Preparation and Characterization of Anhydrothrombin†

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ABSTRACT: Anhydrothrombin, a catalytically inactive derivative of thrombin in which dehydroalanine replaces the active-site serine, was prepared by a novel method. The active-site serine of thrombin was modified to dehydroalanine by promoting the β-elimination of phenylmethylsulfonic acid from phenylmethylsulfonyl fluoride-inactivated thrombin under conditions in which the enzyme is unfolded. After the elimination reaction was quenched, the resulting anhydrothrombin was folded by diluting the denaturant, Gdn·HCl, to nondenaturing concentrations. Anhydrothrombin was purified by PAB affinity chromatography. Both native thrombin and anhydrothrombin were digested by cyanogen bromide, and the peptides from the region of the active-site serine (S205) were isolated by reverse-phase high-pressure liquid chromatography. Serine was present in the native thrombin peptide but absent from the anhydrothrombin peptide, as shown by amino acid analysis. This anhydrothrombin peptide was found to be 18.7 ± 1.6 lower in mass units than the native peptide by electrospray mass spectrometry, in accord with the elimination of a water molecule. The anhydrothrombin preparation was monomeric, as determined by sedimentation equilibrium. Anhydrothrombin was used in a competitive titration of the complex of native thrombin with the leech saliva protein hirudin, a potent thrombin inhibitor, as measured by the recovery of thrombin amidolytic activity. This demonstrated that anhydrothrombin is capable of nativelike binding interactions with macromolecular ligands.

Thrombin plays a central role in thrombosis and hemostasis, with involvements in both the activation and inhibition pathways of blood coagulation. Its primary role is as a catalyst in the first of three *reversible* steps that lead to clot formation (Scheraga & Laskowski, 1957) in which it cleaves four Arg−Gly bonds of fibrinogen, releasing two fibrinopeptides A (FpA)[1] and two fibrinopeptides B (FpB) to form fibrin monomer. Fibrin monomer polymerizes spontaneously to form the fibrin gel, a fibrous scaffold within which platelets and blood cells are incorporated to form a clot, the hemostatic plug that prevents blood loss from a wound. Thrombin also activates factors V, VIII, and XIII in its coagulation promoting and stabilizing roles (Lorand & Konishi, 1964; Colman, 1969; Fass et al., 1982). Thrombin activation of protein C provides a feedback mechanism for regulation of thrombin activation and clot formation (Esmon et al., 1982). In addition to these activities, thrombin exhibits several hormonelike interactions with a variety of cell types (Davey & Luscher, 1967; Chen & Buchanan, 1975; Baker et al., 1980).

Bovine thrombin is a glycoprotein consisting of two polypeptides linked by a single disulfide bond. The 259-residue B-chain contains all the residues required for catalysis (Hageman et al., 1975) and is largely homologous with chymotrypsin and trypsin, its most significant distinguishing features being a number of loop insertions (Bode et al., 1989). The 49-residue A-chain has no known direct involvement in the binding interactions of thrombin with other proteins.

An important feature of thrombin is the presence of two binding sites, an active site, or primary binding site where catalysis occurs, and a secondary binding site that is distinct from the active site (Fenton et al., 1988). Disruption of this secondary site by autoproteolytic activity (Lundblad et al., 1979) has little effect on the ability of thrombin to cleave small-molecule substrates, but is critical for the specificity of thrombin for macromolecular substrates such as fibrinogen (Lundblad et al., 1984; Elion et al., 1986). Further evidence for the ability of thrombin to interact with substrates at a site distinct from its active site was the finding that near-physiological specificity for thrombin cleavage of a fibrinogen-like peptide substrate could be achieved with Aα(1−52) [the numbering scheme being that for the Aα-chain of human fibrinogen (van Nispen et al., 1977)], a peptide substrate that extends well beyond the Arg 16−Gly 17 bond cleaved by thrombin, but not with shorter peptides (Hageman & Scheraga, 1974). An X-ray crystal structure of thrombin complexed with hirudin, a 65-residue medicinal-leech protein that is a potent thrombin inhibitor (Markwardt, 1970), has provided atomic details of the interactions of thrombin with this inhibitor at both the active site and the secondary binding site of thrombin (Grütter et al., 1990; Rydel et al., 1990; Vitali et al., 1992).

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[1] Abbreviations: CNBr, cyanogen bromide; FpA, fibrinopeptide A; FpB, fibrinopeptide B; AT, anhydrothrombin; PAB, *p*-aminobenzamidine; PEG, poly(ethylene glycol); PPACK, D-Phe-Pro-Arg chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; PMS, phenylmethylsulfonic acid; PMS-thrombin, phenylmethylsulfonylthrombin; RP-HPLC, reverse-phase high-pressure liquid chromatography; ES/MS, electrospray mass spectrometry; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; NOE, nuclear Overhauser effect; NMR, nuclear magnetic resonance; BPTI, bovine pancreatic trypsin inhibitor; DTT, dithiothreitol; Gdn·HCl, guanidine hydrochloride; TFA, trifluoroacetic acid; BSA, bovine serum albumin; TLCK, tosyllysine chloromethyl ketone; $NaBH_4$, sodium borohydride; NTSB, disodium 2-nitro-5-thiosulfobenzoate.

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In previous work in this laboratory, transferred NOE NMR experiments were carried out to examine the complex formed between thrombin and a peptide derived from the Aα-chain of fibrinogen, Aα(7−16) (Ni et al., 1989a,b). It was found that the bound peptide adopts a turn structure that brings the residues Phe 8, Leu 9, and Val 15 into proximity with each other to form a hydrophobic cluster that interacts with a nonpolar binding site on thrombin and lies near the cleavable Arg−Gly bond. This study provided a structural basis for the observation that the highly conserved Phe 8 residue, which is nine residues away from the cleavable Arg−Gly bond, must be present to obtain optimal thrombin specificity for cleavage of this bond (Blombäck et al., 1969; Marsh et al., 1982). X-ray crystallographic studies (Martin et al., 1992) of thrombin complexes with Aα(7−16) have since confirmed these findings.

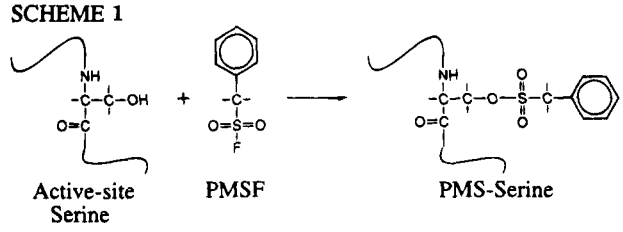
These studies have provided important structural information about the interactions of thrombin with the Aα-chain residues on the N-terminal side of the cleavable Arg−Gly bond of fibrinogen. It is now important to extend these studies to the examination of interactions of thrombin with the regions of fibrinogen and fibrinogen-like substrates that extend in the C-terminal direction beyond the Arg−Gly bond cleaved by thrombin. Also, despite the wealth of information that has been gained from recent studies, the structural details of the interactions of thrombin with fibrinogen at the secondary binding site are lacking.

