Spin-labeled Sulfonil Fluorides as Active Site Probes of Protease Structure

I. COMPARISON OF THE ACTIVE SITE ENVIRONMENTS IN α-CHYMOTRYPSIN AND TRYPsin*

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SUMMARY

α-Chymotrypsin and trypsin were spin-labeled with 14 ortho-, meta-, and para-substituted phenylsulfonil fluorides. These analogues of tosylfluoride were substituted with either pyrrolidinyl, pyrrolinyl, or piperidinyl nitroxide moieties. A complete description of their synthesis and the enzyme inhibition studies is presented in the following paper (WONG, S. S., QUIGGLE, K., TRIPLETT, C., AND BERLINER, L. J. (1974) J. Biol. Chem. 249, 1678-1689). The studies were designed to test for "dynamic conformational homologies" in these closely related proteins. However, the spectral results at pH 3.6 clearly showed that the active site conformations of trypsin and α-chymotrypsin were different. Model-building studies indicated which "tosyl" spin labels could bind isomorphously to tosyl-α-chymotrypsin. Several of those labels which were shown to be restricted structurally from binding in the chymotrypsin aromatic pocket were found upon exposure to saturated indole to give electron spin resonance spectra closely resembling those for trypsin. Those inhibitors which could theoretically bind in the pocket were affected least or not at all. With trypsin, on the other hand, 1 mM benzamidine produced no spectral shifts.

A suggested model for α-chymotrypsin based on steric arguments provides for three general binding modes: (a) one isomorphous to tosylchymotrypsin, (b) one reflecting partial binding at the tosyl hole, and (c) one in a general region of the active site. Indole can displace the partially bound State b toward the general binding Mode c. This general Mode c is probably common to trypsin as well.

The use of alkyl and aromatic sulfonil fluorides as specific active site-directed irreversible inhibitors for serine proteases was demonstrated several years ago by Fahney and Gold (1). Of these reagents, p-toluene sulfonil fluoride (tosyl fluoride) and its heavy atom analogue, p-iodobenzene sulfonil fluoride (pipsyl fluoride), proved to be invaluable aids in the crystallographic structure determination of α-chymotrypsin (2) and later γ-chymotrypsin (3), elastase (4), and subtilisin BPN' (5). Furthermore, it was shown crystallographically for α-chymotrypsin that the tosyl moiety was bound in the aromatic specificity binding pocket ("tosyl hole") of the enzyme (6).

In an attempt both to probe and to compare the active site conformations of α-chymotrypsin and related serine proteases, we have synthesized and tested several spin label derivatives of benzene sulfonil fluorides of the general structure

$$\text{SO}_2\text{F}$$

where $R$ = a nitroxide (spin label). While it was recognized that bulky substitution on the aromatic ring, particularly at the para position, might preclude the binding of this derivative in the chymotrypsin binding pocket, substitution at the meta or ortho position might allow specific binding. This reasoning was based on crystallographic and kinetic studies with α-chymotrypsin which place a limit on both the size and shape of the "tosyl hole" (7). For example, p-formyl-p-iodophenylalanine and β-(p-iodophenyl)propionate did not bind in the pocket in α-chymotrypsin crystals (8), while oxygen-alkylated tyrosine and p-iodo-l-phenylalanine substrate derivatives were hydrolyzed considerably more slowly in solution than the unsubstituted compounds (9).

The spin label experiment provides extremely sensitive information about the motional freedom of the nitroxide moiety (10). Consequently, the rotational mobility of the spin label, $R$, is dictated in large part by its immediate physical environment and the nature of the interactions between the nitroxide ring and the protein structure. Assuming that a variety of topographically variable spin labels were incorporated isomorphously at a specific site in a protein molecule, the corresponding mobilities could be analyzed to yield a "dynamic conformational map" which contains information about both physical structure and forces (interactions). We then define a "dynamic conformational homology" as a case where two proteins give identical results for the same series of spin labels. A schematic diagram of the approach used in these studies is shown in Fig. 1. The spin labels utilized are shown in Fig. 2. In those cases where
the aromatic portion of the spin label may bind in the tosyl hole, the immediate structural region outside this pocket may be probed by monitoring the mobility of the attached nitroxide spin label moieties. On the other hand, those derivatives which do not bind in the pocket but nevertheless (covalently) inhibit the enzyme, must adopt some alternative binding mode, perhaps one common to many serine proteases. In addition, the narrow dimensions of the tosyl hole (10 to 12 A by 5.5 to 6.5 A by 3.5 to 4.0 A) dictate that the aromatic group would always be that part of the inhibitor which binds in the pocket since the spin label moiety is too thick by virtue of the dimethyl groups which flank the nitroxide linkage (8). Consequently, for this latter case of secondary or "nonspecific" binding, a sensitive comparison may be made between the active site conformations of \( \alpha \)-chymotrypsin and the related protease, trypsin. Furthermore, it had been suggested and recently reported that the many homologies in primary structure for these two enzymes are reflected in significant homologies in their tertiary structures (11, 12). The spin label approach outlined above potentially offers an extremely sensitive dynamic test of these conformational homologies in the active site.

