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

II. SPIN LABEL SYNTHESSES AND ENZYME INHIBITION*

SHAN S. WONG, KEVIN QUIGGLE,† CARL TRIPLETT,§ AND LAWRENCE J. BERLINER¶

From the Department of Chemistry, The Ohio State University, Columbus, Ohio 43210

SUMMARY

The syntheses and enzyme inhibition results for the derivatives discussed in the preceding paper are given. Spin-labeled sulfonyl fluorides containing six-membered piperidinyl nitroxide rings were particularly susceptible to intramolecular hydrolysis. In some cases, however, this hydrolysis was retarded significantly by the enzyme environment.

The preceding paper has described the sensitivity to active site structure of the series of spin-labeled sulfonyl fluorides specifically

![Chemical Structure](https://example.com/structure.png)

incorporated in α-chymotrypsin and trypsin (1). The spectral results also show the power of applying such a method to structural comparison of active sites of new proteases whose three-dimensional structures are not yet known. We present here the detailed syntheses and purifications of these spin labels and some of the specific problems encountered in preparing the inhibited sulfonylated enzyme derivatives.

EXPERIMENTAL PROCEDURE

Enzymes

α-Chymotrypsin (Lot CDI 8LK) and trypsin (Lots TRL 2DA, 2EA, 1GA, 2HX) were purchased from Worthington Biochemical Corp.

* This research was sponsored in part through grants from the National Science Foundation (GB 16437), Research Corporation, and the Public Health Service (GM 19364). A preliminary report of this work has been presented at the 14th International Biophysics Congress, Moscow, August 7 to 14, 1972.
† Present address, The Dracket Company, Cincinnati, O.
§ Present address, Department of Chemistry, Michigan Cancer Foundation, Detroit, Mich.
¶ To whom all correspondence should be addressed.

Chemicals

Electron spin resonance (ESR) measurements were taken at X band on a Varian E-4 spectrometer at 20 ± 2°C. Activity and protein concentration measurements were carried out on a Unicam SP 1800 spectrophotometer. For protein concentrations the absorbivities used at 280 nm were 5.0 × 10^4 M⁻¹ cm⁻¹ and 3.7 × 10^4 M⁻¹ cm⁻¹ for α-chymotrypsin (3) and trypsin (4), respectively. The extinction coefficients were increased by less than 1% at 280 nm as a result of labeling. Activity measurements were made with N-acetyltyrosine ethyl ester (ATEE) and p-tosyl-L-arginine methyl ester (tosyl-AME) for α-chymotrypsin (5) and trypsin (6), respectively, at 25°C. The stoichiometry of spin label incorporation was measured by hydrolyzing the spin label with strong base from a known concentration of each enzyme sample and comparing the resultant free nitroxide spectrum with that of a standard.

Preparation of Spin-labeled Enzymes

α-Chymotrypsin—The enzyme (∼10 mg per ml) was reacted at room temperature, pH 7 (0.1 M potassium phosphate buffer), with a 2 to 5 fold molar excess of an acetoniitride solution of spin label. After 30 min a similar concentration of inhibitor was added, as several of these spin labels were found to decompose in aqueous solution over this time course (probably via hydrolysis). The final organic solvent concentration in the reaction mixture was 9% (v/v). After 30 min the solution was clarified where necessary by filtration through a Swinney filter, then dialyzed exhaustively at 4°C against pH 7.5 dilute acetate acid (∼0.006 M), and finally against pH 3.5 (acetate acid) 0.1 M NaCl. Indole was added by dialyzing the spin label derivatives against saturated indole at pH 3.5, 0.1 M NaCl at 4°C.

Trypsin—In a typical experiment approximately 28 mg of...
enzyme were dissolved in 2 ml of 0.1 M Tris-Cl buffer, pH 7.7, containing 0.02 M CaCl₂. Spin label was added in a 2- to 5-fold molar excess in two 0.5-ml dioxane portions over a 30-min period. This high concentration of organic solvent was necessary in order to ensure sufficient solubility of the spin label. It should be noted that dioxane concentrations up to 50% (v/v) do not affect enzyme activity, as shown by past workers (7). The room temperature inhibition reaction was stopped after 1 to 2 hours and subjected to dialysis at 4°C for 6 to 10 hours against dilute acetic acid (∼0.006 M), pH 3.5, containing 0.02 M CaCl₂. The enzyme was then chromatographed on a Sephadex SP-50 column (1.5 × 11 cm, 30 ml per hour flow rate) with 0.1 M Tris-Cl, pH 7.1, containing 0.02 M CaCl₂ and none or 1 mM benzamidine as in earlier trypsin studies (2, 8). The fractions were collected and mixed immediately with 0.1 M citrate buffer (final pH was about 3.5) to reduce the probabilities of both desulfonylation and autolysis. The latter half of the overlapping α- and β-trypsin peaks in the elution profile was consolidated and concentrated about 10-fold in a collodion bag apparatus (Schleicher and Schuell). A final protein concentration of 2 to 7 mg per ml resulted. In cases where benzamidine was included in the eluting buffer, the resulting solution remained at approximately 1 mM benzamidine after concentration. All of the above purification steps were done at 4°C. ESR spectra were taken immediately after the final concentration step.

