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SPECIFIC SPIN-LABELING AT TRYPSIN ACTIVE SITE

APPLICATION OF 'INVERSE SUBSTRATE' TO THE STRUCTURAL ANALYSIS OF THE ACTIVE SITE

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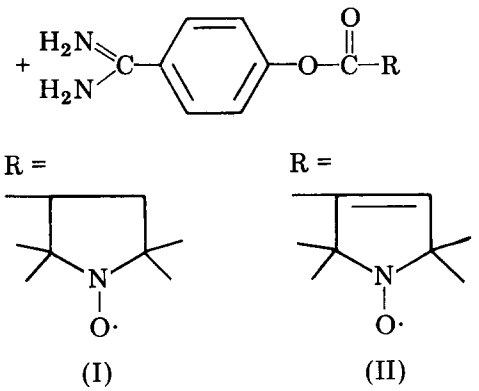
Summary

Specific and reversible spin-labeling of trypsin (EC 3.4.21.4) active site was carried out by 'inverse substrate', 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyl-oxyl and 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolinyloxyl-*p*-amidinophenyl esters. The paramagnetic resonance spectra of the labeled trypsin in the form of acyl enzyme intermediate were investigated. The $2T$ value, separation of the outer hyperfine extrema, was observed to be sensitive to pH of the medium. These results are discussed in terms of pH-dependent conformational change at the vicinity of active site.

Introduction

Esters of *p*-amidinophenol were demonstrated to undergo efficient and specific tryptic hydrolysis [1]. These esters are characterized by their linkage, i.e., the site-specific cationic center is included in the leaving group instead of being in the acyl moiety. The concept, 'inverse substrate', was proposed for these esters with regard to their specific binding and efficient acylation comparable to those for normal-type substrates. A facile procedure for the preparation of acyl enzymes carrying nonspecific residues was also demonstrated in the previous study [1]. This approach may enable us to provide a general method for the specific introduction of reporter groups into the active site of trypsin (EC 3.4.21.4) without recourse to a cationic acyl moiety inherent to conventional substrates.

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Bes, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid.



SCHEME I.

Spin-labeling of proteins provides sensitive information about the motional freedom of the radical moiety reporting the nature of the environment in which it resides. Spin-labeling at the active center of chymotrypsin and trypsin has been achieved using phosphorylating [2—4] or sulfonylating reagents [5,6], which label the enzymes irreversibly. On the contrary, reversible labeling was unsuccessful except for a few cases in chymotrypsin [7,8]. For trypsin, none of such a labeling has been reported because the design of the reagents which have strong affinity to the enzyme was considered not feasible. In the present report, the facile method for the preparation of spin-labeled acyl trypsins and their spectroscopic behavior are described.

Materials and Methods

Synthesis of spin-labeled p-amidinophenyl esters. DL-3-Carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl and 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolinyloxyl were prepared from 3-carbamoyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl and 3-carbamoyl-2,2,5,5-tetramethyl-1-pyrrolinyloxyl (Aldrich Chemical Company, Inc.), respectively, according to the procedure in Ref. 9. *p*-Hydroxybenzamidine hydrochloride and its *p*-toluenesulfonate were prepared as described in the previous paper [1]. The coupling of *p*-hydroxybenzamidine with 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl or 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolinyloxyl was carried out following the procedure reported by Rozantsev [10]. To a solution of 3-chloroformyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl in anhydrous tetrahydrofuran was added a solution of equimolar amount of *p*-hydroxybenzamidine hydrochloride in anhydrous pyridine. The mixture was kept at room temperature overnight. For synthesis of the pyrroline derivative *p*-hydroxybenzamidine *p*-toluenesulfonate was used instead of the hydrochloride. The products were purified by silica gel column chromatography ($CHCl_3/CH_3OH$, 4 : 1) and recrystallized from CH_3CN. DL-3-Carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl-*p*-amidinophenyl ester hydrochloride (I): m.p. 206—208°C (decompn.). 3-Carboxy-2,2,5,5-tetramethyl-1-pyrrolinyloxyl-*p*-amidinophenyl ester *p*-toluenesulfonate (II): m.p. 198.5—201.5°C (decompn.).