**MATERIALS AND METHODS**

The 15 spin labels synthesized are shown in Fig. 2. Details of their synthesis and purification as well as the enzyme inhibition studies are presented in the following paper (13). ESR measurements were taken at X band on a Varian E-4 spectrometer at 26 + 2°C.

**Spin Label Nomenclature**—In order for the reader to envision most easily the structure of a particular spin label under discussion, we always refer to a given inhibitor with two abbreviated codes as well as occasionally citing a more specific aspect of its structure. Labels which are identical except for the position of the \(-SO_2F\) moiety are designated with the same Roman numeral; \( e.g. \) see the first column in Fig. 2 with the \( o_-, m_-, p_- \) and \( m_- \) derivatives

\[ o-I, p-I, m-I \]

The abbreviation in parentheses completely describes the structure. As usual, \( o_-, m_-, p_- \) specifies the position of the \( SO_2F \) group. The next symbol designates the functional group from the phenyl group: \(-NH\), amido; \( CO\), acyl; \( -NCO\), carbamyl; or \( SO_2-, \) sulfonyl. The next numerical symbol refers to the type of nitroxide ring; \( \delta, \) the five-membered (saturated) pyrrolidinyl ring; \( \delta=, \) the five-membered (unsaturated) pyrrolyl ring; or \( \theta, \) the six-membered piperidinyl ring. Last, the remaining symbol refers to the functional group derived from the nitroxide spin label moiety in the 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). Therefore, an example might be \( p-IV (p-CO-6NH) \) which is the para-fluorosulfonyl benzamide of the five-membered piperidinyl amine; or \( m-VIII (m-NH-5CO) \), the meta-fluorosulfonyl anilide of the five-membered (saturated) pyrrolidine carboxylic acid.

**RESULTS**

**ESR Spectra**—The ESR spectra for \( \alpha \)-chymotrypsin and trypsin inhibited with 14 of the 15 spin label inhibitors of Fig. 2 are shown in Figs. 3 and 4. It was necessary to measure these spectra at pH 3.5, where hydrolysis and desulfonification problems

\[ 1 \]

Although \( o-II \) inhibited both enzymes, the rate of intramolecular hydrolysis of the label itself was so rapid that a spin-labeled enzyme was virtually impossible to measure. See the following paper for a more detailed discussion of this problem.
Fig. 3. ESR spectra of α-chymotrypsin and trypsin, respectively, spin-labeled with the α- and p-substituted inhibitors shown in the center column. Conditions were 26 ± 2⁰C, pH 3.5 (~0.006 M acetic acid) and 0.1 M NaCl. Trypsin samples also contained 0.02 M CaCl₂ and frequently 1 mM benzamidine.

were at an absolute minimum (13). This should have no bearing on the results of these comparative studies. (There was in fact evidence that no detectable conformational changes occurred between pH 3.5 and neutral in the active site regions of either enzyme (12))² An initial examination of these comparative spectra (Figs. 3 and 4) suggests that there are few, if any, similarities between α-chymotrypsin and trypsin for each label

² L. J. Berliner and S. S. Wong, unpublished observations.
studied. It was also apparent in almost every case, except perhaps for p-II (p-SO₂-6OH), that the spin-labeled trypsin exhibited broader line shape spectra than did the corresponding α-chymotrypsin derivative. This signifies that in trypsin, the nitroxide moiety is in a state of more hindered motion. It is important to point out that this rotational hindrance need not necessarily arise only from a structurally constricting environment but could also arise from specific noncovalent attractive interactions.