The high-resolution X-ray crystallographic studies of inhibited thrombins have greatly increased our understanding of the interactions of thrombin (Bode et al., 1989; Grütter et al., 1990; Rydel et al., 1990). More information can be obtained, however, by using thrombin derivatives that retain the ability to bind intact *substrates* in a physiological-like manner, without cleavage of the peptide bonds normally targeted by thrombin. To obtain such a thrombin derivative, we have prepared anhydrothrombin, a catalytically inactive thrombin derivative in which the active-site serine is chemically modified to dehydroalanine. The resulting amino acid residue is an analog of alanine with a double bond between the C^{α} and C^{β} carbons. This modification renders anhydro derivatives of serine proteases incapable of cleaving peptide bonds.

Anhydro derivatives of chymotrypsin and trypsin have been prepared by promoting the β-elimination of PMS[1] from the PMSF-inactivated enzyme under alkaline conditions, as shown in Figure 1 (Strumeyer et al., 1963; Ako et al., 1972). The utility of this reaction is enhanced by the absolute specificity of the PMSF modification for the active-site serine (Gold & Fahrney, 1964). The end result of these chemical modifications is the *removal* of a water molecule from the active-site serine of thrombin. Hence, the derivatization will not sterically hinder ligand binding to the primary binding site of the modified thrombin.

Studies of anhydrotrypsin and anhydrochymotrypsin indicate that these derivatives retain binding properties similar to those of the native precursors for both protein inhibitors and small substrate-like ligands (Weiner & Koshland, 1965; Feinstein & Feeney, 1966; Ako et al., 1972, 1974; Vincent et al., 1974; Ishii & Yokosawa, 1977; Schultz et al., 1979). An X-ray crystal structure at 1.9 Å resolution of BPTI bound to anhydrotrypsin (Huber et al., 1975) found no significant structural differences between this complex and the complex of native trypsin and BPTI.

SCHEME 1



SCHEME 2

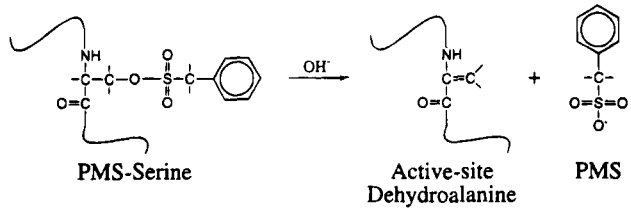


FIGURE 1: Scheme 1: inactivation of thrombin by PMSF. Scheme 2: PMS elimination reaction.

In this paper, we present the preparation of bovine anhydrothrombin by a novel method, and its characterization. This alternative method was developed because previous attempts to prepare anhydrothrombin using literature procedures for the preparation of trypsin or chymotrypsin had resulted in denatured thrombin preparations. Also, we examine the binding of hirudin to anhydrothrombin, as a probe of interactions at both the primary and secondary binding sites of anhydrothrombin. This method of preparation of anhydro derivatives may be generally applicable to a variety of serine proteases, including human thrombin, provided that they can be refolded after denaturation in Gdn·HCl. Tomono and Sawada (1986) have previously published a procedure for the preparation of human anhydrothrombin, but we were not able to adapt it to bovine thrombin for reasons discussed herein.

Bode et al. (1989) have devised a canonical numbering scheme that aligns the homologous regions of the serine proteases, human thrombin, bovine thrombin, bovine trypsin, and bovine chymotrypsinogen. This scheme identified the active-site serine of these proteases as Ser 195. The numbering scheme used in the present paper labels the thrombin B-chain residues sequentially from Ile 1 through Ser 259. Thus, the active-site serine is identified here as Ser 205, which is Ser 195 in the scheme of Bode et al.

MATERIALS AND METHODS

Materials. The following items were purchased from Sigma Chemical Co.: Tris, poly(ethylene glycol) (8000 MW), *Echis carinatus* snake venom, phenylmethylsulfonyl fluoride, BSA, 2-mercaptoethanol, and the SDS-7 molecular weight standard. *p*-Aminobenzamidine−Sepharose was purchased from Pharmacia. PD-10 desalting columns were purchased from Bio-Rad. D-Phe-Pro-Arg chloromethyl ketone was purchased from Bachem. Ultrapure guanidine hydrochloride was purchased from ICN. Protogel [premixed polyacrylamide and bis(acrylamide) for gel electrophoresis] was purchased from National Diagnostics. HPLC grade acetic acid and acetonitrile were purchased from Fisher Chemical Co. Trifluoroacetic acid was purchased from J. T. Baker. The chromogenic substrate S-2238 was a generous gift from Kabi. NTSB was prepared in our laboratory. Recombinant hirudin was a generous gift from Ciba-Geigy.

Bovine Thrombin. Bovine thrombin was prepared by the procedure of Ghosh and Seegers (1980) and purified as reported previously (Ni et al., 1989a).

Protein Concentration. Concentrations of thrombin and anhydrothrombin stock solutions were determined by absorbance measurements using an extinction coefficient of $E_{280}^{1\%} = 19.5$ (Winzor & Scheraga, 1964). It was assumed that the modification of the thrombin active-site serine to dehydroalanine had no effect on the absorbance of anhydrothrombin relative to that of thrombin. The concentration of the hirudin stock solution was determined at the Cornell Biotechnology facility by amino acid analysis using Waters Pico-Tag chemistry.

Phenylmethylsulfonylthrombin and PPACK-Thrombin. PMS-thrombin was prepared by the addition of 26 μL of 7% (w/v) PMSF in methanol, 3 times at 30-min intervals, to 24 mg of thrombin in 50 mM sodium phosphate buffer, pH 6.5, which was 0.15 M in NaCl and 0.1% in PEG. During and between additions of PMSF, the protein solution was allowed to sit at room temperature (23 °C). The PMS-thrombin was concentrated to 3 mL by using an Amicon concentration cell fitted with a YM10 membrane, and then applied to a Bio-Rad PD-10 desalting column equilibrated with 10 mM sodium phosphate buffer, pH 6.5, containing 0.1 M NaCl and 0.1% PEG.

PPACK-thrombin was prepared by the method of Kettner and Shaw (1979). Excess PPACK was removed by dialysis by using a Pierce microdialyzer.