All other chemicals used were Koso and Nakarai Chemicals, analytical grade.

Determination of the kinetic parameters and preparation of the acyl enzyme. Bovine trypsin was purchased from Worthington Biochemicals (TRL 36S799). Enzyme concentration was measured spectrophotometrically at 280 nm ($E^{1\%}_{1cm}$ = 15.5 [11]). The acylation rates of trypsin by I and II were analyzed by using a Union Giken Corp. RA/401 stopped-flow spectrophotometer, and steady-state rate constant (k_{cat}) were determined by using a Shimazu UV-200 double-beam spectrophotometer. The reactions were monitored by measuring the liberation of amidinophenolate ion at pH 8.0 ($\Delta\epsilon_{305nm}$ = 14 000).

Spin-labeled acyl enzyme was prepared by the method previously reported [1]. 120 mg trypsin was dissolved in 15 ml of 0.05 M Tris/0.02 M $CaCl_2$ (pH 8.0). To this solution, 5 equiv. of I (7 mg) or II (10.5 mg) in 0.3 ml of ethanol was added and the mixture was incubated at 25°C for 5 min. The pH was adjusted to 2.0 by addition of 1 N HCl and the resultant solution was gel filtered on a Sephadex G-25 column (2.7 × 46 cm) using HCl (pH 2.0) as an eluent, and lyophilized. For further purification, the lyophilized acyl enzyme, 55 mg, was redissolved in 3 ml of 1 N HCl and gel filtered on a Sephadex G-75 column (2.7 × 90 cm) using HCl (pH 2.0) as an eluent and lyophilized.

Reactivation of acyl enzyme resulting from deacylation was carried out by incubation of the acyl enzyme preparation in 0.1 M Mes/0.02 M $CaCl_2$ at pH 6.0, 25°C. The time course of reactivation was obtained by measuring the residual activities of the aliquots toward *N*-α-benzyloxycarbonyl-L-lysine-*p*-nitrophenyl ester hydrochloride (Aldrich Chemical Company, Inc.) by the method of Bender et al. [12]. The substrate concentration was $4.87 \cdot 10^{-4}$ M and the acyl enzyme concentration was $1.43 \cdot 10^{-6}$ M.

Electron spin resonance (ESR) measurements. The ESR spectra of spin-labeled acyl enzyme were obtained at X band on a JEOL Model ME-3X spectrometer at 25°C or 77 K. The measurements were performed within 30 min from dissolution. The concentration of acyl enzyme solution was 10^{-4} M. Buffer solution: pH 2.4, 0.1 M KCl/HCl; pH 2.4—5.0, 0.1 M citrate; pH 5.0—6.0, 0.1 M acetate; pH 6.0—6.5, 0.1 M Mes; pH 6.5—7.0, 0.1 M Bes. Each $2T$ value was determined by duplicated or triplicated measurements. They agreed to less than 0.2 G from the mean. Experimental errors for the rotational correlation time (τ_R), therefore, are calculed to be less than $1.0 \cdot 10^{-9}$ s.

The spectral changes associated with deacylation were monitored by measuring the spectra of the aliquots incubated in 0.1 M Mes/0.02 M $CaCl_2$ at pH 6.0, 25°C, at appropriate intervals after lowering pH to 3.0 by the addition of 1 N HCl.

Results and Discussion

Kinetic parameters and acyl enzyme preparation

Kinetic parameters for trypsin-catalyzed hydrolysis of amidinophenyl esters are listed in Table I. As shown in the table, both compounds I * and II have strong affinity to trypsin. The K_s values are comparable to those of *p*-acetoxy-

* The compound I used in the experiment is a racemic mixture, and the enantiomeric specificity has not been examined.

