The Location of the Calcium Ion Binding Site in Bovine α-Trypsin and β-Trypsin Using Lanthanide Ion Probes†

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ABSTRACT: The effect of Gd^{3+} on the nuclear magnetic resonance (NMR) relaxation rates, T_{1m}^{-1} and T_{2m}^{-1}, of inhibitor protons in metal–inhibitor–trypsin ternary complexes has been measured. The Solomon–Bloembergen equations have been used to calculate distances of 10.0 ± 0.5, 8.8 ± 0.5, and 9.5 ± 0.5 Å between the metal ion and the methyl and ortho protons of *p*-toluamidine, and the methyl protons of acetamidine, respectively. Essentially the same results are obtained for both α-trypsin and β-trypsin. Binding constants of 3.3×10^3 and 4.1×10^3 M^{-1} for the association of Gd(III) with α-trypsin and β-trypsin, respectively, in the presence of *p*-toluamidine at pH 6.0 have been obtained by equilibrium dialysis. Calcium binding constants of 260 and 3700 M^{-1} at pH 6.0 and 8.0, respectively, with β-trypsin have also been obtained. Calcium ion and gadolinium ion compete for the same site on the protein. Calcium has been shown to protect α-trypsin from further autolytic degradation to ψ-trypsin. From examination of the crystal structure of the enzyme we propose that the calcium ion binding site of bovine trypsin is comprised of the side chains of Asp-194 and Ser-190 (based on the chymotrypsin sequence numbering system). This seems to be the only site which is comprised of at least one carboxyl group, which fits our distance requirements and which is consistent with other chemical data.

Bovine pancreatic β-trypsin, a proteolytic enzyme with a molecular weight of 23985, is a single polypeptide chain containing 223 amino acid residues whose sequence is known (Walsh and Neurath, 1964), and whose crystal structure has been well defined (Stroud et al., 1971, 1974). Trypsin catalyzes the hydrolysis of esters and peptides containing lysine and/or arginine residues. Various amidines and guanidines competitively inhibit the enzyme and have been shown to possess a high affinity for the specificity site of trypsin located near Asp-189[1] (Mares-Guia and Shaw, 1965; Mares-Guia, 1968; Mares-Guia and Figueiredo, 1970). Binding at this site by an amidine inhibitor serves to block the substrate from entering the active site at His-57 and Ser-195 and prevents their participation in hydrolysis. The position of benzamidine relative to the specificity site and active site has been determined by Krieger et al. (1974).

Bovine trypsin binds one calcium ion (Delaage and Lazdunski, 1967) which does not appear to alter the catalytic activity of trypsin toward synthetic substrates. The binding of calcium ion does serve to retard denaturation and degradation of the protein by autolysis (Green and Neurath, 1953; Delaage and Lazdunski, 1967; Gabel and Kasche, 1973; Lazdunski and Delaage, 1965). Although the crystal structure for trypsin has been reported earlier, the position of the calcium binding site was not determined and relatively little is known about the metal binding site.

Recently the paramagnetic properties of the lanthanide ions have been used to increase the relaxation rates of substrate protons as well as water protons bound to several metalloproteins (Butchard et al., 1972; Furie et al., 1974; Abbott et al., 1975; Darnall et al., 1975). Among the lanthanide series gadolinium(III) and europium(II) have proved to be the best probes for nuclear magnetic resonance relaxation investigations. Gadolinium(III) is an S-state ion with a relatively long electronic relaxation time compared with the remainder of the trivalent lanthanide ions (Bloembergen and Morgan, 1960). Gadolinium(III) has been used in a manner analogous to manganese(II) as a paramagnetic probe for studies involving the effect on the relaxation rate of solvent nuclei upon metal binding to macromolecules (Dwek et al., 1971; Reuben, 1971a; Sherry and Cottam, 1973). By observing changes in the relaxation rates of suitable nuclei on substrates (or inhibitors) bound in a ternary protein–metal–substrate complex, Butchard et al. (1972) and Furie et al. (1974) have been able to calculate metal–substrate distances in lysozyme and staphylococcal nuclease, respectively.