Thrombin Activity. Thrombin activity was measured with the Kabi chromogenic substrate S-2238. A 10 μL aliquot of thrombin solution was added to 940 μL of 50 mM Tris-HCl buffer, pH 8, with 0.15 M NaCl and 0.1% PEG. A 50 μL aliquot of 1 mM S-2238 was added, and the rate of increase in absorbance at 405 nm was followed at room temperature (23 °C).

Preparation of Anhydrothrombin. The early stages of the preparation were carried out in a cold room at 4 °C. Buffer and Gdn·HCl solutions used in the procedure were filtered through a 0.22 μm Millipore filter. All solutions were cooled to 0 °C in an ice/water bath prior to use. A 12 mL volume of 6 N Gdn·HCl was added to a 50 mL steel centrifuge tube (for rapid temperature equilibration) and placed on ice. Then 120 μL of 6 N NaOH was added with stirring. A 2.4 mL volume of PMS-thrombin (24 mg) in 10 mM sodium phosphate buffer, pH 6.5, containing 0.1 M NaCl, and 0.1% PEG, was added rapidly, with stirring. The elimination reaction was allowed to proceed for 10 min, and then quenched by the rapid addition, with stirring, of 15 mL of 0.4 M sodium phosphate buffer, pH 6, previously chilled on ice.

The following steps were subsequently carried out at room temperature. The protein solution was added dropwise, with stirring, to 300 mL of 50 mM sodium phosphate buffer, pH 6.5, containing 0.75 M NaCl and 0.1% PEG. This solution was concentrated to 6 mL using Amicon concentration cells fitted with YM10 membranes. The concentrated protein solution was desalted on a Bio-Rad PD-10 column equilibrated with 25 mM sodium phosphate buffer, pH 6.5, containing 0.1 M NaCl, and 0.1% PEG. The resulting 8 mL of solution (containing 24 mg of protein) was purified by two runs of 4 mL each, applied to a *p*-aminobenzamidine (PAB) affinity column (1.6 × 10 cm) connected to an LKB HPLC system. The column was equilibrated with 5 mM sodium phosphate buffer, pH 6.5, containing 0.1% PEG and flowing at 1 mL/min. After application of a 4 mL aliquot of protein solution, the column was washed with the same buffer, and unbound protein was eluted in the first 16 mL. After a total wash of 22 mL, a 5 mL volume of 1 M arginine in 50 mM sodium phosphate buffer, pH 6.5, containing 0.1 M NaCl, and 0.1% PEG, was applied to elute bound anhydrothrombin. The protein fractions eluted with arginine were pooled and desalted on a PD-10 column. It was necessary to remove arginine, prior to concentrating the solution, because it caused the protein to aggregate on the membrane of the concentration cell. The protein preparation was concentrated to 2.5 mL containing 16 mg of protein. A 150 μL aliquot of 2 mM PPACK was added to the protein solution to inactivate thrombin amidolytic activity (toward S-2238) arising from the 0.3% residual activity in the PMS-thrombin and the 9% of activity recovered due to desulfonylation (Gold & Fahrney, 1963) during the treatment with alkali. This solution was allowed to sit at room temperature for 30 min, and then overnight at 0 °C.

The anhydrothrombin preparation was dialyzed against 25 mM sodium phosphate buffer, pH 6.5, containing 0.1 M NaCl, and 0.1% PEG, in a Pierce microdialyzer, prior to a second application to the PAB column, as above. The eluted fractions of bound protein were pooled, desalted, and concentrated. The anhydrothrombin solution was then applied to an HP Genenchem G-2000 SW preparative gel filtration column (2.1 × 30 cm) connected to the LKB HPLC system described above. This column, which was obtained from HP Genechem (San Francisco, CA), was packed with TSK Gel G2,000SW. The column was equilibrated with 50 mM sodium phosphate buffer, pH 6.5, containing 0.1 M NaCl, and 0.1% PEG, at a flow rate of 3 mL/min. Leading and trailing edges of the eluting anhydrothrombin peak were cut out to remove high and low molecular weight contaminants. The fractions containing anhydrothrombin were pooled and concentrated to 1.2 mL containing 5 mg of protein. Residual thrombin activity (10^{-3}%) was inactivated by the addition of 50 μL of 2 mM PPACK to the 1.2 mL of anhydrothrombin, as above. The protein was dialyzed against 50 mM sodium phosphate buffer, pH 6.5, containing 0.1 M NaCl, and 0.1% PEG, in the Pierce microdialyzer to remove PPACK. The anhydrothrombin was stored frozen at −80 °C. As a control experiment, native thrombin was subjected to the alkali treatment and refolding procedures used in the preparation of anhydrothrombin from PMS-thrombin. The thrombin sample was then purified by PAB affinity chromatography.

PMS Elimination. The release of PMS from PMS-thrombin during the incubation at alkaline pH was demonstrated by RP-HPLC. Samples for the RP-HPLC analysis were prepared in a cold room at 4 °C. A 60 μL volume of PMS-thrombin (4.8 mg/mL) in 10 mM sodium phosphate buffer, pH 6.5, containing 0.1 M NaCl, and 0.1% PEG, was mixed with 180 μL of 8 M Gdn·HCl, and then a 60 μL aliquot of 6 M Gdn·HCl with 0.25 N NaOH was added, with stirring. At various times, 30 μL aliquots were removed, added to tubes containing 5 μL of 3 M perchloric acid, mixed, and placed on ice. Zero-time samples were prepared by adding 6 M Gdn·HCl without NaOH. Infinite-time samples were prepared by placing samples in a boiling water bath for 1 min prior to perchloric acid precipitation. Denatured protein was pelleted by centrifugation. The

supernatants were collected, and 20 μL aliquots of each were injected onto an Aquapore RP-300 column (0.2 × 10 cm) connected to the LKB HPLC system described above. The column had been equilibrated with a buffer of 10 mM HPLC grade acetic acid adjusted to pH 6 with triethylamine. PMS was eluted isocratically at a flow rate of 1 mL/min and was detected by UV absorbance at 205 nm. The retention time for the PMS peak was 5.83 min. Solutions of acid-hydrolyzed PMSF were used to demonstrate that the peak area was linear with PMS concentration.

Alternative Preparation Procedure. We also attempted the preparation of anhydrothrombin by the method of Tomono and Sawada (1986). PMS-thrombin (2 mg in 0.5 mL) was dialyzed at 4 °C for 36 h, in a Pierce microdialysis unit, against 50 mM Tris-HCl buffer, pH 9, containing 0.1 M NaCl and 0.1% PEG. After the incubation, the protein was assayed for PMS using the RP-HPLC assay described above. This preparation was applied to the PAB affinity column to recover anhydrothrombin.

