta-substituted reagents, but not with para-substituted reagents. The most notable difference, however, between the thrombins and trypsin was the strong immobilization of all spin-labeled α- or γ-thrombins upon exposure to ligands which bind to the basic specificity pocket, while the ESR spectra of the corresponding spin-labeled trypsin derivatives were totally unaffected by ligand binding to its structurally homologous binding pocket. These differences were attributed to both critical substitutions and/or deletions in the sequence lining the thrombin specificity pocket. From analysis of CPK models, a topographical map was derived describing the relationship of the indole and α–γ difference sites to other loci in the catalytic center. These two regions were approximated by oblate ellipsoids of revolution (minor axis 4 Å, major axis 5 Å) which extended out from the phenylsulfonyl ring. The indole site ellipsoid was attached to the para position of the phenyl ring by its minor axis; the α–γ difference site ellipsoid was attached to the meta position of the phenyl ring, also by its minor axis (i.e., at an orientation relative to the indole site by a 60° rotation in the plane of the phenyl ring). This "double ellipsoid" model overlapped in a region approximately 2.8 Å wide. These data provide direct physical evidence for tertiary structural differences in certain active-site regions of γ-thrombin, which may be related to its loss of clotting and other biologically important activities.

Coagulant α-thrombin (EC 3.4.21.5) is a serine protease generated in the final preclotting events of blood coagulation and has several bioregulatory functions in hemostasis [see Fenton et al. (1979)]. Although the human and bovine enzymes have been crystallized (McKay et al., 1977; Tsernoglou & Petsko, 1977), the three-dimensional structure has thus far been unobtainable, and thrombin structural features have been inferred only from amino acid sequence homologies between its larger B chain (Figure 1) and the pancreatic serine proteases (Magnusson et al., 1975; Elion et al., 1977). In the human system, autolytic or limited tryptic fragmentation of the α-thrombin B chain gives rise to two and subsequently three B-chain fragments of β- and γ-thrombins, respectively (Figure 1). These enzyme forms lack fibrinogen clotting activity and certain other biological functions but retain similar estero- and amidolytic and most other proteolytic activities of the parent form. Because the functional residues of the catalytic triad (His-43, Asp-99, and Ser-205) are contributed by the noncovalently bound B-chain fragments in γ-thrombin, the fragments remain in noncovalent association in the functional enzyme form (Fenton et al., 1979). Comparisons between α- and γ-thrombins have shown that the noncovalent form (i.e., γ-thrombin) more readily denatures (Bauer et al., 1980; Chang et al., 1980), yet displays remarkably similar intrinsic fluorescence and other properties as dansyl-, anthraniloyl-, or spin-label-active serine conjugates (Berliner & Shen, 1977a–c).

In this work, the complete fluorosulfonylphenyl spin-label series of Berliner & Wong (1974) has been employed to examine active-site topography and differences between human α- and γ-thrombins. The method involves the isomorphous incorporation of several topographically sensitive spin-labels in both α- and γ-thrombins and subsequent comparisons of nitroxide mobility both between the two thrombin forms and each structural class of spin-label. The nitroxide tumbling

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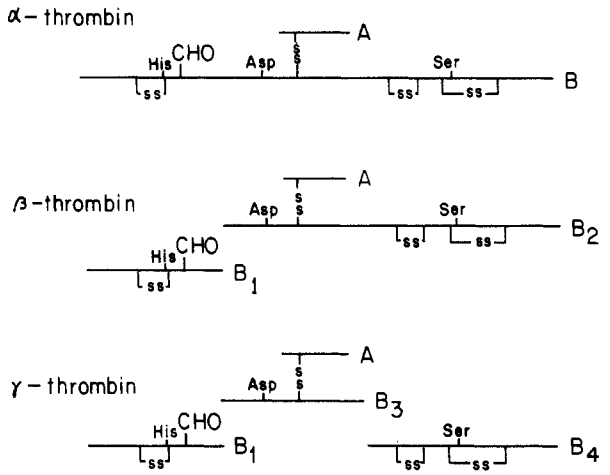

FIGURE 1: Schematic polypeptide structures of human α-, β-, and γ-thrombins. While all three forms have estero- and amidolytic activity, only α-thrombin will catalyze fibrinogen cleavage in the clotting event. [From Bauer et al. (1980) with permission. Copyright 1980, American Society of Biological Chemists.]

motion, as manifested in its ESR[1] spectrum, reflects specific structural interactions (hydrophobic, steric hindrance, etc.) which are a dynamic conformational monitor of the protein environment where the label situates (Berliner & Wong, 1974). Concerns about structural perturbations of a label are eliminated by virtue of the comparative nature of this technique (Berliner & Wong, 1974). In addition to reagents which detected the previously described indole site of α-thrombin in γ-thrombin as well (Berliner & Shen, 1977a), reagents were found which detected specific structural differences between the thrombin forms, consistently showing more immobilized ESR spectra when conjugated to γ-thrombin as compared with α-thrombin (Figure 2).

CPK models of the spin-labels revealed that the *indole-site* reagents were essentially linear (predominantly para-substituted reagents) whereas the α–γ difference reagents were bent (all meta-substituted reagents). These α–γ difference reagents provide the first physical evidence for a structural obstruction (or increased hydrophobicity) neighboring the catalytic site on noncoagulant thrombin.