TABLE I

KINETIC PARAMETERS FOR SPONTANEOUS AND TRYPSIN-CATALYZED REACTIONS OF AMIDINOPHENYL ESTERS

0.05 M Tris-HCl, 0.02 M $CaCl_2$, pH 8.0, 25°C.

Substrate	K_s (M, $\times 10^5$)	k_2 (s^{-1}, $\times 10$)	k_{cat} (s^{-1}, $\times 10^4$)	k_{spont} (s^{-1}, $\times 10^6$)
I	57.8	0.79	18.4	35.0
II	2.45	1.87	87.0	6.00
p-Acetoxybenzamidine *	3.87	170	92.6	26.0
p-Trimethylacetoxybenzamidine *	16.2	11.5	2.59	2.27

* Ref. 1.

benzamidine and *p*-trimethylacetoxybenzamidine [1]. In addition to the binding affinity, much faster acylation rate than the deacylation rate facilitates the production of acyl trypsin. In fact facile preparation of spin-labeled acyl enzyme was carried out using I or II following the procedure [1]. The remaining activity of the acyl trypsin preparation was less than 2% of that of the native enzyme, while substantial reactivation (80%) was observed as a result of deacylation after incubation at pH 6.0, 25°C.

ESR spectra

The ESR spectra of the spin-labeled acyl enzyme are shown in Fig. 1. The spectra of acyl enzyme derived from racemic compound (I) are identical to those from non-racemic II. These spectra have both sharp resonance lines and broad lines. Similar sharp resonance lines were reported by Berliner and Wong [4] in their spin-labeled trypsin preparation obtained by phosphorylating reagents. These lines were estimated to be originated from autolyzed fragments of the spin-labeled trypsin, which could not be removed by Sephadex G-25 chromatography or dialysis. The sharp line components of our sample could not be removed by Sephadex G-25 chromatogaphy at pH 3.0 (Fig. 1a), but they were removed by Sephadex G-75 to a negligible extent (Fig. 1c). In the meanwhile, sharp resonance lines appeared again from the acyl trypsin preparation purified by Sephadex G-75 after incubation at neutral pH. The appearance of these lines was accompanyed with increase of the enzymic activity (Fig. 2). The deacylation rate constant, k_3, obtained from the recovery of the enzymatic activity coincided with that obtained from the peak height of the sharp resonance lines. It is evident that the sharp lines are originated from the corresponding *N*-oxyl acids liberated from trypsin active site. The spectrum resembles those of spin-labeled trypsin obtained from the phosphorylation [2—4] or those of spin-labeled chymotrypsin [7]. These spectra demonstrate that the spin component is highly immobilized. The $2T$ values, the separation of the high and low-field hyperfine components, are one of measures of the microenvironments of the spin-labeled site, because the values are dependent on the rotational correlation times. These values for spin-labeled trypsins were found to be variable with pH (Fig. 3). The pH-inherent dependency was confirmed by the measurements overlapping the pH coverage of respective buffers. It is noted that the parent *N*-oxyl acids exhibit no such changes in this pH