SDS−PAGE. A Bio-Rad Mini-PROTEAN minigel apparatus was used to carry out SDS−PAGE on 12% polyacrylamide gels according to the method of Laemmli (1970). Protein samples were reduced with 2-mercaptoethanol. Gels were stained with Coomassie Brilliant Blue R. SDS−PAGE was carried out on anhydrothrombin and also on an anhydrothrombin sample that had been incubated with $NaBH_4$ to reduce the active-site dehydroalanine to alanine. To prepare the $NaBH_4$-treated anhydrothrombin, a 50 μL aliquot of 1 mg/mL $NaBH_4$ in 20 mM sodium borate buffer, pH 9, was added to a 50 μL aliquot of the same buffer containing 147 μg of anhydrothrombin, and the solution was placed on ice, in the dark, for 3 h prior to being prepared for SDS−PAGE. Sigma SDS-7, a preparation of premixed molecular weight standards, was used to provide molecular weight markers.

CNBr Digestion and Analysis. One milligram each of native thrombin and anhydrothrombin was dissolved in 2 mL of 0.1 N acetic acid and concentrated to less than 100 μL with Amicon Centricon 10 concentrators. The samples were dried in a Speed Vac connected to a Virtis Freezemobile lyophilizer. Two milligrams of CNBr was added to 100 μL of 70% formic acid, and 25 μL aliquots were added to the dried protein samples. They were incubated in the dark at room temperature (23 °C) for 36 h, with mixing. The digests were lyophilized. The dried digests were redissolved in 0.5 mL of 0.1 M acetic acid and lyophilized again. They were then dissolved in 100 μL of 50 mM Tris-HCl, pH 8, containing 2 M Gdn·HCl and 15 mM reduced DTT. The solutions were allowed to sit for 3.5 h at room temperature, and then were made up to 1 mL with 0.09% TFA in water. Separation of the CNBr digest peptides was carried out by RP-HPLC on a Waters C_{18} Radial-PAK (8 × 10) column connected to the LKB HPLC system. The mobile phase buffers were A (0.09% TFA in water) and B (0.09% TFA in acetonitrile). The column was equilibrated with buffer A, and 25 μL aliquots of the CNBr digest solutions were injected onto the column. The digest peptides were eluted with a gradient of 0−45% B in 45 min and collected separately for further analysis.

Disulfide/thiol analyses of the reduced CNBr digest peptides were also carried out on-line during RP-HPLC chromatography as described by Thannhauser et al. (1985). This procedure identifies both cystine- and cysteine-containing peptides.

Peptides isolated from the CNBr digests by RP-HPLC were characterized by amino acid analysis at the Cornell Biotechnology facility using the Waters Pico-Tag chemistry. Samples of native thrombin and anhydrothrombin were also submitted for amino acid analysis.

The CNBr digest peptides from thrombin and anhydrothrombin corresponding to residues 186−211, as determined by amino acid analysis, were examined by ES/MS with a SCIEX TAGA 6,000E (upgraded to API-III) mass spectrometer in the laboratory of Professor J. D. Henion. Samples of native thrombin, anhydrothrombin, and PPACK-thrombin were submitted to the Cornell Biotechnology facility where matrix laser desorption-ionization time of flight mass spectrometry was carried out using a Finnigan MAT Lasermat mass spectrometer to determine molecular weights for the proteins.

Sedimentation Equilibrium. The sedimentation equilibrium experiment was carried out with 2 mg of anhydrothrombin in 0.5 mL of 25 mM sodium phosphate buffer containing 0.1 M NaCl. PEG had been removed from this sample by ion-exchange chromatography on a Pharmacia analytical Mono S column. The experiment was carried out on a Beckman Model E ultracentrifuge for 24 h at 23.5 °C and 15 000 rpm.

Hirudin Binding. The ability of anhydrothrombin to compete with thrombin for hirudin binding was examined. Hirudin activity in solution was determined by its ability to inhibit the amidolytic activity of thrombin. The methods used are similar to those described previously by Vincent et al. (1974) in their study of anhydrotrypsin interactions with bovine pancreatic trypsin inhibitors. In our experiments, thrombin activity was measured with the Kabi chromogenic substrate S-2238, as described above. All steps were carried out at 23 °C. Anhydrothrombin and hirudin were mixed to give concentrations of 133 ± 4 nM and 312 ± 30 nM, respectively, in 90 μL of 50 mM Tris-HCl buffer, pH 8, containing 0.15 M NaCl, 0.1% PEG, and 1 mg/mL BSA (incubation buffer). The BSA was added in addition to the PEG as a further precaution against the loss of proteins on vessel surfaces. The protein solution was allowed to sit for 3 min to allow the anhydrothrombin−hirudin complex to form. A 10 μL aliquot of thrombin was then added, giving final concentrations for thrombin, anhydrothrombin, and hirudin of 160 ± 5 nM, 120 ± 5 nM, and 280 ± 30 nM, respectively, in a solution volume of 100 μL. The mixture was again allowed to sit for 3 min. Then a 10 μL aliquot was removed and assayed for thrombin amidolytic activity. This experiment was repeated with increasing final concentrations of anhydrothrombin of 120, 150, 180, 210, and 240 nM. Thrombin activity was measured in the absence of added anhydrothrombin and hirudin to obtain values for 100% thrombin activity.

RESULTS

Preparation of Anhydrothrombin. Table 1 lists the yield of protein and the specific activity at each stage of the anhydrothrombin preparation. The affinity chromatography of anhydrothrombin on PAB−Sepharose in 5 mM sodium phosphate (pH 6.5) resulted in two peaks (data not shown). The first peak contained protein that eluted from the column in the wash buffer. Anhydrothrombin and recovered native thrombin (from desulfonylation) were then eluted from the

Table 1: Preparation of Anhydrothrombin

thrombin treatment	protein (mg)	% specific activity	% yield
none	24	100	100
PMSF inactivation	24	0.3	100
denatured, base treated, and refolded	21	9	88
first PAB affinity chromatography	16	15	67
first PPACK inactivation	16	7×10^{-4}	67
second PAB affinity chromatography	6	1×10^{-3}	25
gel filtration	5	1×10^{-3}	21
second PPACK inactivation	5	none detected[a]	21

[a] At approximately 700 times the concentration of enzyme normally used in the assay, no activity was detected at 0.1 absorbance unit full scale for 1 h. See text for details of assay.

column with the arginine buffer in 5 mM sodium phosphate (pH 6.5). We found that anhydrothrombin was not bound by the PAB affinity column as tightly as thrombin. Therefore, a significant reduction in the ionic strength of the buffer (5 mM sodium phosphate rather than 25 mM sodium phosphate/0.1 M NaCl) was required to prevent anhydrothrombin from beginning to elute from the affinity column before the application of the arginine buffer. However, in a separate experiment, even if a high ionic strength wash buffer containing 1 M NaCl was used, the eluted anhydrothrombin was still well resolved from the early-eluting protein that lacked any binding affinity for the PAB column, although the anhydrothrombin now eluted as an extremely broadened peak.