Syntheses—All elemental analyses were done by Chemalytics Inc., P. O. R. Inc. (Gainesville, Fla.). All melting points are uncorrected. The structures of the starting fluorosulfonyl derivatives and nitroxides are shown in Figs. 1 and 2, respectively. Refer to Fig. 2 of the previous paper for the structures of the inhibitors themselves. Details of these syntheses are presented in the “Appendix” immediately following this paper.1

RESULTS AND DISCUSSION

Synthetic Methods—The synthetic procedures used were relatively simple and straightforward procedures for ester and amide linkages as described in the “Appendix.” On the surface, the syntheses appear to be quite easily accomplished; however, we encountered great difficulties in syntheses involving the six-membered piperidinyl group in I or 2. We found that the esters, amides, carbamide, and urethans containing this ring structure were extremely labile to hydrolysis. The secondary aliphatic group J has also been reported by several groups to be quite sensitive to hydrolysis in ester and glycosidic linkages (10). Nevertheless, we were able to isolate the pure crystalline products in most instances by multiple purifications on silica gel columns and careful drying of the pure products.

1 The synthetic details are presented as a miniprint supplement immediately following this paper. Material published in miniprint form can be easily read with the aid of a large field reading glass of a type readily available at most opticians. For the convenience of those who prefer to obtain supplementary material in the form of a microfiche or full size photocopies, these same data are available as JBC Document No. 73M-1010. Orders for supplementary material should specify the title, authors, and reference to this paper and the JBC Document Number, the form of a microfiche or full size photocopy of eight pages, and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $2.50 for microfiche or photocopy.

2 T. Frey and L. J. Berliner, unpublished results.

3 A similar difficulty was encountered with a pyrrolidinyl secondary alcohol in phosphatidyl esters (11).

Unfortunately, during an enzyme inhibition reaction in aqueous solution we were faced with this hydrolysis problem in that a significant fraction of inhibitor had decomposed to (paramagnetic) sulfonyl fluoride and nonreactive nitroxide. This created no problem in the electron spin resonance (ESR) spectral analysis, since the technique observes only those enzymes modified with the intact paramagnetic inhibitor. Second, due to the quite limited solubility of all of the labels synthesized, and due to the minimal stability of several in aqueous solution, accurate kinetic measurements of the enzyme inhibition reaction were abandoned. Nevertheless, we were able at pH 3.5 to isolate stable spin-labeled sulfonylated chymotrypsin and trypsin in every case but one.

Enzyme Inhibition—A typical α-chymotrypsin inhibition reaction required about 1½ to 2 hours, compared to 30 min for phenylmethylsulfonyl fluoride under similar reaction conditions (12).
The reactivity toward trypsin was much poorer, yet this was expected from similar results obtained by earlier workers (12). The price paid for allowing longer reaction times for trypsin inhibition was the extensive autolysis of (already) labeled trypsin species (2). Under any set of labeling conditions, there was always a significant degree of autolysis of sulfonylated trypsin. This appears to be consistent with results obtained by other workers, suggesting that a serine-substituted trypsin was conformationally more susceptible to autolysis than native trypsin (5). In any case, it was necessary to separate these autolyzed forms from the intact forms by the specific procedures of Schroeder and Shaw (8). Since most of the labeled trypsin samples still contained some free enzyme, particular care was necessary in the final ultraconcentration step, as the enzyme would still undergo limited autolysis at presumably stable pH (3.5) values (2). In the cases where we included 1 mM benzamidine, a potent reversible inhibitor, this autolysis problem was reduced significantly.

From the previous work of Gold and Fahney (12, 14, 15) and Cardinaud and Baker (16) we can confidently assume that the inhibition occurs specifically by modifying the active Ser 195 of α-chymotrypsin or trypsin. Furthermore, both diisopropylphosphoryl (DIP)-chymotrypsin and chymotrypsinogen A fail to react with a spin-labeled sulfonyl fluoride under the identical conditions.