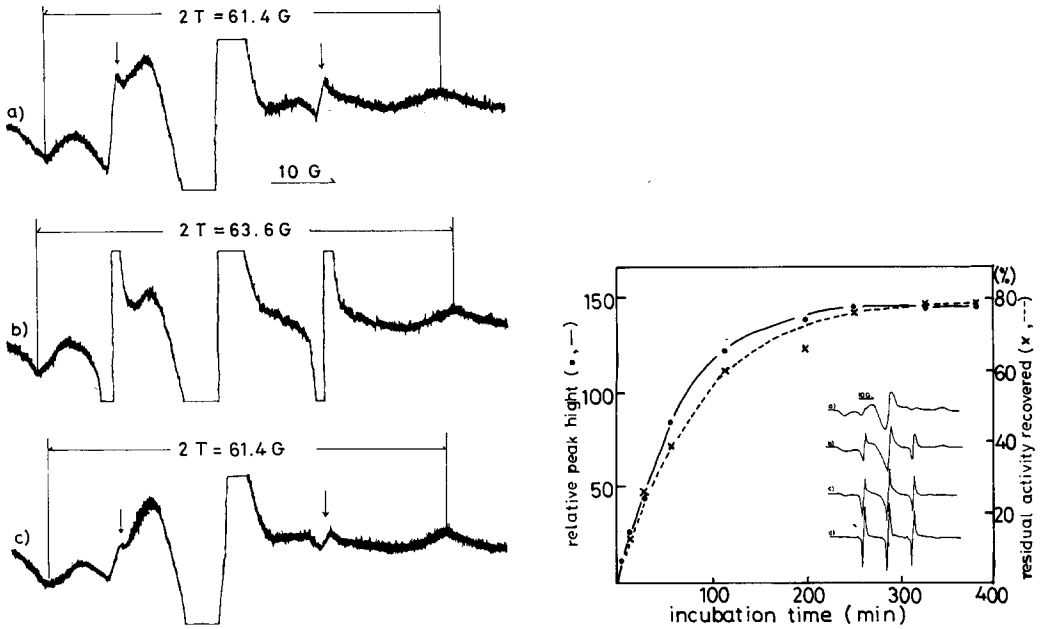


Fig. 1. The ESR spectra of acyl trypsin derived from I. (a) Measured at pH 3.0, after gel filtration on Sephadex G-25; (b) measured at pH 7.0, after gel filtration on Sephadex G-25, and (c) measured at pH 3.0, after consecutive gel filtration on Sephadex G-25 and G-75 (see Materials and Methods). Resonance lines originated from autolyzed trypsin (↓) were greatly diminished in the spectrum (c). Sharp resonance lines observed in the spectrum (b) were originated from free *N*-oxyl followed deacylation during the period of the measurement.

Fig. 2. ESR spectral changes of acyl trypsin associated with deacylation. Acyl trypsin derived from I was gel filtered consecutively by Sephadex G-25 and G-75 column. Spectra were measured at pH 3, 25°C. Before the measurement, the samples were incubated in 0.1 M Mes, pH 6.0. Intensities of sharp resonance lines at the high-field peak were plotted as a function of the incubation period. Residual activities toward *N*-α-benzyloxycarbonyl-L-lysine-*p*-nitrophenyl ester hydrochloride were compared to that of native trypsin. ESR spectra resulting from the incubation for 0 (a), 15 (b), 30 (c) and 200 min (d) were shown.

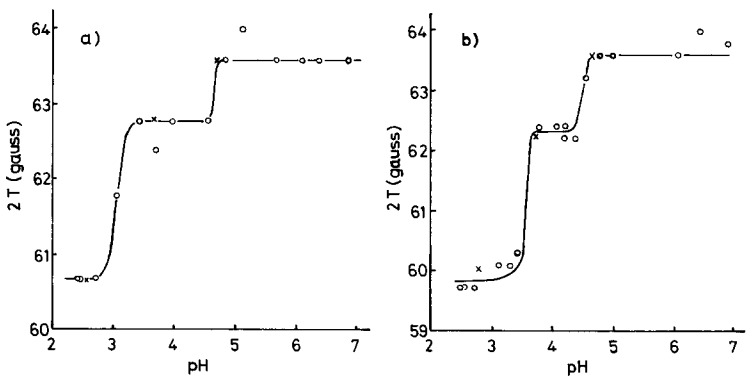


Fig. 3. The pH profile of the 2*T* values of acyl trypsin. (a) Derived from I, $pK_{a(1)}$; 3.1, $pK_{a(2)}$; 4.75 are determined; (b) derived from II, $pK_{a(1)}$; 3.5, $pK_{a(2)}$; 4.5 are determined. ×, in the presence of 0.02 M Ca^{2+}; ○, in the absence of Ca^{2+}.

range. From the pH dependency of $2T$ values observed for the acyl enzyme, the involvement of the functional groups with pK_a values 3.1—3.5 and 4.5—4.75 are suggested for the constituents of active site vicinity. On the contrary, it was reported, by Berliner and Wong [4], that the spin-labeling by phosphorylating reagents resulted in the observation of the identical conformational environment at either neutral and acidic pH. The different response between our labeling and the labeling by phosphorylation would arise from the difference in the chain length between the attaching group and the NO group.