Materials and Methods

Spin-Labels and Nomenclature. The 13 spin-labeling reagents which are shown in Figure 2 were synthesized as reported earlier (Wong et al., 1974). We have adopted the shorthand notation of Berliner & Wong (1974) which describes the fluorosulfonylphenyl and nitroxide moieties in each label. Labels which are identical except for the ring position of the SO_2F group are designated with the same Roman numeral; e.g., *p*-I (*p*-NH-5=CO), in the first row (Figure 2) is a position isomer of *m*-I (*m*-NH-5=CO), which is in the second row of labels. The parenthetical abbreviation completely describes the structure: *o*-, *m*-, or *p*- specifies the position of the SO_2F group on the ring; the next symbol specifies the linking functional group to the nitroxide, thus -NH, amido, -CO, acyl, -NCO, carbamoyl, or -SO_2, sulfonyl. The next numerical symbol specifies the nitroxide ring: 5, the five-membered (saturated) pyrrolidinyl ring; 5=, the five-membered (unsaturated) pyrrolinyl ring; or 6, the six-membered piperidinyl ring. The last remaining term describes the functional group derived from the nitroxide moiety in the covalent linkage: CO, acyl (from the carboxylic acid); OH, an ester (from the alcohol); NH, amido (from the amine); and CH_2OH, ester (from the primary alcohol). For example, label *m*-I (*m*-NH-5=CO) is the *m*-fluorosulfonylanilide of the unsaturated pyrrolinecarboxylic acid nitroxide.

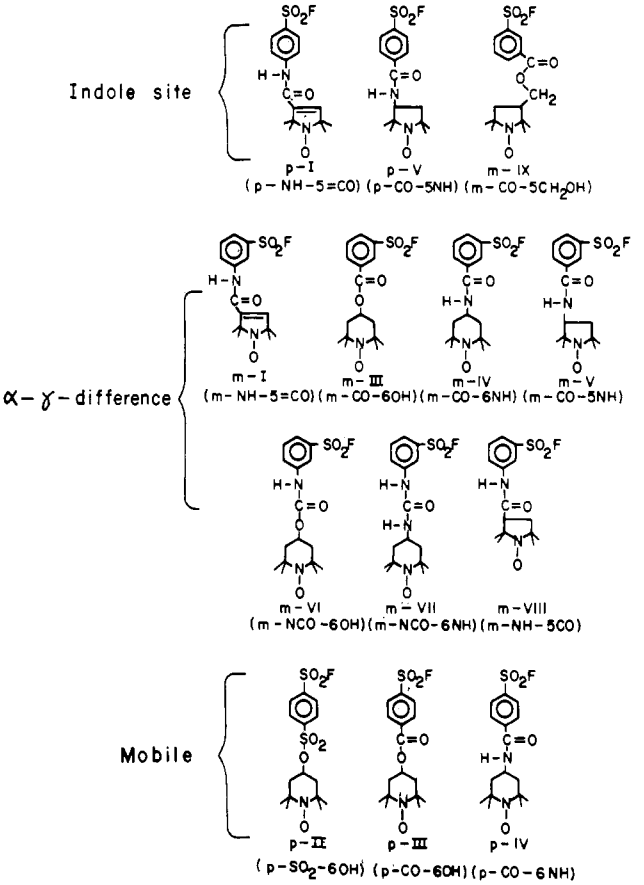

FIGURE 2: Structures of spin-labeled sulfonyl fluorides. Compounds which are isomers of the same structure are designated by the same Roman numeral. The three classes of labels, indole site, α–γ difference, and mobile, respectively, are related to the tumbling domains in space of each nitroxide group relative to the sulfonyl substituent on the ring.

Materials. The following chemicals were obtained commercially: benzamidine hydrochloride hydrate and the HCl salt of *p*-CB, Aldrich Chemical Co.; indole and Tos-Arg-OMe, Sigma Chemical Co.; NPGB, ICN-NBC; bovine fibrinogen (Lot No. 21, 90% clottable), Miles Research Laboratories, Inc. Valerylamidine was a generous gift of W. B. Lawson, New York State Department of Health, Albany. Highly purified α-thrombin (~3000 NIH units/mg, 95–98% active by NPGB titration) and tryptic γ-thrombin were prepared in Albany as described previously (Fenton et al. 1977a,b, 1979) and evaluated before and after shipment for specific clotting activity, active-site concentration (by NPGB titration), and polypeptide fragment homogeneity and α-, β-, and γ-thrombin content (by gel electrophoresis).

Methods. Human α- or γ-thrombin was spin-labeled with the fluorosulfonyl nitroxide spin-labels (Figure 2) by the procedure of Berliner & Shen (1977a) and then dialyzed exhaustively vs. 0.75 M NaCl and 0.05 M sodium phosphate, pH 6.5. Thrombin (M_r 36 600) concentration was determined spectrophotometrically by its absorbance at 280 nm, E_{280nm} = 1.83 mL mg^{-1} cm^{-1} (Fenton et al., 1977b), with a Unicam SP 1800 spectrophotometer. The ESR spectra were measured in quartz flat cells near ambient temperature (26 ± 1 °C) on a Varian E-4 spectrometer.