The anhydrothrombin preparation recovered in the first PAB affinity chromatography step had significant thrombin amidolytic activity (Table 1). PPACK was used to inactive active thrombin prior to carrying out a second PAB affinity chromatography. Residual activity remaining after this step was inactivated by a second addition of PPACK (Table 1). Amino acid analyses of thrombin and anhydrothrombin (data not shown) showed no significant differences, indicating that the procedure for the preparation of anhydrothrombin has not caused any gross changes in the amino acid content of the anhydrothrombin.

As a control, native thrombin was subjected to the alkali treatment and refolding steps used for the preparation of anhydrothrombin from PMS-thrombin. The protein was then purified by PAB affinity chromatography. The recovered protein had a specific activity for S-2238 that was 72% of that of the starting protein. The loss of amidolytic activity may be a result of the autoproteolytic activity of native thrombin

When PMS-thrombin was incubated according to the method of Tomono and Sawada (1986) for preparing anhydrothrombin and applied to the PAB affinity column, all the protein eluted with the water buffer. This lack of affinity for the PAB column indicated that PMS was not eliminated from the PMS-thrombin or that the incubation had severely degraded the protein.

PMS Elimination. The data from the RP-HPLC assay measuring the rate of release of PMS from PMS-thrombin were best fit by two simultaneous first-order processes (Figure 2). The larger PMS release constituted 87% of the total, with a half-life of 2.2 min. The remaining 13% of the PMS was released more rapidly with a half-life of 0.12 min. Weiner et al. (1966) also observed a burst for the release of the tosyl group from tosylchymotrypsin under alkaline

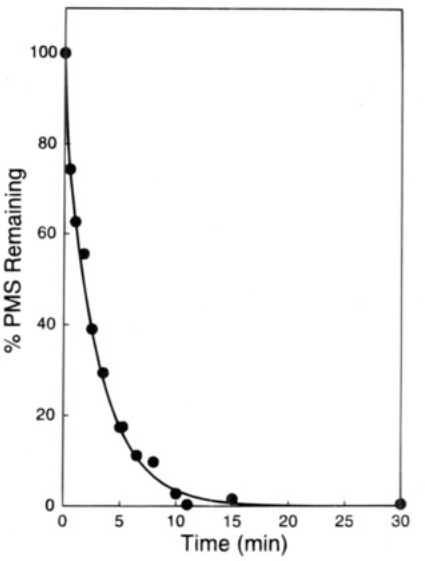


FIGURE 2: Release of PMS from PMS-thrombin was fitted to a model with two first-order processes occurring simultaneously. See text for a discussion.

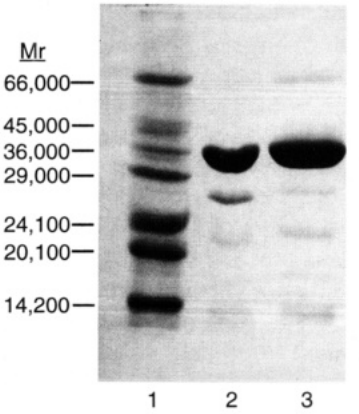


FIGURE 3: SDS−PAGE of anhydrothrombin. Lane 1, molecular weight standards. Lane 2, anhydrothrombin. Lane 3, anhydrothrombin treated with $NaBH_4$.

conditions. These workers suggested that the burst was due to a conformational change occurring upon the exposure of the protein to alkali. We have, however, observed that approximately 10−15% of the original thrombin specific activity can be recovered as s result of desulfonylation (Gold & Fahrney, 1963) during the alkaline incubation. It is possible that the faster release of PMS is due to desulfonylation, while the slower release of PMS is due to the β-elimination reaction. It is not apparent, however, why the faster process is limited to only 10−15% of the total reaction. It is possible that conformational changes in the protein do play a role in the rate of release of PMS or in the nature of the reaction (elimination or desulfonylation) leading to its release.

Using the RP-HPLC assay for PMS, we found that 95 ± 3% of the PMS remained linked to the protein after PMS-thrombin was incubated according to the method of Tomono and Sawada (1986) for the preparation of anhydrothrombin. This explains why the protein from this preparation was not bound by the PAB affinity column as discussed above.

SDS−PAGE. The major band for anhydrothrombin in SDS−PAGE had an M_r of 33 800, the expected value for the thrombin B-chain (Figure 3). The diffuse band at approximately M_r = 14 000 at the bottom of the gel lanes

(Figure 3) probably arises from the presence of a small amount of γ-thrombin (Kingdon et al., 1977; Martin et al., 1992), a thrombin derivative resulting from autoproteolysis. Because the anhydrothrombin sample used for the $NaBH_4$ reduction was not fully inactivated with a second PPACK incubation, it appears that a small amount of additional proteolysis occurred during the $NaBH_4$ reduction, as indicated by the darker bands. The faint band of M_r = 23 500 (lane 2) is consistent with the presence of a small amount of the anhydro form of the thrombin autoproteolytic product, β-thrombin (Lundblad et al., 1979). The presence of a band of M_r = 26 500, however, suggested that some of the anhydrothrombin B-chain was cleaved at the dehydroalanine residue during the denaturation step prior to SDS–PAGE. Under extremes of pH or at elevated temperatures, dehydroalanine undergoes hydrolysis to pyruvate and ammonia. When present in a peptide, it renders that peptide susceptible to nonenzymatic cleavage at its site of incorporation (Patchornik & Sokolovsky, 1964). The complementary cleavage peptide of approximately M_r = 6400 is too small to be resolved on these gels.

This nonenzymatic cleavage is prevented by reduction of dehydroalanine with $NaBH_4$. Ordinarily, $NaBH_4$ does not reduce carbon–carbon double bonds. It can, however, reduce a carbon–carbon double bond conjugated with a carbonyl, as is the C^{α}–C^{β} double bond of dehydroalanine (Carey & Sundberg, 1990). After subjecting anhydrothrombin to reduction with $NaBH_4$, the M_r = 26 500 band is significantly reduced (Figure 3, lane 3), confirming that nonenzymatic cleavage at dehydroalanine had occurred during the preparation of anhydrothrombin for SDS–PAGE. Knowing that the band at M_r = 26 500 was an artifact of the SDS–PAGE experiment, we determined from a densitometric scan of the gel (data not shown) that the preparation was approximately 95% anhydrothrombin, with the remaining protein most likely being anhydro-β-thrombin and anhydro-γ-thrombin. The band at $M_r \approx 66\,000$ is an artifact of the $NaBH_4$ reduction. It is probably a dimer of anhydrothrombin formed in a competing reaction of $NaBH_4$, viz., the reduction of disulfide bonds. Such reduction of disulfide bonds would allow, e.g., an SH group from one anhydrothrombin molecule to attack the dehydroalanine double bond of a second anhydrothrombin molecule.