On the other hand, differences in conformations of trypsin between at neutral and acidic pH were analyzed by ESR spectra obtained from double-labeled phosphorylating reagent [3]. It was shown that the immobilization of nitroxide groups decreased with increasing pH, just the opposite to ours. The spectral changes from acidic to neutral form were very slow and it is irreversible [3]. These observations seem different in the mechanistic basis from those of ours where the pH-dependent transition is completely reversible and instantaneous.

The ESR of a nitroxide-free radical is sensitive to rotational motions with rotational correlation times, τ_R, in the 1—1000 ns range [13]. For the slow-motional region, it was pointed out that shifts in the positions at the outer extrema of the spectrum ($2T$ value) yield a quantitative measure of τ_R [14]. The τ_R values at 25°C were determined by a simplified method by Goldman et al. [15], to use the outer extrema at 77 K for the rigid-limit values, and they are listed in Table II. It is shown that the motional freedom decreases with the increasing pH. Consistently, the transitions of $2T$ values in Fig. 3 are ascribed to the results from the change of the enzyme structure where rotational motion of the spin components are mainly influenced. The rotational correlation time of spin-labeled α-chymotrypsin was reported to be $12 \pm 2 \cdot 10^{-9}$ s at 20°C [14].

For native trypsin, pH-dependent reversible conformational changes participating ionization groups of the pK_a values, 1.2, 3.0 and 5.2, were detected by observing the absorbance change at 293 nm [16]. A gradual conformational transition over the pH range from pH 3.0 to 5.0 was also reported in the optical rotation study of native trypsin [17]. In contrast with these observations where the site of the structural transition was not specified, our results described above are the measures of the structural change occurred at a limited area, active site vicinity. Our observations are incidentally very close to those for native trypsin.

TABLE II

ROTATIONAL CORRELATION TIME (τ_R) OF ACYL TRYPSIN AT 25°C

τ_R as determined by the use of the outer extrema in the spectra at 77K for the rigid-limit values.

Acyl trypsin derived from	τ_R (ns)		
	pH 3.0 *	pH 4.0 *	pH 6.0 **
I	5.2	6.4	7.5
II	5.2	6.6	7.8

* 0.1 M citrate.

** 0.1 M acetate and 0.1 M Mes.

Acceleration at the deacylation rate by the addition of cationic molecules was observed in the tryptic hydrolysis of 'inverse substrate' of which an acyl group is spacially small [18]. The result was analyzed as a cooperative effect of acyl enzyme and the cationic molecule which coexist within the active site. In the hydrolysis of I and II, no such an acceleration was observed by the addition of *p*-hydroxybenzamidine at the concentration used. The acyl groups introduced by these substrates, are perhaps too bulky for the production of sufficient concentration of acyl trypsin-ligand complex in such a manner as small acylgroups do. This observation is consistent with the fact that the ESR spectra of acyl trypsins were not perturbed by the presence of *p*-hydroxybenzamidine. pH-dependency of the $2T$ values in the presence of 1 mM of *p*-hydroxybenzamidine was also examined. This dependency curve was found identical to those in the absence of the cation shown in Fig. 3. All these observations could support the previous assumption that the accomodation of specific ligand in the acylated trypsin active center suffers spacial restriction arised from the bulkyness of both acyl group and ligand [18].

The binding of the specific ligand to the acyl (or modified) enzymes which carry nonspecific residues has been the subject of considerable study [5,8,19, 20]. Requirements for the binding of the cationic ligand to acyl trypsin derived from 'inverse substrates' have been studied [21]. Systematic analysis of the dissociation constants for acyl trypsin-cationic ligand complexes revealed that the binding was decreased mainly with increasing the sterical size of the acyl group. Dissociation constants for the acyl trypsin derived from I and II with *p*-hydroxybenzamidine can be estimated to be more than 0.1 M.

Work is in progress to approach the topography of the active center by utilizing the labels with various chain length between the attaching CO group and the NO group.

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