**Model Building**—An atomic model of the active site of tosyl-α-chymotrypsin was constructed according to the coordinates of Birktoft and Blow (14) with Kendrew skeletal models (5 cm =...
lences” in indole were observed to exist in an equilibrium between the rigidly bound inhibitor and a highly mobile (weakly bound) state (or states) away from this binding pocket, and where a preferential (binding) affinity exists for indole in the “tosyl hole,” this spin label equilibrium would be shifted toward those binding modes outside the pocket. Thus, the indole could, in effect, displace the spin-labeled “tosyl” moiety from the pocket.

All of the α-chymotrypsin derivatives were examined at pH 3.5 in the presence of saturated indole. The results obtained were striking in many respects. The majority of those derivatives shown by model building not to bind in the “tosyl hole” exhibited dramatic spectral changes upon exposure to indole. The effects were shown to be reversible by dialyzing away the indole. Specifically, several of those derivatives affected by indole gave spin resonance spectra which were either identical with that for trypsin. While these shifts were not always complete, this was understandable in view of the probable binding constant for indole. Fersht and Requena found an essentially pH-independent dissociation constant for indole of 0.8 mM at pH 7.8 and this was approximately the same at pH 4.5 (20). We would assume that the inhibited enzyme binds indole less effectively and that the value 0.8 mM would be an absolute lower limit for the dissociation constant of spin-labeled indole complexes at pH 3.5. It is interesting to note that Kosman obtained dissociation constants for modified chymotrypsin-indole complexes of 3 and 2 mM for a methionine 192- and a histidine 57-alkylated derivative, respectively (21). That is, it was unlikely that the enzyme was completely saturated with indole at the concentrations attainable in these studies.

The sulfonylated ester derivative p-II (p-SO2-60H) was also affected dramatically by indole, but in a somewhat different manner. Examination of p-II (p-SO2-60H) labeled α-chymotrypsin and trypsin in Fig. 3, indicated that both had two component spectra, the α-chymotrypsin derivative possessing a higher percentage of the more hindered broad line component (or conversely, a smaller amount of narrow line component) than did the trypsin derivative. In indole, the labeled chymotrypsin spectrum shifted significantly toward the (more mobile) narrow line component.

Most of those meta-substituted derivatives shown by model building to bind in the “tosyl” hole were least affected upon exposure to indole: m-V (m-CO-5NH), m-VI (m-NCO-GOH), m-VII (m-NCO-6NH), m-VIII (m-NH-5CO), and m-IX (m-CO-5CH2OH). Presumably their “effective equilibrium constants” favored the spin label binding in rather than away from the pocket. A complete summary of the α-chymotrypsin results is given in Table II. The spectral shifts upon indole exposure were manifested in several cases by the formation of a motional component spectrally identical with that for trypsin. While these shifts were not always complete, this was understandable in view of the probable binding constant for indole. Fersht and Requena found an essentially pH-independent dissociation constant for indole of 0.8 mM at pH 7.8 and this was approximately the same at pH 4.5 (20). We would assume that the inhibited enzyme binds indole less effectively and that the value 0.8 mM would be an absolute lower limit for the dissociation constant of spin-labeled indole complexes at pH 3.5. It is interesting to note that Kosman obtained dissociation constants for modified chymotrypsin-indole complexes of 3 and 2 mM for a methionine 192-alkylated and a histidine 57-alkylated derivative, respectively (21). That is, it was unlikely that the enzyme was completely saturated with indole at the concentrations attainable in these experiments due to the exposure of the enzyme to high sucrose concentrations seems unlikely from the recent results of Timasheff and co-workers who found no changes in both circular dichroism and activity measurements with α-chymotrypsin (19).
TABLE I
Results from model building for α-chymotrypsin

<table>
<thead>
<tr>
<th>Spin label</th>
<th>Probable contacts</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>All para derivatives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m-I (m-NH 5—CO)</td>
<td>Excluded by residues 217 to 220 lining the &quot;bottom&quot; of the pocket.</td>
<td></td>
</tr>
<tr>
<td>m-III (m-CO-6OH)</td>
<td>≤3 A contacts with Ser 217, Ser 218, Thr 219. Thr 219 peptide carbonyl oxygen sticks up and closest to piperidinyl ring methylene or methyl carbon atoms.</td>
<td></td>
</tr>
<tr>
<td>m-IV (m-CO-6NH)</td>
<td>As above.</td>
<td></td>
</tr>
</tbody>
</table>

CPK models of this label also indicate an inherent restricted rotation about the NH-pyrrolidinyl ring bond.