CNBr Digestion and Analysis of Active-Site Peptides. CNBr digestions of thrombin and anhydrothrombin and the subsequent analyses of the digest peptides were carried out in order to isolate the serine-containing active-site peptides and determine if the active-site serine was modified to dehydroalanine in anhydrothrombin. There are five methionines in the bovine thrombin B-chain, and none in the A-chain. A CNBr digestion results in two forms of each digest peptide (except the C-terminal peptide), one in which methionine is transformed to homoserine upon cleavage, and another in which the homoserine lactone is formed. Not all of the 11 possible peptide fragments of the B-chain were identified in the RP-HPLC chromatograms of the CNBr digests of native thrombin and anhydrothrombin, detected at 205 nm and shown in Figures 4A and 5A, respectively. However, peptides corresponding to residues 186–211, the CNBr digest peptide that spans the region of the active-site serine (S205), were among the peptides identified for both thrombin and anhydrothrombin. The identities of the

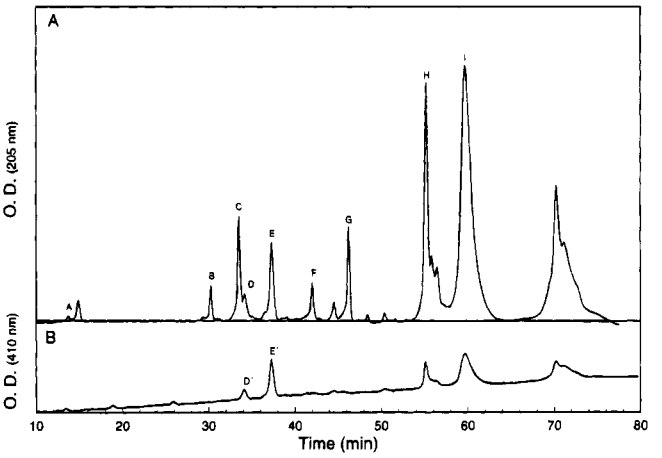


FIGURE 4: RP-HPLC separation of CNBr digest peptides of native thrombin. Thrombin sequences were identified by amino acid analysis. The A-chain contains residues 1–49, and the B-chain contains residues 1–259. Duplicate peptides result from the isolation of homoserine and homoserine lactone forms. (A) Peptides detected at 205 nm: A, 74–80 (this peptide is a digest product of a small amount of β-thrombin present); B, 212–222; C, 212–222; D, 186–211; E, 186–211; F, 1–17; G, 1–17; H, A-chain; I, 18–80; and unidentified peptides. Mass spectral analyses identified peak D as the homoserine derivative of 186–211, and peak E as the homoserine lactone form of 186–211. (B) Simultaneous on-line detection of cysteine-containing peptides.

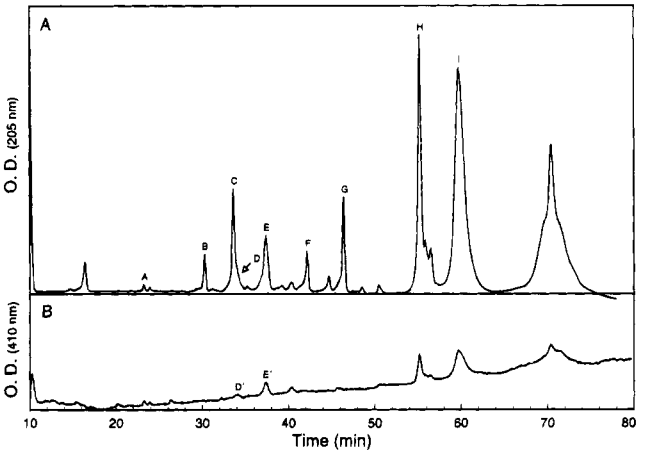


FIGURE 5: RP-HPLC separation of CNBr digest peptides of anhydrothrombin. Methods are as discussed in the legend to Figure 4A. (A) Peptides detected at 205 nm: A, 186–204 (this peptide results from the hydrolysis of dehydroalanine in 186–211); B, 212–222; C, 212–222; D, 186–211; E, 186–211; F, 1–17; G, 1–17; H, A-chain; I, 18–80; and unidentified peptides. Mass spectral analysis identified peak E as the homoserine lactone form of 186–211. (B) Same as Figure 4. The areas of peaks D′ and E′ are smaller relative to the other cysteine-containing peaks, particularly in comparison to their counterparts, peaks D′ and E′ in Figure 4A.

peptides, as determined by amino acid analyses, are given in the figure legends.

As discussed above, peptides containing dehydroalanine are susceptible to nonenzymatic cleavage at extremes of pH and elevated temperatures. Apparently, the conditions of the CNBr digest were not stringent enough for significant hydrolysis of the AT-186–211 peptide, which was recovered intact, except for a small amount of the AT-186–204 N-terminal hydrolysis fragment (peak A in Figure 5A). The amino acid compositions of the recovered T-186–211 and AT-186–211 peptides (the E peaks in Figures 4A and 5A) are given in Table 2. The native thrombin peptide, T-186–211, contains a single serine that is absent in the anhydro-

Table 2: Amino Acid Analysis of Peptides T-186−211 and AT-186−211[a,b]

amino acid	T-186−211	AT-186−211
Asx (Asp + Asn)	2.5(2)	2.0
Glx (Glu + Gln)	2.2(2)	2.0
Ser	1.3(1)	
Gly	6.7(7)	7.9
His		
Arg	1.2(1)	1.0
Thr		
Ala	1.8(2)	2.1
Pro	2.6(2)	2.5
Tyr	1.3(1)	0.97
Val	0.83(1)	1.0
Met	*c*	1.6[d]
Cys	0.43(2)	0.49
Ile		
Leu		
Phe	1.6(2)	2.0
Lys	1.7(2)	1.9
homo-Ser	0.85(1)	0.66

[a] Theoretical recoveries are in parentheses. [b] Recoveries were summed and divided by 26, the number of residues in the peptide, to give the average recovery for a single residue. Recoveries for each amino acid were divided by this value. [c] Met is not present because of its conversion to homo-Ser. [d] Since Met is transformed to homo-Ser in a CNBr digest, this is an unidentified species with the same retention time as Met. See text for a discussion.

thrombin peptide, AT-186−211, as expected. However, although methionine is reported as being present in AT-186−211, it cannot be present because this residue is modified to homoserine and homoserine lactone as a consequence of the CNBr digestion. It is known from the amino acid sequence of thrombin that T-186−211 contains two cysteine residues (MacGillivray & Davie, 1984). It is likely that the unidentified residue with the same retention time as methionine in the amino acid analysis was a lanthionine formed by the nucleophilic addition of thiol from one of the two cysteine residues to the double bond of dehydroalanine (Masri & Friedman, 1982), after the reduction of the CNBr digestion peptides with DTT. Because it is difficult to obtain a quantitative analysis of cysteine by amino acid analysis, we carried out the disulfide/thiol analyses.