[1] Abbreviations used: ESR, electron spin resonance; Tos-Arg-OMe, tosylarginine methyl ester; NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; *m*-V-α, α-thrombin spin-labeled with fluorosulfonylphenyl nitroxide *m*-V (*m*-CO-5NH in Figure 2); *m*-V-γ, the corresponding γ-thrombin derivative; *p*-CB, *p*-chlorobenzylamine; CPK, Corey–Pauling–Koltun.

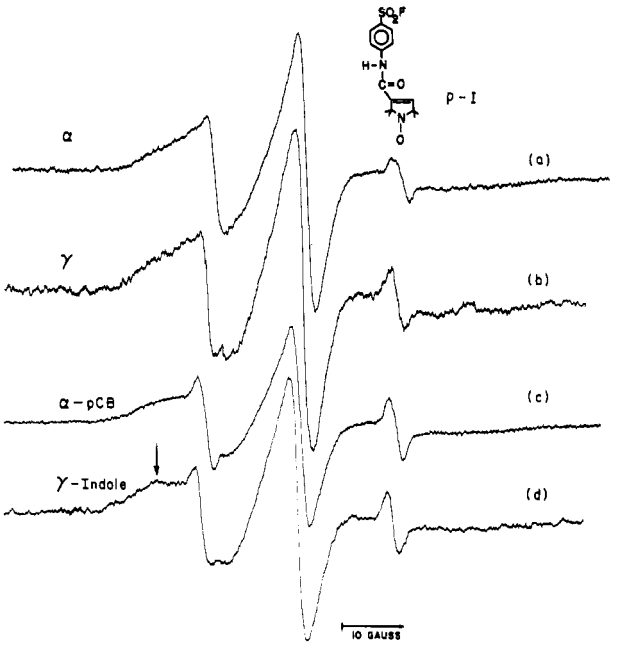

FIGURE 3: X-Band ESR spectra of human α- and γ-thrombin spin-labeled at the active serine with *p*-I (*p*-NH-5=CO). All conditions were identical with those in Figure 4. (a) *p*-I-α; (b) *p*-I-γ; (c) *p*-I-α plus 50 mM *p*-chlorobenzylamine (*p*-I-γ under the same conditions gave an identical effect); (d) *p*-I-γ in saturated (~20 mM) indole.

Results

Some example ESR spectra for the spin-labeled thrombin samples are shown in Figures 3 and 4. As observed in our earlier studies with α-chymotrypsin and trypsin, several of these labels undergo a slow intramolecular hydrolysis in aqueous solution which results in some fraction of the fluorosulfonyl inhibitor becoming devoid of a paramagnetic (nitroxide) moiety, yet remaining an efficient active-site serine-directed inhibitor (Wong et al., 1974). It is important to note that while some of the derivatives had a relatively low apparent incorporation of spin-label ("spin count"), it was only those molecules with covalently bound nitroxide which were observed in the ESR experiment. Figures 3 and 4 depict examples of spectra obtained with each thrombin form and their interaction with specific active-site ligands. The spectrally (and also topographically) different spin-labels were distributed into three general classes.

Indole-Site Reagents. Since Berliner & Shen (1977a) had found that a group of three topographically isomorphous spin-labels, *p*-I, *p*-V, and *m*-IX (first row of Figure 2), specifically sensed the binding of apolar ligands near the active center of α-thrombin, we examined the γ form for the same binding site. Figure 3 shows comparative results for α- and γ-thrombins labeled with *p*-I (*p*-NH-5=CO) in the absence and presence of specific ligands. First, note that labeled α- and γ-thrombins alone (Figure 3a,b) gave identical spectra, as did each pair with the other two labels, *p*-V and *m*-IX. Upon exposure to near-saturating concentrations of indole (Figure 3d), a distinct increase in immobilization was observed as for the other two γ-thrombin derivatives, *m*-IX-γ and *p*-V-γ (not shown), exactly as found earlier for the corresponding α-thrombin derivatives (Berliner & Shen, 1977a). On the contrary, no "indole effect" was found for any of the other labels in Figure 2 bound to γ-thrombin, in complete agreement with the results for α-thrombin. Furthermore, basic ligand binding to this indole-site class of labeled derivatives gave identical strongly immobilized spectra for both α- and γ-thrombin forms, again consistent with the results found earlier