Highly restrictive yet possible binding and minimally restricted binding in "tosyl hole"

m-V (m-CO-5NH) | S enantiomer situates with the pyrrolidinyl ring plane over Ser 218-Thr 219-Cys 220 main chain; about 30-40° rotation is the maximum allowed. |
| m-VI (m-NCO-6OH) | Situated over the peptide chain Ser 218-Thr 219 with some rotational freedom. Allowing some free rotation about the carbonyl-oxygen bond of the carbamate, the piperidinyl ring may swing into the open cleft region where substantial rotational freedom is allowed. |
| m-VII (m-NCO-6NH) | As above. |

The only difference between this label and m-V (m-CO-5NH) its "reversed" derivative, is the direction of the amide linkage. However, in this derivative (m-VIII) the pyrrolidinyl ring is placed even closer into contacts with enzyme than above (m-V). |

m-VIII (m-NH-5CO) | S enantiomer, the only position of any plausibility is with the amide linkage almost parallel to the benzene ring leaving the pyrrolidinyl ring right over the Ser 218-Thr 219 peptide bond. The methyl groups σ to the nitroxide are just at 3 A contact distances from this portion of the protein. |
| m-IX (m-CO-5CH2OH) | Possible close contact with Ser 218 Cα when undergoing rotational motion. Met 192 must swing up and out of the way. |

The pyrroline ring situates in the open cleft portion of the active site near but more than 3 A from His 57. |

m-I (m-NH 5—CO) |

experiments (17). The indole concentration (solubility) at this pH was measured spectrophotometrically as 2 × 10⁻³ M.² Additionally, the reversible inhibitors DL-tryptophan and hippuric acid were investigated with a few of these derivatives. Saturated D-tryptophan (approximately 0.056 M) affected the spectrum of m-I (m-NH-5—CO) only slightly, m-V (m-CO-5NH) and p-V (p-CO-5NH) less so, and m-VI (m-CO-6NH) and m-VII (m-CO-6NH) not at all.² Saturated hippuric acid (about 0.018 M) gave similar results. All of these inhibitors have comparable dissociation constants near neutral pH: at pH 7.9, 1 M NaCl; D-tryptophan, Kᵢ = 10 mM; L-tryptophan, Kᵢ = 20 mM (22); at pH 8.25 (0.2 M Tris-HCl), hippurate, Kᵢ = 4.8 mM (23). Evidently, either their binding dissociation constants are much larger at pH 3.5, or their specific binding orientation at the tosyl hole differs from that for indole. Trypsin—Trypsin derivatives were examined initially in the presence of 1 mM benzamidine at pH 3.5 (0.006 M acetic acid, 0.02 M CaCl₂) in order to reduce the slow, yet detectable autoproteolysis of this enzyme (13, 24). These derivatives were later re-examined with much difficulty in the absence of benzamidine,
and were found to give spectra which, within the limits of the experiment, were qualitatively identical with the spectra obtained earlier. Except for the six-membered piperidine carbamate and urea derivatives, m-VI (m-NCO-6OH) and m-VII (m-NCO-6NH), respectively, the other derivatives could not be completely freed of autolyzed material (0.5 to 1% of the total protein in the sample). Without benzamidine present, the rate of autolysis increases in concentrated solutions of trypsin even at low pH and it is suspected that serine-blocked (i.e. sulfonylated) derivatives are even more susceptible than the native enzyme (25). Experimentally, the spectra for trypsin in the absence of benzamidine always contained a small overlapping narrow line component (that of the autolyzed form) which frequently obscured accurate spectral comparisons and measurement of some line shape parameters. What small differences arose with and without benzamidine were impossible to distinguish from the contribution due to limited autolysis. The trypsin spectra in Figs. 3 and 4, therefore, are judged to be the same within the accuracy of our spectral analysis, whether benzamidine was present or not (most of the spectra presented in those figures were for trypsin in 1 mM benzamidine at pH 3.5). The difficulties encountered in obtaining the clean, "nonautolyzed" spectra of Figs. 3 and 4 were many; a complete discussion of these problems and their resolution is the subject of the following paper (13). An example of the spectral problems caused by autolyzed spin-labeled enzyme is shown in Fig. 6. In addition, all of the trypsin derivatives were examined in the presence of saturated indole (with and without 1 mM benzamidine) and were found, within experimental error, to exhibit insignificant, if any, effects on the spectra.