In Figures 4A and 5A, peaks D and E are the homoserine and homoserine lactone forms of the 186−211 peptides, respectively, as determined by amino acid analysis and ES/MS analysis (see discussion below). Figures 4B and 5B are the same chromatograms, developed with the disulfide/thiol assay and detected at 410 nm. Peaks D′ and E′ correspond to D and E in Figures 4A and 5A, and identify them as cysteine-containing peptides. The peak areas are directly proportional to the cysteine content of corresponding peptides. It is evident from the chromatograms in Figures 4A, 4B, 5A, and 5B that the amount of measurable thiol in the AT-186−211 peptides is lower than that in the T-186−211 peptides. Examination of the other cysteine-containing peptides in the four chromatograms shows that this large difference is not observed for the other cysteine-containing CNBr digestion peptides obtained from native thrombin or anhydrothrombin. This indicates either the partial loss or the partial blocking of cysteine in the AT-186−211 peptide. Blocking of a cysteine residue would occur upon the nucleophilic attack of one of the cysteines in the AT-186−211 peptide on the dehydroalanine double bond to form lanthionine.

Mass Spectrometry. ES/MS was carried out on the T-186−211 and AT-186−211 peptides to determine if a water molecule was removed by the β-elimination of PMS from the active-site serine of thrombin to form dehydroalanine in the AT-186−211 peptide. Amino acid analysis had identified peaks D and E from the chromatograms in Figures 4A and 5A as the 186−211 peptides. We report here only the results of the ES/MS analysis of the E peaks. The molecular weights of the T-186−211 and AT-186−211 peptides (the E peaks in Figures 4 and 5) were found to be 2588.3 ± 0.6 mass units and 2569.6 ± 1.5 mass units, respectively. The difference of 18.7 ± 1.6 mass units between the native and anhydrothrombin peptides was the expected result for the removal of a water molecule from the side chain of serine 205 due to the β-elimination of PMS. The molecular weights obtained here uniquely identified these peptides as the homoserine lactone species of T-186−211 and AT-186−211, which have a lower molecular weight, by 18 mass units, than the homoserine-containing species. It should be noted that there are no glutamines or asparagines in the 186−211 peptide, eliminating deamidation as a possible cause for the lower molecular weight of AT-186−211.

Given the molecular weight obtained, it is evident that AT-186−211 was isolated intact. Therefore, neither cysteine was lost from this peptide. This supports the hypothesis that an intramolecular cyclization occurred in AT-186−211 with a cysteine thiol adding to the double bond of dehydroalanine to form lanthionine (Masri & Friedman, 1982). This would prevent the reaction of the disulfide/thiol assay reagent, NTSB, with the blocked cysteine, resulting in a lowered yield for the cysteine content of the AT-186−211 peptide, as we observed.

The matrix laser desorption-ionization time of flight mass spectrometry measurements gave M_r values of 37 635 for thrombin, 38 084 for PPACK-thrombin, and 37 622 for anhydrothrombin. Single measurements were made for the first two proteins, but an error of ±83 mass units for several anhydrothrombin measurements indicated that the experiment cannot distinguish between thrombin and anhydrothrombin. However, PPACK-thrombin is clearly differentiated by its larger M_r. This eliminates any concern that PPACK can modify anhydrothrombin covalently. Yokosawa and Ishii (1977) made a similar observation that TLCK, a chloromethyl ketone inhibitor of trypsin, does not modify anhydrotrypsin covalently.

Sedimentation Equilibrium. The results of the sedimentation equilibrium experiment indicated that the anhydrothrombin preparation was homogeneous and monomeric (data not shown). The correlation coefficient of a linear fit to the log C vs r^2 data was 0.9999. The computed molecular weight for anhydrothrombin was 38 000, using estimated values of 0.71 mL/g for the partial specific volume of thrombin, and 1 g/mL for the solution density. The use of estimates for these quantities prevents a comparison of the accuracy of this experiment and the matrix laser desorption-ionization time of flight mass spectrometry experiment.

Hirudin Binding. In the competitive assay, anhydrothrombin and thrombin were found to bind to hirudin in approximately a 1:1 ratio. The precise stoichiometry is not reported because of the unknown rates of formation of the complexes of hirudin with anhydrothrombin and thrombin, respectively.

DISCUSSION

Procedures for the preparation of anhydrotrypsin and anhydrochymotrypsin require the incubation of the respective PMSF-inactivated serine protease at 0 °C at a pH above 11 (Strumeyer et al., 1963; Ako et al., 1972). Under these conditions, the β-elimination is likely to be accompanied by alkali-induced denaturation of the protein, and desulfonylation, which leads to recovered enzyme activity, resulting in autoproteolysis from residual and recovered proteolytic activity. Indeed, only a fraction of the starting protein is recovered as the anhydro derivative using these procedures. These denaturing processes are apparently even more injurious to thrombin than they are to trypsin or chymotrypsin. Thrombin undergoes autoproteolysis at neutral and higher pHs, forming β-thrombin (Lundblad et al., 1975). Thrombin is also inactivated when incubated at an alkaline pH (Koehler & Magnusson, 1974; Fenton et al., 1977). The hysteresis observed upon back-titration of thrombin solutions from high to low pH (Winzor & Scheraga, 1964) is further evidence for a pH-dependent, irreversible denaturation of thrombin.

Hydrophobic loop insertions in thrombin, features not present in trypsin or chymotrypsin, may also render it more susceptible to irreversible pH-induced denaturation. The unfolding of these loops at extremes of pH may provide sites for aggregation when the unfolded protein is returned to a solution pH closer to its isoelectric point where repulsive interactions due to excess charge do not predominate (Tanford, 1968).

Thus, it was not surprising that our earliest attempts to prepare anhydrothrombin, using literature procedures for the preparation of anhydrotrypsin and anhydrochymotrypsin, resulted in visible protein aggregation. Soluble protein obtained from these preparations by affinity chromatography purification could not be concentrated without the occurrence of further protein precipitation. Tomono and Sawada (1986), recognizing the lability of thrombin at pH values above 10, attempted to carry out the β-elimination of PMS from human PMS-thrombin at a solution pH of 9. We did not, however, observe a significant release of PMS when we incubated bovine PMS-thrombin under similar conditions.