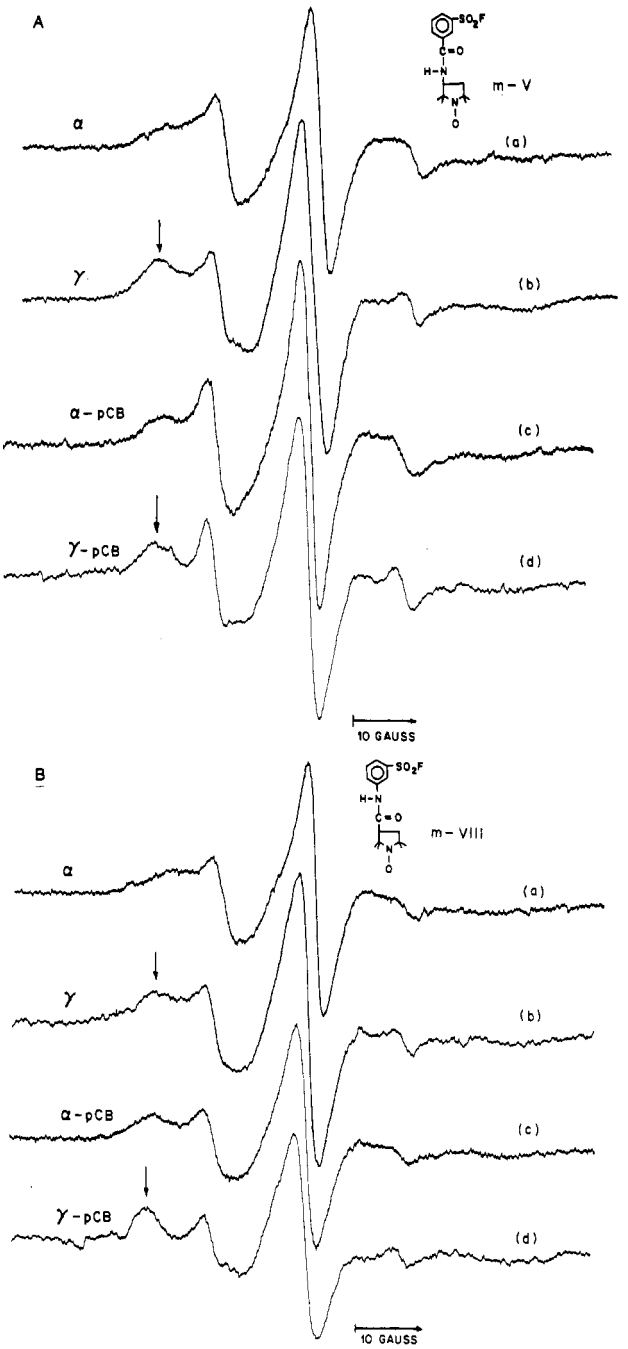

FIGURE 4: X-Band ESR spectra of human α- and γ-thrombin conjugated at the active serine with (A) *m*-V (*m*-CO-5NH) and (B) *m*-VIII (*m*-NH-5CO). (a) Spin-labeled α-thrombin; (b) spin-labeled γ-thrombin; (c) spectrum a in the presence of 50 mM *p*-chlorobenzylamine; (d) spectrum b in the presence of 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.

with α-thrombin (Berliner & Shen, 1977a). For exemplary purposes, the spectra in Figure 3 show *p*-I-γ in the presence of indole (Figure 3d) and *p*-I-α in the presence of *p*-chlorobenzylamine (Figure 3c), respectively.

α–γ Difference Reagents. As shown in Figure 4, the labels *m*-V (*m*-CO-5NH) and *m*-VIII (*m*-NH-5CO) were distinctly more immobilized in their respective γ-thrombin derivatives than in their α-thrombin derivatives. This difference in immobilization was most clearly seen from the position of the low-field lines for *m*-V-α and *m*-V-γ, respectively, and *m*-VIII-α and *m*-VIII-γ, respectively, in Figure 4 where the latter (γ) was broader and lower field shifted than the former (α) (see arrows). The characteristic of stronger immobilization

for serine-labeled γ- vs. α-thrombin was found consistent with all of the following labels (whose structures are grouped in Figure 2): *m*-I, *m*-III, *m*-IV, *m*-V, *m*-VI, *m*-VII,[2] and *m*-VIII. When a basic competitive inhibitor was bound to these spin-labeled thrombins, such as benzamidine, *p*-CB, or valeryl-amidine, the nitroxide moiety became almost completely immobilized for all spin-labeled γ-thrombin derivatives with *identical* spectral behavior with that observed for the α form (Berliner & Shen, 1977a). Spectra c and d for each label in Figure 4 compares the α and γ derivatives in the presence of 50 mM *p*-chlorobenzylamine, although identical spectral results were observed with benzamidine. While we did not examine every spin-labeled α- or γ-thrombin derivative with the straight-chain basic inhibitor valerylamidine, or the dye proflavin, every spin-labeled thrombin spectrum examined shifted to that of a strongly immobilized nitroxide environment upon ligand binding. These basic ligand binding effects were also reversible.

Mobile Reagents. Last, we found a group of three spin-labels, *p*-II, *p*-III, and *p*-IV (bottom row of Figure 2), which gave spectrally indistinguishable ESR spectra as the α- or γ-thrombin derivatives. Furthermore, while we could not distinguish any changes in label immobilization upon exposure to indole, an increase in immobilization of the label was observed upon exposure of any α- or γ-thrombin derivatives to *p*-CB or benzamidine. The spectra for these derivatives (not shown) were characteristically more mobile (i.e., a more freely tumbling nitroxide moiety) than those observed for the former two groups of spin-labels. The spin-labels of this class have been termed "mobile" labels since the nitroxide moiety was less sensitive to its protein structural environment than in the former cases. On the other hand, the fluorosulfonylphenyl moiety was responsive to structural effects similar to that observed in our previous studies (Berliner & Shen, 1977a) in that all spin-labels were sensitive to basic ligand binding.