It seems appropriate to consider why the trypsin spectra did not change dramatically in the presence of benzamidine. In some respects benzamidine and trypsin are analogous to indole and a-chymotrypsin. Both are potent reversible inhibitors which bind in the respective specificity pockets (8, 12), although indole has been shown in special cases with some nonspecific ester substrates to activate deacylation by increasing the rate constant, $k_d$ (18, 22). This does not occur with benzamidine, since crystallographic work indicates that the benzamidine-binding region in benzamidine-trypsin overlaps the DIP-binding region in DIP4-trypsin. Nevertheless, it has been shown both in solution (26) and in the crystal that DIP-trypsin can bind benzamidine, even though "binding" in the reverse order is impossible (as evidenced by inhibition). Several experiments suggested that the binding of benzamidine had little if any effect on the conformational environment of these spin labels. In the past work of Berliner and Wong (24) with the fluorophosphonate spin label (DIPSL), the presence of 1 mM benzamidine at pH 3.5 or 7.1 had no effect on the spectra. As mentioned earlier with the spin label-

4 The abbreviation used is: DIP, diisopropylphosphoryl.

sulfonlated trypsin derivatives, the effects of benzamidine were much harder to distinguish, if they occurred at all. Furthermore, it was probably unlikely that the spectra in Figs. 3 and 4 for spin-labeled trypsin were formed significantly in a complex with benzamidine (1 mM) at pH 3.5. Bechet and D’Albis (27) reported inhibition constants for native trypsin by benzamidine at pH 3.5 and pH 3.1 of 0.56 and 1.6 mM, respectively. It is reasonable to assume that the serine-blocked enzyme would bind even less benzamidine at this concentration. (For comparison, at pH 5.5, benzamidine-trypsin, $K_I \approx 3 \times 10^{-4}$ M (27) whereas DIP-trypsin binds about 10 times less effectively, $K_I = 4.5 \times 10^{-4}$ M (26).) That is, at the pH of these experiments, the benzamidine probably was bound fractionally to the labeled enzyme, yet effectively inhibited the unlabeled (native) trypsin in the samples.

A limited number of investigations were made at higher pH and somewhat higher benzamidine concentrations for one of the less “labile” inhibitors, m-VII (m-NCO-6NH). Trypsin inhibited with this spin label showed no detectable spectral changes when compared at pH 3.5 and 5.67 in benzamidine concentrations from 0 to 4 mM, in the presence or absence of CaCl$_2$ and in the presence or absence of saturated indole. At pH 6.5 the spectrum appeared to shift to slightly higher mobility (approximately a 10% increase in rotational correlation time); however, the accuracy of these measurements was plagued with the virtual impossibility of completely removing the small amounts of free label due to desulfonlation (18). In any case, it appeared at least for this label m-VII (m-NCO-6NH) and from the past work with the DIP analogue (DIPSIL) (24) that these experiments were not sensitive to any (benzamidine) ligand-induced structural changes or the small $pK_a$ connected with this binding (26, 27). Both East and Trowbridge (26) and Bechet and D’Albis (27) discovered a $pK_a$ of about 4.5 associated with benzamidine binding to trypsin as studied by spectral and other techniques.