Tables I and II show the results obtained for some typical samples of labeled α-chymotrypsin and trypsin, respectively. The data for chymotrypsin (Table I) exemplify its high reactivity toward aromatic sulfonyl fluorides, as all of the derivatives were completely inhibited in the 1-hour reaction. However, the problem of intramolecular label hydrolysis (discussed earlier) is strikingly obvious when one compares the stoichiometry of spin label incorporation. Those labels containing the six-membered piperidinyl moiety (o-II, m-III, m-IV, m-VI, m-VII, p-II, p-III, p-IV) showed significantly reduced spin label incorporation since, as we believe, a substantial percentage of hydrolyzed (diamagnetic) sulfonyl fluoride inhibited the enzyme as well. Most of the other sulfonyl fluorides exhibited equimolar stoichiometry between inhibition and spin label incorporation. In the specific case of the ortho-substituted piperidinyl sulfonyloate o-II (o-SO4-6OH) intramolecular hydrolysis proceeded rapidly on the enzyme as well. This produced a large sharp line free spectrum which totally precluded direct observation of the labeled enzyme species alone. The results for trypsin (Table II) are quite similar, although the results display the significantly lower reactivity of this enzyme toward aromatic fluorosulfonyl compounds.

A second problem that occurred to a much lesser degree was the hydrolytic desulfonylation of the inhibitor from the enzyme.
This problem was studied in some detail by Gold for phenyl-methane sulfonyl fluoride (12, 15). He found that the rate of desulfonylation was slow and independent of pH over the range 2 to 8.5, but increased significantly at higher pH values. This phenomenon showed up at pH 3.5 as a finite but slow desulfonylation, which also resulted in the production of a sharp line spectrum for the liberated spin label sulfonate. In order to eliminate this spectral impurity, it was necessary to measure the ESR spectrum of a labeled enzyme immediately after removal from the dialysis cell.

Both of these phenomena could, of course, occur simultaneously as well. In the only examples examined in any detail, we monitored the increase of free spin label with the increase in enzyme activity for two labeled chymotrypsin derivatives, m-IV (m-CO-6NH) and p-II (p-SO$_2$-60H), respectively. If desulfonylation of diamagnetic sulfonates can also occur with no corresponding increase in spin label signal). If intramolecular spin label hydrolysis is predominant, then the enzyme activity should remain rather constant. The results are shown in Fig. 3. The derivative m-IV (m-CO-6NH) was remarkably stable to intramolecular hydrolysis on the enzyme (filled circles and triangles, Fig. 3). From the close correspondence of the ESR and activity curves, it was evident that this hydrolysis was comparable to, or only slightly faster than desulfonylation. This was a particularly remarkable result when one notes that the stoichiometry data in Table I for this label showed that only 40 to 60% of the fully inhibited enzyme was paramagnetic. Presumably, the environment of this highly vulnerable amide linkage was well protected from the solvent in its enzyme environment. On the other hand the para-sulfonate derivative p-II (p-SO$_2$-60H) underwent intramolecular hydrolysis to a much greater extent than desulfonylation while on the enzyme (open circles and triangles, Fig. 3). Although we did not broaden this investigation to all of the derivatives in Tables I and II, we believe that, except for the case of o-II (o-SO$_2$-60H), all of the hydrolytically susceptible labels were sufficiently well protected from intramolecular hydrolysis when on the enzyme. This is borne out by the fact that the free label spectrum appeared at a slow enough rate to obtain a "clean" ESR spectrum in the 5- to 10-min spectral scanning times.

This approach to studying chemical decomposition on an enzyme may be valuable in the future as a convenient tool for comparing the chemical similarities of specific regions of enzyme active sites with the medium or other organic solvents.

Acknowledgment—We wish to thank Dr. J. Morrisett, Baylor College of Medicine, for a gift of the primary alcohol nitroxide 6.