Commercial trypsin contains a mixture of proteins which are designated as α-trypsin, β-trypsin, and "inert proteins". β-Trypsin has a single polypeptide chain. The cleavage of the peptide bond between Lys-145[1] and Ser-146 of β-trypsin produces α-trypsin (Schroeder and Shaw, 1968). The "inert proteins" comprising anywhere from 20 to 40% of the commercial preparation are a mixture containing various autolytic products of β-trypsin.

Earlier we reported metal–inhibitor distances for a commercial preparation of bovine trypsin which was largely a mixture of α-trypsin and β-trypsin (Abbott et al., 1975; Darnall et al., 1975). Herein we extend our previous studies and report data which lead to calculations of metal ion–inhibitor (*p*-toluamidine) distances for pure α-trypsin and pure β-trypsin.

Theory

The longitudinal and transverse relaxation times, T_{1m} and T_{2m}, respectively, of resonating nuclei bound in a paramagnetic complex are given by the Solomon–Bloembergen

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[1] Throughout this discussion we shall use the chymotryptic numbering system for the amino acid sequence of trypsin (Stroud et al., 1971).

equations (Solomon, 1955; Bloembergen, 1957) as modified by Connick and Fiat (1966) (for general review of these equations, see Dwek, 1973). Assuming there is no scalar contribution to the relaxation times, the relationships contain an r^{-6} (where r is the distance between the paramagnetic ion and the proton under observation) distance dependence between spin centers, and the dipolar interaction is modulated by the correlation times τ_{c1} and τ_{c2}.

The correlation time for the dipolar interaction is:

$$\frac{1}{\tau_{ci}} = \frac{1}{\tau_m} + \frac{1}{\tau_r} + \frac{1}{\tau_{si}} \quad (1)$$

with $i = 1, 2$, respectively. τ_m is the lifetime of the nuclei in the paramagnetic complex, and τ_r is the rotational correlation time, or "tumbling" time of the complex. τ_{si} is the electronic relaxation time, reflecting the possible differences between the spin-lattice ($i = 1$) and spin-spin ($i = 2$) relaxation times.

In order to obtain r, the distance between spin centers, τ_c must be obtained explicity. Evaluation of τ_c from eq 1 is frequently impossible; however, under conditions where the scalar contribution to the relaxation rates is small, advantage may be taken of the fact that T_{1m}^{-1} and T_{2m}^{-1} have different functional dependence upon the correlation time, τ_c. When the scalar terms are negligible and $\tau_{c1} = \tau_{c2}$ (i.e., $\tau_{s1} = \tau_{s2}$, Reuben et al. (1970)), the ratio of T_{1m}/T_{2m} becomes:

$$\frac{T_{1m}}{T_{2m}} = \frac{4\tau_c + \frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{13\tau_c}{1 + \omega_s^2\tau_c^2}}{\frac{6\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{14\tau_c}{1 + \omega_s^2\tau_c^2}} \quad (2)$$

and only one relevant correlation time need be considered. The scalar interaction should be negligible here since the inhibitor is not bound directly to the paramagnetic ion and thus no delocalization of electron spin density is expected.

Since $\omega_s = 658\omega_I$ and $\omega_I = 6.28 \times 10^8$ rad sec^{-1} at 100 MHz, the last term in the numerator and denominator will be small when $\tau_c > 10^{-10}$. The ratio, T_{1m}/T_{2m}, is constant below values of $\tau_c \simeq 8 \times 10^{-10}$, but begins to increase as τ_c becomes larger. Thus τ_c values can be obtained directly from the ratio T_{1m}/T_{2m} when τ_c values greater than 8×10^{-10} sec determine the relaxation properties of the system.