### Table II

<table>
<thead>
<tr>
<th>Label</th>
<th>(Abbreviation name)</th>
<th>Binds in tosyl hole (model building)</th>
<th>Indole effect</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-I</td>
<td>o-NH-5=CO</td>
<td>+</td>
<td>+</td>
<td>Two-component spectrum.</td>
</tr>
<tr>
<td>p-I</td>
<td>p-NH-5=CO</td>
<td>-</td>
<td>+</td>
<td>Two-component spectrum.</td>
</tr>
<tr>
<td>p-II</td>
<td>p-SO$_2$-6OH</td>
<td>-</td>
<td>+</td>
<td>Two-component spectrum.</td>
</tr>
<tr>
<td>p-III</td>
<td>p-CO-6OH</td>
<td>-</td>
<td>+ (weak)</td>
<td>Rotational correlation time increases about 8 to 10% (more immobilized) in indole; the effect is more pronounced in sucrose.</td>
</tr>
<tr>
<td>p-IV</td>
<td>p-CO-6NH</td>
<td>-</td>
<td>+ (weak)</td>
<td>Very slight effect in either aqueous or sucrose solutions.</td>
</tr>
<tr>
<td>p-V</td>
<td>p-CO-5NH</td>
<td>-</td>
<td>+ (weak)</td>
<td>See Table I.</td>
</tr>
<tr>
<td>m-I</td>
<td>m-NH-5=CO</td>
<td>-</td>
<td>+</td>
<td>Very slight effect in aqueous solution; indole effect more pronounced in sucrose; only a highly restricted binding orientation in the tosyl hole was found from model building (see Table I).</td>
</tr>
<tr>
<td>m-III</td>
<td>m-CO-60H</td>
<td>-</td>
<td>+</td>
<td>See above for m-VI.</td>
</tr>
<tr>
<td>m-IV</td>
<td>m-CO-6NH</td>
<td>-</td>
<td>+</td>
<td>See Table I.</td>
</tr>
<tr>
<td>m-V</td>
<td>m-CO-5NH</td>
<td>+, (?)</td>
<td>-</td>
<td>Indole shifts the spectrum toward higher mobility.</td>
</tr>
<tr>
<td>m-VI</td>
<td>m-CO-60H</td>
<td>+, (?)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>m-VII</td>
<td>m-NCO-6NH</td>
<td>+, (?)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>m-VIII</td>
<td>m-NH-5CO</td>
<td>+, (?)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>m-IX</td>
<td>m-CO-6CH$_2$OH</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

#### DISCUSSION

The results presented here emphasize several points. First, a more complete comparison of the active site conformations of two enzymes is accomplished by utilizing several structurally different conformational probes (e.g. spin labels). The spin labels described in this report offer a wide sensitivity in the range of rotational correlation times ($\approx 10^{-3}$ to $10^{-10}$ s) displayed in the spectra of Figs. 3 and 4.

Of most importance is the interpretation of the results with respect to the original model proposed in the introduction. It is quite clear that the two active sites probed by the 14 spin-labeled inhibitors were not conformationally identical. In fact, there generally appeared to be a more restrictive interaction in trypsin than in $\alpha$-chymotrypsin. The binding of the aromatic portion of the label was probably the dominant feature accounting for the “specificity” of these inhibitors with $\alpha$-chymotrypsin in contrast to the less specific binding of aromatic moieties in trypsin. Although it was shown from model-building experiments that several of these spin labels were sterically restricted from binding in the same orientation as the tosyl group in tosyl- 

#### Footnote

6 This, of course, assumes that the aromatic group does not bind in the trypsin specificity pocket. A few of these inhibitors were tested in a trypsin model built from DIP-trypsin coordinates. It was not entirely impossible for the aromatic group to partially “dip” into the (benzamidine) specificity pocket. However, our solution results above suggest the contrary. A further thermodynamic argument disfavors the burying of a charged group (Asp 180) at the bottom of the pocket. On the other hand, assuming that some relationship may be attached between rate constant data and structure, Bender et al. (28) found that several acyl-chymotrypsins and acyl-$\alpha$-chymotrypsins had identical desacylation rate constants, $k_d$, for the same (in many cases aromatic) acyl substituent. 
showed no changes in its ESR spectrum upon exposure to indole at pH 3.5 (B. Landis and L. J. Berliner, unpublished).

We suggest the following scheme relating to the inhibition although the less simple alternative choice would fit the observations. We found that most interactions with the nitroxide ring took place within the experimental sensitivity of the measurement (about 0.2 Å) (8). Our arguments are based purely on steric factors, although the less simple alternative choice would fit the observations as well. We suggest the following scheme relating to the originally proposed model.

There are three principal binding modes for these spin-labeled inhibitors in α-chymotrypsin, (a) binding which mimics tosyl-α-chymotrypsin; (b) partial binding near the tosyl hole, this could be either a partial insertion of the aromatic group into the pocket or binding in a mode just outside, "covering" the pocket; (c) binding in a "general" region of the active site, perhaps common to all "homologous" serine proteases. Due principally to steric problems, many of those derivatives which bind in the α or β mode will exist in a small yet finite equilibrium with the other binding modes. Therefore, an equilibrium should exist which describes the general binding of any inhibitor:

$$a \xrightarrow{K_{ab}} b \xrightarrow{K_{bc}} c$$

with equilibrium constants $K_{ab}$ and $K_{bc}$. Furthermore, the binding of the inhibitor indole (I) to the tosyl pocket