Other Reagents. An ortho-substituted spin-labeled sulfonyl fluoride, *o*-I (*o*-NH-5=CO), would not react with thrombin, as evidenced by no inhibition of esterase activity or a resultant ESR spectrum after dialysis of the inhibition reaction mixture. While Berliner & Wong (1974) showed that this label (*o*-I) specifically inhibited α-chymotrypsin while just slightly reactive with trypsin (only under high excesses of inhibitor), the apparent lack of reactivity with human thrombin suggests some unique structural features in the thrombin active site which prevents this label from binding.

Discussion

Loss of clotting activity is directly related to α-thrombin degradation, where formation of β-thrombin precedes γ-thrombin formation, suggesting that proteolysis at the β cleavage site(s) causes structural changes which destroy clotting activity and expose the γ-cleavage site. Although the intermediate human β-thrombin form[3] cannot be obtained with sufficient purity, γ-thrombin has been exceedingly useful for assessing requirements for clotting activity, since both (β and γ) thrombins exhibit very similar properties with synthetic substrates (Fenton et al., 1979), the plasma protease inhibitors, antithrombin III, and α_2-macroglobulin. In contrast to α-thrombin, nonclotting γ-thrombin further has ~200-fold reduced affinity for hirudin (Landis et al., 1978) and does not appear to interact with fibrinogen (Chang et al., 1979) or incorporate into fibrin clots (Wilner et al., 1979). These differences provide further evidence for tertiary structural changes, as presently demonstrated with our topographically sensitive phenylsulfonyl spin-labeling reagents. Additionally, all spin-labeled γ-thrombins gave completely immobilized spectra upon binding benzamidine, *p*-CB, or other basic ligands and gave the identical spectral evidence for an apolar (indole) binding site[4] as described earlier for the α form (Berliner & Shen, 1977a).

Model Building. Upon examining and comparing CPK space filling models for all of the labels in Figure 2, we found an excellent correlation between the three spectrally discriminated groups. As noted in our earlier paper (Berliner & Shen, 1977a), the three spin-labels which we have termed indole-site reagents (*p*-I, *p*-V, and *m*-IX) occupied overlapping volumes in space relative to bond rotations between the nitroxide moiety and a fixed phenyl group. This specific volume was peripheral to the para position of the phenylsulfonyl ring and was sensitive to indole binding. These indole-site reagents were of an overall linear conformation extending about 15–16 Å (including van der Waals radii) from the sulfonyl sulfur to the nitroxide moiety. The seven labels which detected structural differences between α- and γ-thrombins (*m*-I, *m*-III, *m*-IV, *m*-V, *m*-VI, *m*-VII, and *m*-VIII) also occupied mutually overlapping volume in space for the same rotational constraints. This volume was peripheral to the meta position of the phenylsulfonyl ring and was sensitive to structural differences brought about in the α- to β- to γ-thrombin conversion as manifested by more immobilized ESR spectra for the γ-thrombin derivatives.[5] Thus, if the sulfonylphenyl group is fixed in one orientation, the nitroxide moieties of the spin-label classes α–γ difference site and indole site clearly segregate into two tumbling loci in space (see Figure 5 which compares CPK models of labels from each class). We have described these binding loci more quantitatively in the "topographical map" (Figure 6), which is based on the maximum-allowed tumbling volume for a label in each class. This model, which includes van der Waals distances, was constructed from the label in each class of smallest tumbling volume in which the corresponding structural feature was detected (i.e., indole binding or γ-thrombin immobilization). These labels were *m*-I and *p*-I for the α–γ difference and indole sites, respectively. That volume in space which coincides with the α–γ difference site approximates an oblate ellipsoid whose minor axis of revolution (r = 4 Å) extends out from the meta position of the phenyl ring, with a major axis of r = 5 Å. The corresponding dimensions for the locus which defines the indole site would also

[2] This reagent was an exception in that it also detected indole binding to both thrombin forms, although the indole complex of *m*-VII-γ was more immobilized than the corresponding α-thrombin complex. Since this label partially overlapped the indole site, the ESR spectra in the presence of indole were likely complex mixtures of several nitroxide tumbling modes (T.-L. Chang and L. J. Berliner, unpublished results).

[3] Human and bovine β-thrombins are homologous. However, bovine thrombin does not have a form corresponding to human γ-thrombin with the consequence that bovine α-thrombin converts only to the homologues of human β-thrombin.

[4] γ-Thrombin gives the identical activation of Tos-Arg-OMe hydrolysis as found for α-thrombin by indole and related analogues (B. G. Conery and L. J. Berliner, unpublished data).

[5] Since loss of clotting occurs at the α- to β-thrombin conversion, it is possible that the α–γ structural differences detected in these experiments were reflecting principally the same structural difference which occurs between α- and β-thrombin. While human β-thrombin cannot be isolated as a single species, we spin-labeled with the α–γ difference reagent *m*-V (*m*-CO-5NH) one preparation of composition: 1% α, 64% β, and 35% γ form. The ESR spectrum of this predominantly β-thrombin mixture was remarkably similar to that of a corresponding γ-thrombin derivative, this experiment suggesting that the structural features of the α to β conversion were detected in both β- and γ-thrombin spin-labeled conjugates.