REFERENCES
1. BERLINER, L. J., AND WONG, S. S. (1974) J. Biol. Chem. 249, 1668-1677
2. BERLINER, L. J., AND WONG, S. S. (1973) J. Biol. Chem. 248, 1118-1120
3. LASKOWSKI, M. (1961) in Biochemist's Handbook (LONG, C., ed) p. 304, Van Nostrand-Reinhold Books, Princeton
4. TROWBRIDGE, C. W., KREHBIHL, A., AND LASKOWSKI, M., Jr. (1963) Biochemistry 2, 843-850
5. SCHWEITZ, G. W., AND TAKENAKA, Y. (1955) Biochim. Biophys. Acta 16, 570-577
6. HUMMEL, B. C. W. (1959) Can. J. Biochem. Physiol. 37, 1393-1399
7. COLETTI-PREVIERO, M-A., PREVIERO, A., AND ZUCKERKANDL, E. (1969) J. Mol. Biol. 40, 493-501
8. SCHOEDER, D. D., AND SHAW, E. (1968) J. Biol. Chem. 243, 2943-2949
9. ROZANTSEV, E. G. (1970) Free Nitroxyl Radicals. Ch. 9, Plenum Press, New York
10. WIES, W. W., MORRISETT, J. D., AND McCONNELL, H. M. (1972) Biochemistry 11, 3704-3716
11. THREE, R., AND DAVIES, A. P. (1970) Chem. Phys. Lipids 4, 60-71
12. GOLD, A. M, AND FAHRNEY, D. (1964) Biochemistry 3, 763-791
13. MORRISETT, J. D., AND BROOKFIELD, C. A. (1972) J. Biol. Chem. 247, 7224-7231
14. FAHRNEY, D. E., AND GOLD, A. M. (1963) J. Amer. Chem. Soc. 85, 997-1000
15. GOLD, A. M. (1965) Biochemistry 4, 897-901
16. CARDINAUD, R., AND BAKER, B. H. (1970) J. Med. Chem. 13, 467-470, references therein
17. STROUD, R. M., KAY, L. M., AND DICKERSON, R. E. (1972) Cold Spring Harbor Symp. Quant. Biol. 35, 125-140
Supplemental Material to 3-Phenyl-3-hydroxypropionic acid as Active Site Inhibitor of Proteinase

STRUCTURE II. 3-Phenyl propionate and ampergite pigments

The reaction procedure was identical to that of 5 (288 mg). The 3-phenyl propionate was obtained by the addition of 2.5 ml (0.5 mmol) of 3-phenyl propionic acid (0.25 mmol) to 4 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.01 M acetic acid. The reaction mixture was allowed to stand at room temperature for 24 h, and then the reaction product was precipitated by the addition of 5 ml of acetone. The precipitate was collected by centrifugation and dried under vacuum. The yield was 90% of the theoretical yield. The reaction product was dissolved in 0.1 M phosphate buffer (pH 7.4) and then dialyzed against distilled water. The dialyzed solution was lyophilized to give the pure product. The product was then purified by thin-layer chromatography (TLC) on silica gel. The purified product was subjected to 5 (288 mg) and then further purified by recrystallization from 0.1 M phosphate buffer (pH 7.4) using 5 ml of pure methanol. The yield was 90% of the theoretical yield. The product was then subjected to 5 (288 mg) and then further purified by recrystallization from 0.1 M phosphate buffer (pH 7.4) using 5 ml of pure methanol. The yield was 90% of the theoretical yield.

Elemental analysis for C_{15}H_{18}O_2P

Theory: C, 56.84; H, 6.82; P, 10.77

Found: C, 56.85; H, 6.80; P, 10.80

For p-32 Glucose-6-phosphate-3-dehydrogenase

The reaction procedure was identical to that of 5 (288 mg). The 3-phenyl propionate was obtained by the addition of 2.5 ml (0.5 mmol) of 3-phenyl propionic acid (0.25 mmol) to 4 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.01 M acetic acid. The reaction mixture was allowed to stand at room temperature for 24 h, and then the reaction product was precipitated by the addition of 5 ml of acetone. The precipitate was collected by centrifugation and dried under vacuum. The yield was 90% of the theoretical yield. The reaction product was dissolved in 0.1 M phosphate buffer (pH 7.4) and then dialyzed against distilled water. The dialyzed solution was lyophilized to give the pure product. The product was then purified by thin-layer chromatography (TLC) on silica gel. The purified product was subjected to 5 (288 mg) and then further purified by recrystallization from 0.1 M phosphate buffer (pH 7.4) using 5 ml of pure methanol. The yield was 90% of the theoretical yield. The product was then subjected to 5 (288 mg) and then further purified by recrystallization from 0.1 M phosphate buffer (pH 7.4) using 5 ml of pure methanol. The yield was 90% of the theoretical yield.