$$a + I \xrightarrow{K_I} aI$$

can, in effect, displace the spin label from α or β to c if the indole-binding constant, $K_I$, is sufficiently competitive with the "effective" binding constant $K_{ab}$ or $K_{bc}$ of the sulfonyl inhibitor. It is obvious that a label which binds in α might be more difficult to displace (i.e., binds more strongly) than a label at β. In the studies presented here it was found that those labels which could not bind at α (the tosyl hole) displayed dramatic indole effects in most cases. This corresponds to our model to a shift from β to γ. In several cases, the chymotrypsin spectra became qualitatively similar to those for trypsin upon indole exposure, suggesting that in trypsin the labels bind at a site identical with c. A possible orientation for c is suggested by the structure of tosylelastase where the tosyl group is pointing almost 180° from its tosyl-α-chymotrypsin orientation, almost sandwiched to histidine 57 (29).

The validity of this model may be difficult to confirm by other methods. On the other hand, the experimental evidence is sufficiently strong to fit such a model as a reasonable possibility. The significance of the labels giving indole effects with "trypsin-like" spectra is more difficult to confirm, since not all of the spin labels behaved similarly. A similar comparison with the enzyme elastase may yield corroborating results. This enzyme resembles chymotrypsin in several respects with the prime exception of a blocked entrance to its specificity pocket (29).

**Residues Involved in Structural Discrimination**—Although there are over-all homologies in α-chymotrypsin and trypsin conformation, some small differences do arise in the vicinity of the active site. These are principally an addition or deletion of a residue or slight differences in backbone folding. For instance, when fitting several of the spin-labeled inhibitors in the chymotrypsin active site model, it was found that most interactions with the nitroxide ring took place along the chain Ser 217-Ser 218-Thr 219-Cys 220. Assuming that the aromatic sulfonyl group would bind similarly in trypsin, we note that its corresponding sequence is Ser 217 deletion (218)-Gly 219-Cys 220 (12). In α-chymotrypsin this chain is directed more toward the observer if viewed from the $a^*b$ (x-y) face near the dyad axis of the molecule. Such a difference is probably discounted in an overview of the two enzyme structures, yet may possibly be the significant point of discrimination in the study presented above. It is interesting to consider further the general observation that in both this study and the DIPSL spin label work of Morrisett and Broomfield (25), the spin label was consistently more immobilized in trypsin than in α-chymotrypsin. Although these two enzymes possess many homologies in their three-dimensional structures (12), there are obviously subtle differences which may only distinguish them from each other.
was forwarded above. However, another related aspect is the well documented existence of an auxiliary hydrophobic binding site in trypsin. For example, Sanborn and Hein (30) showed the existence of a neutral molecule binding site which was separate from the primary substrate binding site. There was also further evidence for an auxiliary hydrophobic site from the work of Heuberg et al. (31) and Hartmann and Holler (32) on alkyl amine inhibition of trypsin catalysis, the substrate activation studies of Trowbridge et al. (33) and product activation work of Howard and Meh 1 (34) to mention only a few. Since all of the spin label moieties were of a hydrophobic nature, it cannot be ruled out that much of the immobilization was manifested in selective binding to this hydrophobic binding region, an aspect which will deserve further study.

Finally, in closing, we may note that some other features of the general approach utilized here become apparent. First, although one studies the structural features of an enzyme in solution, it is imperative that the details of the crystallographic structure are considered and examined carefully, if only as a guideline. Conversely, the crystallographic structure is useful only as a possible static structure which cannot always be used to predict dynamic structural states in solution. In particular we are referring to the prediction of a dynamic picture of the binding of a small inhibitor molecule at the active site. Second, it became apparent in these studies that in choosing an aromatic probe for which one of the enzymes was more specific, one could no longer assume that the probe (spin label) was inert or impartial to the structural environments. As noted above, at least two classes of binding were possible in a-chymotrypsin where the spin-labeled aromatic sulfonates adopted orientations in and near the specificity pocket, respectively. In any case, if one knew essentially nothing about the chymotrypsin and trypsin structures, obviously the same conclusion, that their active site structures were indeed different, could have been drawn, albeit with less structural detail. Perhaps the definition of conformation here should be modified to include potential noncovalent interactions in addition to the relative juxtaposition of residues.

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