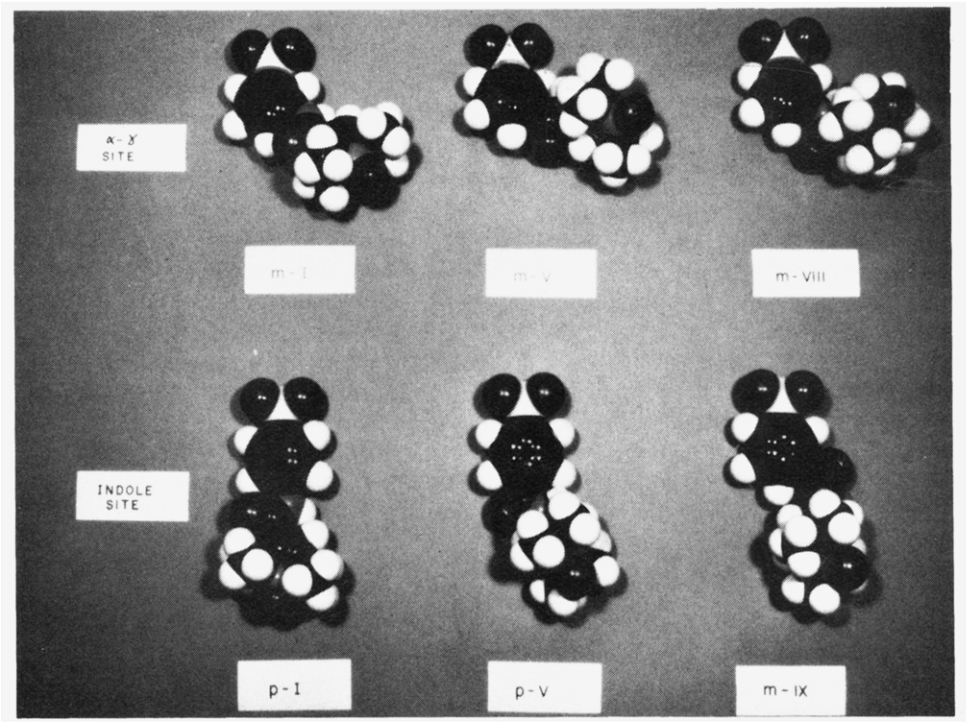

FIGURE 5: Example CPK models of *α–γ difference site* and *indole site sensitive labels*, respectively. The labels shown are (top row) *m*-I (*m*-NH-5=CO), *m*-V (*m*-CO-5NH), and *m*-VIII (*m*-NH-5CO) and (bottom row) *p*-I (*p*-NH-5=CO), *p*-V (*p*-CO-5NH), and *m*-IX (*m*-CO-5CH_2OH). The sulfonylphenyl moiety is fixed in the same orientation in all cases while the nitroxide moieties are placed in a common orientation for each label class. Where amide bonds existed between moieties, a planar, trans conformation was chosen.

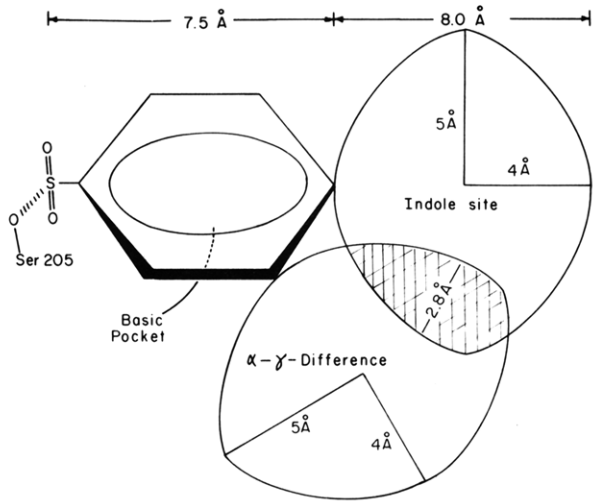

FIGURE 6: Two-dimensional representation of human thrombin active-site topography as a "map" constructed from the accessible volume of rotation of each nitroxide moiety with respect to the phenylsulfonyl group according to the constraints detailed in the text and Figure 5 legend. These distances, which include van der Waals radii, were derived from CPK models of the spin-labels in Figure 2. The distance marker at the phenyl ring bisects the covalent bond between the ring and the nitroxide moiety. The two oblate ellipsoids of revolution approximates those for the smallest spin-label which detects each structural feature. Their volume of intersection thus *excludes* either the indole or α–γ difference sites.

be an oblate ellipsoid whose minor axis extends out from the para position of the phenyl ring of the same minor and major axis dimensions as above, 4 and 5 Å, respectively. The active-site topography is then described by the "double ellipsoid" model shown in Figure 6. While the tumbling volume of any nitroxide moiety does not obviously determine a perfect oblate ellipsoid of revolution, this approximation was very conservative; in fact, the width of the maximum plane of overlap of the two ellipsoids should be slightly less.[6]

Apolar (Indole) Site. The maximum overall distance from the sulfonyl sulfur atom to the furthest nitroxide atom (15–16 Å) is within the range of the longest dimension of the proflavin molecule (~12.6 Å, including van der Waals radii) which Berliner & Shen (1977a) concluded must overlap both the basic binding pocket and the indole site simultaneously. While its exact location in the (yet unknown) thrombin three-dimensional structure is impossible to pinpoint at this juncture, we can confidently specify its distance from the active serine to the basic specificity pocket as well as its orientation relative to the α- to β- (γ-) thrombin structural conversion as shown in Figure 6.