Since Bauer et al. (1980) showed that Gdn·HCl- or urea-denatured thrombin can be refolded by diluting the protein solution to nondenaturing concentrations of these reagents, this led us to carry out the β-elimination of PMS from PMS-thrombin under denaturing conditions because any residual thrombin in the PMS-thrombin preparation, or thrombin recovered by desulfonylation, would be unfolded and unable to carry out autoproteolysis. Also, unfolding caused by incubation of the protein at a basic solution pH would be inconsequential because the protein was already unfolded and would be refolded at lowered pH, after quenching the elimination reaction. Our recovery of 21% of the starting protein as anhydrothrombin with this approach is a significant improvement over our previous attempts. Further work is needed, however, to determine if the alkaline conditions are responsible for modifications to the protein that resulted in the 79% loss.

Our recovery of anhydrothrombin on a PAB affinity column was a demonstration of the ability of anhydrothrombin to display thrombin-like binding interactions with a small substrate-like ligand, in addition to being a convenient purification method. PAB binds at the active site of thrombin (Evans et al., 1982), most likely in the S_1 subsite,[2] similar to the interaction of PAB with trypsin. The need to decrease the buffer ionic strength when carrying out the affinity chromatography with anhydrothrombin indicates that the strength of its PAB binding interaction is weaker than that of thrombin. Evidently, the affinity of anhydrothrombin for benzamidine-like ligands is lower than that of thrombin. This is also true of anhydrotrypsin, which has a dissociation constant for benzamidine binding an order of magnitude larger than that of trypsin (Yokosawa & Ishii, 1977).

When we reduced and denatured anhydrothrombin for SDS−PAGE, a polypeptide with an M_r of 26 500 was observed. This was consistent with the partial nonenzymatic cleavage of the B-chain into two polypeptide chains because of the hydrolysis of dehydroalanine at position 205 at elevated temperatures (Patchornik & Sokolovsky, 1964). Treating anhydrothrombin with $NaBH_4$ prior to SDS−PAGE essentially eliminated the 26 500 M_r cleavage fragment, because of the reduction of the dehydroalanine to alanine. Apparently, dehydroalanine is stabilized against hydrolysis as long as it is confined within the active site of the folded anhydrothrombin. It was only during the denaturation steps prior to SDS−PAGE that the dehydroalanine was significantly degraded.

Apparently, the conditions of the CNBr digestion were not stringent enough to hydrolyze the dehydroalanine residue of AT-186−211 significantly. Amino acid analysis showed that the peptide was recovered intact and was identical to the native peptide, T-186−211, except for the absence of serine and the presence of an unknown residue eluting with the same retention time as methionine. ES/MS analysis showed that we had recovered the homoserine lactone forms of these peptides and that they differed by a molecular weight of 18.7 ± 1.6 mass units, the expected result for the *removal* of a water molecule.

The disulfide/thiol analyses provided evidence for an intramolecular cyclization having occurred in the reduced AT-186−211 peptide, with one of the two thiols in this peptide having added to the dehydroalanine double bond to form a lanthionine residue (Masri & Friedman, 1982). The cysteine residue, C201, four residues away from the active-site serine (dehydroalanine) in the N-terminal direction is a more likely candidate for this cyclization than C187, not only because it is in closer in the sequence but also because it is brought into the proximity of the active-site serine (dehydroalanine) by a β-turn in which the D204 amide nitrogen is hydrogen bonded to the C201 carbonyl (Bode & Huber, 1991). Although this mechanism is consistent with our observations, the proof requires identification of the unknown residue in the amino acid analysis of the AT-186−211 CNBr digest peptide as lanthionine.

We have shown that anhydrothrombin is able to bind to hirudin, the potent leech thrombin inhibitor, by assaying remaining hirudin activity in solution by its ability to inhibit thrombin. During the course of these experiments, which were carried out under conditions of pH 8, ionic strength 0.17, and 23 °C, anhydrothrombin remained bound in a complex with hirudin in the presence of added thrombin, demonstrating effective competition with thrombin for binding to hirudin. The bovine thrombin−recombinant hirudin

[2] Nomenclature of Schechter and Berger (1967).

complex has a dissociation constant of 19.5 pM at conditions of pH 8.3, ionic strength 0.23, and 25 °C (Dodt et al., 1990). The recombinant hirudin differs from the native protein obtained from leeches by the lack of a sulfate group on tyrosine 63. The solution conditions of the competitive titration experiment are not very different from those in which the K_d of the thrombin−hirudin complex was determined. It is, therefore, unlikely that anhydrothrombin could compete effectively with thrombin for hirudin binding, unless it retained physiologically relevant binding interactions with hirudin at both its active site and its secondary binding site that enabled it to compete with thrombin for hirudin binding since the thrombin−hirudin complex has a picomolar dissociation constant.

Huber et al. (1975) carried out an X-ray crystallographic study of the complex of anhydrotrypsin and BPTI with a refinement to 1.9 Å resolution. They observed no major differences between this complex and that with the native enzyme other than the absence of the serine O^{γ} and the movement of the dehydroalanine C^{β} into the plane formed by N195, C^{α}, and the serine carbonyl carbon. Warshel et al. (1989) have discussed the changes around a carbonyl carbon of a substrate molecule undergoing a transition from sp^2 to sp^3 geometry. They point out that this change is associated with displacements of less than 0.3 Å for the atoms involved and that the flexibility of proteins allows these minor perturbations to be easily accommodated. This is evidently also true for the changes occurring in the active site of serine proteases when the active-site serine is transformed to dehydroalanine.

In summary, we have prepared anhydrothrombin, a new derivative of bovine thrombin, using a novel methodology. We took advantage of the ability of thrombin to be refolded after denaturation in Gdn·HCl solutions, and carried out an elimination reaction on unfolded PMS-thrombin, because the alkaline conditions necessary for the elimination of PMS denature the folded protein. We then refolded the modified protein and recovered it in good yield. The procedure also takes advantage of the extraordinary specificity of PMSF for the nucleophilic active-site serines of serine proteases. We have applied a number of physical characterizations to our anhydrothrombin preparations, and all indicate that our procedure has yielded a thrombin derivative with properties that will make it a useful tool for further studies in the thrombin−fibrinogen system. In a future paper (R. W. Ashton and H. A. Scheraga, in preparation), we will report the results of experiments in which we examined the binding of anhydrothrombin to fibrinogen, fibrin, and the peptides Aα(1−20) and Aα(1−16).

As a final note, a sample of our preparation of anhydrothrombin has been crystallized in the laboratory of Professor A. Tulinsky at Michigan State (private communication).

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