Elemental analysis for C_{15}H_{18}O_2P

Theory: C, 56.84; H, 6.82; P, 10.77

Found: C, 56.85; H, 6.80; P, 10.80

For p-32 Glucose-6-phosphate-3-dehydrogenase

The reaction procedure was identical to that of 5 (288 mg). The 3-phenyl propionate was obtained by the addition of 2.5 ml (0.5 mmol) of 3-phenyl propionic acid (0.25 mmol) to 4 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.01 M acetic acid. The reaction mixture was allowed to stand at room temperature for 24 h, and then the reaction product was precipitated by the addition of 5 ml of acetone. The precipitate was collected by centrifugation and dried under vacuum. The yield was 90% of the theoretical yield. The reaction product was dissolved in 0.1 M phosphate buffer (pH 7.4) and then dialyzed against distilled water. The dialyzed solution was lyophilized to give the pure product. The product was then purified by thin-layer chromatography (TLC) on silica gel. The purified product was subjected to 5 (288 mg) and then further purified by recrystallization from 0.1 M phosphate buffer (pH 7.4) using 5 ml of pure methanol. The yield was 90% of the theoretical yield. The product was then subjected to 5 (288 mg) and then further purified by recrystallization from 0.1 M phosphate buffer (pH 7.4) using 5 ml of pure methanol. The yield was 90% of the theoretical yield.

Elemental analysis for C_{15}H_{18}O_2P

Theory: C, 56.84; H, 6.82; P, 10.77

Found: C, 56.85; H, 6.80; P, 10.80

For p-32 Glucose-6-phosphate-3-dehydrogenase

The reaction procedure was identical to that of 5 (288 mg). The 3-phenyl propionate was obtained by the addition of 2.5 ml (0.5 mmol) of 3-phenyl propionic acid (0.25 mmol) to 4 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.01 M acetic acid. The reaction mixture was allowed to stand at room temperature for 24 h, and then the reaction product was precipitated by the addition of 5 ml of acetone. The precipitate was collected by centrifugation and dried under vacuum. The yield was 90% of the theoretical yield. The reaction product was dissolved in 0.1 M phosphate buffer (pH 7.4) and then dialyzed against distilled water. The dialyzed solution was lyophilized to give the pure product. The product was then purified by thin-layer chromatography (TLC) on silica gel. The purified product was subjected to 5 (288 mg) and then further purified by recrystallization from 0.1 M phosphate buffer (pH 7.4) using 5 ml of pure methanol. The yield was 90% of the theoretical yield. The product was then subjected to 5 (288 mg) and then further purified by recrystallization from 0.1 M phosphate buffer (pH 7.4) using 5 ml of pure methanol. The yield was 90% of the theoretical yield.

Elemental analysis for C_{15}H_{18}O_2P

Theory: C, 56.84; H, 6.82; P, 10.77

Found: C, 56.85; H, 6.80; P, 10.80

For p-32 Glucose-6-phosphate-3-dehydrogenase

The reaction procedure was identical to that of 5 (288 mg). The 3-phenyl propionate was obtained by the addition of 2.5 ml (0.5 mmol) of 3-phenyl propionic acid (0.25 mmol) to 4 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.01 M acetic acid. The reaction mixture was allowed to stand at room temperature for 24 h, and then the reaction product was precipitated by the addition of 5 ml of acetone. The precipitate was collected by centrifugation and dried under vacuum. The yield was 90% of the theoretical yield. The reaction product was dissolved in 0.1 M phosphate buffer (pH 7.4) and then dialyzed against distilled water. The dialyzed solution was lyophilized to give the pure product. The product was then purified by thin-layer chromatography (TLC) on silica gel. The purified product was subjected to 5 (288 mg) and then further purified by recrystallization from 0.1 M phosphate buffer (pH 7.4) using 5 ml of pure methanol. The yield was 90% of the theoretical yield. The product was then subjected to 5 (288 mg) and then further purified by recrystallization from 0.1 M phosphate buffer (pH 7.4) using 5 ml of pure methanol. The yield was 90% of the theoretical yield.

Elemental analysis for C_{15}H_{18}O_2P

Theory: C, 56.84; H, 6.82; P, 10.77

Found: C, 56.85; H, 6.80; P, 10.80
Spin-labeled Sulfonyl Fluorides as Active Site Probes of Protease Structure: II.
SPIN LABEL SYNTHESSES AND ENZYME INHIBITION
Shan S. Wong, Kevin Quiggle, Carl Triplett and Lawrence J. Berliner

*J. Biol. Chem.* 1974, 249:1678-1682.

Access the most updated version of this article at [http://www.jbc.org/content/249/6/1678](http://www.jbc.org/content/249/6/1678)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/249/6/1678.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/249/6/1678.full.html#ref-list-1](http://www.jbc.org/content/249/6/1678.full.html#ref-list-1)