α–γ Difference Site. The maximum distance from the sulfonyl sulfur to the furthest possible point would be somewhat shorter due to the bent nature of the spin-label reagents in this class, or about 13–14 Å, (including van der Waals distances). While we lack either the α or γ human thrombin X-ray structures, some progress has been recently reported by using interactive computer surface graphics, basing the human thrombin sequence on the α-chymotrypsin three-dimensional structure where the β cleavage at Arg-74 and γ cleavage at Lys-154 of the thrombin B chain were detectable (Bing et al., 1980). Since increased spin-label immobilization (as observed for the γ-thrombin derivatives) may be due either to steric

[6] Actually, the CPK model of the indole site reagent *m*-IX (*m*-CO-5CH_2OH) revealed that, while the nitroxide moiety almost completely overlapped the ellipsoid which defines the α–γ difference region, its maximum possible overlap with the indole site ellipsoid refined this latter site to a smaller volume. This refinement would involve a contraction of the most distant half of this ellipsoid (i.e., the half which does not overlap the α–γ difference ellipsoid) by ~1.8 Å along the major axis. The results obtained with *m*-VII (see footnote 2) would also suggest a contraction of this volume. Furthermore, it is important to remember that a label need not necessarily bind to a particular site even if it were rotationally accessible; here, the label *m*-IX was totally insensitive to the α–γ difference region, most probably due to a lack of binding to that locus.

obstructions to nitroxide tumbling or to an increase in attractive interactions (i.e., hydrophobic) between the nitroxide and the protein surface, a more detailed description of the γ-thrombin surface should enable further refinement of the topographical map in Figure 6.

Last, we must note that all of the spin-labeled derivatives of both α- and γ-thrombin became more immobilized upon the binding of a basic ligand (e.g., *p*-CB, Figures 3 and 4), *regardless* of the structural site sensitivity of the nitroxide moiety. That is, the immobilization of spin-label was imparted through an interaction (mediated by basic ligand binding) at the phenyl ring which is the only structural feature common to all of the labels. This phenomenon of a perturbation in the phenylsulfonyl spin-label motion upon ligand binding to the substrate specificity pocket was also observed earlier for this same spin-label series conjugated to α-chymotrypsin in the presence of a competitive inhibitor (Berliner & Wong, 1974). This latter study placed the phenylsulfonyl moiety directly over (or partially bound in) the specificity pocket; the results with thrombin may be analogous.

There was, of course, an implicit assumption in deriving the model depicted in Figure 6. In order to compare all of the spin-labels as structural classes, we fixed the sulfonyl group in space via a covalent linkage to Ser-205[7] but also maintained an isomorphous orientation of the phenyl group for each label. That is, by fixing the phenylsulfonyl moiety of each label as a suitable reference point, we found that the topographical features of each class of labels in Figure 2 totally correlated with their ESR results.[8] Furthermore, the uniformly consistent observation that spin-label immobilization occurred upon basic ligand binding also strengthens the model in which the structurally common portion, the phenylsulfonyl moiety, interacted at the same basic ligand (sensitive) locus in all cases.

Similarities and Dissimilarities to Bovine Trypsin. Comparison of our spin-labeled α-thrombin conjugates with the corresponding bovine trypsin derivative (pH 3.5, Berliner & Wong, 1974) showed a fairly high degree of similarity in nitroxide motion for most of the meta-substituted labels (Figure 2) as contrasted with the general dissimilarity among the para-substituted labels [see also Berliner & Shen, 1977b; Shen, 1977].[9] On the other hand, ESR spectra for the four-chain γ form of thrombin were dissimilar to that for the single-chain spin-labeled bovine trypsin. While we note that the indole-site reagents (*p*-I, *p*-V, and *m*-IX) gave different nitroxide motion in each comparison between α-thrombin and trypsin, we also recall that neither these nor any of the spin-labeled trypsin derivatives in the series of Berliner & Wong (1974) detected binding of indole concentrations up to ~20 mM, nor did indole displace proflavin from the trypsin binary complex.[10] Yet both α- and γ-thrombins were sensitive to complexation with indole.

Despite the apparent conformational similarities between α-thrombin and trypsin for the meta-substituted labels, the binding of basic ligands to spin-labeled trypsins caused absolutely no *change* in the nitroxide environment for trypsin inhibited with *any* label in Figure 2. This was true for concentrations of benzamidine, *p*-CB, valerylamidine, or *n*-propylamine ranging between 150 to 200 mM, which is almost 10^5-fold greater than the K_I for benzamidine inhibition of trypsin esterase activity (Bechet & D'Albis, 1969). The three-dimensional model of benzamidine–trypsin or native trypsin has a basic ligand pocket depth of 6.5 Å (Laura et al., 1980). If one compares the human α-thrombin sequence in this region, there are several insertions (trypsin numbering system), Glu-187a, Gly-187b, and Gly-188b, as well as major residue changes, 221 (Asp for Ala) and 221a (Arg for Gln). While the thrombin insertions do not actually lie *in* the basic pocket, they occur just adjacent to a serine protease species conserved section of the backbone. From the simplest point of view, it seems reasonable that part of the conformational strain imposed by these insertions and substitutions might be alleviated by a resultant decrease in the depth of the basic pocket, accounting both for the benzamidine and *p*-CB effects with the spin-labeled derivatives and also with the 10^2- to 10^3-fold decrease in thrombin's affinity for these ligands as compared with trypsin. Bing et al. (1980) have noted that the residue at position 190 which neighbors the common Asp-189 in the basic specificity pocket (Krieger et al., 1974) changes from a serine in trypsin to an alanine in the thrombin B chain. In trypsin, this serine may assist ligand binding through hydrogen bonding, attributing its much stronger binding affinity for basic residues as compared with thrombin. Perhaps this replacement with alanine in thrombin could result in a less deeply buried basic ligand, thus accounting for the *p*-CB or benzamidine spectral effects observed with the spin-labeled thrombin derivatives discussed earlier. Drawing upon the above comparisons, we suggest that trypsin similarities may exist in regions sensed by most of our meta-substituted reagents (i.e., α–γ difference site), while the indole site both on α- and γ-thrombins does not appear to be analogous to the active-site structure of trypsin. Lastly, the basic specificity pocket appears to be deeper in trypsin than in the thrombins.

Biological Implications. The loss of clotting and other important biological activities result from the obstruction of protein binding or recognition sites which are independent of the catalytic site. Consistent with this hypothesis, the α–γ difference reagents provide direct spectral evidence for tertiary structural changes, causing such an obstruction and/or increased hydrophobicity in regions of the active site transformed in the α- to β- (γ-) thrombin conversion.

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[7] While the criticism might be made that specific Ser-205 labeling may not be generally assumed, there is only one case to the contrary for a benzamidine *exo-site* affinity label to which a fluorophenylsulfonyl moiety was attached (Bing et al., 1977). Due to the very strong affinity of the basic ligand binding pocket for the benzamidino moiety, which consequently blocked the active-site serine, some sulfonylation of distant serines resulted. On the other hand, the spin-labels reported here as well as several other sulfonyl fluoride irreversible inhibitors react stoichiometrically with human α- or γ-thrombin with concomitant loss of (Ser-205) catalytic activity (Berliner & Wong, 1974).

[8] If the phenylsulfonyl moiety and nitroxide-linking side chains were unrestrained in the enzyme, the label would be completely mobile, as occurs upon urea or guanidine hydrochloride denaturation of the enzyme (Bauer et al., 1980).

[9] Since human α-thrombin has an M_r of 36 600 vs. 24 500 for bovine trypsin, for identical protein environments, the observed nitroxide tumbling rate would be ~1.5 times slower, assuming both enzymes have the same density and spherical conformation.

[10] T.-L. Chang and L. J. Berliner, unpublished results.

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Hydrogen-1 Nuclear Magnetic Resonance Study of the Complexes of Two Diastereoisomers of Folinic Acid with Dihydrofolate Reductase†

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ABSTRACT: The ^1H chemical shifts for the formyl and benzoyl protons of the individual diastereoisomers of folinic acid bound to dihydrofolate reductase have been measured. For the tightly bound biologically active 6*S*,α*S* isomer, the "bound" signals were assigned by using transfer of saturation methods. In this case, only one of the two rotameric states of the formyl group in folinic acid (form I) is bound to the enzyme. The H3′ and H5′ benzoyl protons have identical shifts in the bound state (as do the H2′ and H6′ protons). This equivalence is attributed to flipping of the benzoyl ring about the N10–C4′ and C1′–CO bonds in the bound state. In the case of the biologically inactive 6*R*,α*S* isomer, both rotameric forms (I and II) bind to the enzyme. The "bound" shifts for the formyl and aromatic protons are different in the complexes with the 6*S*,α*S* and 6*R*,α*S* isomers, indicating that the pteridine ring and benzoyl moiety are binding in different environments in their enzyme complexes. The glutamic acid moiety is probably binding at the same site in the two complexes.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the reduction of 7,8-dihydrofolic acid to 5,6,7,8-tetrahydrofolic acid by using NADPH as a coenzyme. The enzyme is of considerable pharmacological interest, being the target for several "antifolate" drugs such as methotrexate, trimethoprim, and pyrimethamine. We have been using high-resolution NMR to study the complexes formed by the enzyme with its coenzyme and substrate analogues with the aim of understanding the factors controlling the specificity of the ligand binding (Birdsall et al., 1977; Feeney et al., 1977, 1980a,b; Roberts et al., 1977, 1978; Cayley et al., 1979; Hyde et al., 1980a,b; Wyeth et al., 1980).

The complexes formed with the substrates folate and 7,8-dihydrofolate can be studied with ease, but complexes formed with the product 5,6,7,8-tetrahydrofolate have proved difficult to characterize because of the instability of the latter com-

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