If the inhibitor undergoes rapid exchange between the bulk solution and the ternary enzyme-metal ion-inhibitor complex, the observed relaxation rate consists of contributions from both environments. Under such conditions the relaxation times are given by:

$$\frac{1}{T_i} = \frac{P}{T_{im} + \tau_m} + \frac{1 - P}{T_{io}} \quad (3)$$

where $i = 1, 2$; P is the mole fraction of inhibitor in the ternary complex; T_i is the observed relaxation time; T_{io} is the diamagnetic contribution; τ_m is the exchange time, and T_{im} is the relaxation time for the nuclei in the ternary paramagnetic complex.

Any possible contribution from the exchange rate, τ_m, can be verified by the temperature dependence of the relaxation rate. If T_i increases with increasing temperature, then the condition of rapid exchange is valid, i.e., $T_{im} \gg \tau_m$. If only a small fraction of inhibitor is bound in the ternary complex, i.e., $1 \gg P$, and fast exchange occurs in the system, then eq 3 becomes:

$$\frac{1}{T_i} = \frac{P}{T_{im}} + \frac{1}{T_{io}} \qquad i = 1, 2 \quad (4)$$

Experimental Section

Twice crystallized, dialyzed, and lyophilized bovine trypsin (Sigma Chemical Co., Type III) was dialyzed against 10^{-3} M hydrochloric acid and lyophilized. β- and α-trypsin were prepared essentially by the method of Schroeder and Shaw (1968). The dimensions of the SP-Sephadex C-50 column were 4.2 × 85 cm and, after bed equilibration, the flow rate was 75 ml/hr. Typically, 1 g of trypsin was dissolved in 50 ml of 0.1 M Tris buffer (pH 7.1) containing 0.02 M calcium and 10^{-3} M benzamidine. After centrifugation (30,000g, 5 min), the protein was applied to the column and the elution profile was monitored by an Isco Model UA-2 uv analyzer. The α-trypsin and β-trypsin fractions were collected. Those fractions containing both α-trypsin and β-trypsin were discarded. The pooled fractions were lyophilized, dissolved in a small amount of water, and dialyzed at 4°, against 10^{-3} M HCl containing 10^{-3} M benzamidine. When the pH of the dialysate remained stable at 3, the benzamidine was removed by exhaustive dialysis against 10^{-3} M HCl. The pure proteins were then lyophilized and stored at −20°. As expected, end group analysis by carboxypeptidase B showed the presence of C-terminal lysine for α-trypsin, but no C-terminal residue for β-trypsin under the same conditions.

The extinction coefficients at 278 nm of commercial trypsin (after extensive dialysis against 10^{-3} M HCl), α-trypsin, and β-trypsin were determined by measuring the absorbances of solutions whose concentrations were determined by the dry weight method. The extinction coefficients were 1.67, 1.59, and 1.70 cm^{-1} mg^{-1} ml for the commercial trypsin, α-trypsin, and β-trypsin, respectively.

Activities of the enzyme were measured as described previously (Gomez et al., 1974) using Tos-ArgOMe[2] as a substrate. Specific activities of the commerical trypsin, α-trypsin, and β-trypsin were 190, 380, and 280 μmol per min per mg, respectively.

Gadolinium(III) chloride was prepared by adding 0.1 N HCl to an excess of the sesquioxide (99.9+% from Kerr McGee Corp. and Molybdenum Corp. of America), filtering the solution, and evaporating the filtrate to dryness. Stock gadolinium chloride solutions were analyzed by complexometric titration in a pH 6 acetate buffer with an EDTA solution standardized against fired La_2O_3. Xylenol Orange was used as the indicator (Lyle and Rahman, 1963).

p-Toluamidine hydrochloride (Columbia Organic Chemicals Co.), acetamidine hydrochloride (Aldrich Chemical Co., Inc.), and Mes buffer, Sigma Chemical Co., were used as received. Stock solutions of p-toluamidine and Mes buffer were prepared at pH 6 in distilled, deionized water.

Metal ion-trypsin binding constants were determined by equilibrium dialysis under the following conditions: ~2 × 10^{-4} M protein, 0.2 M p-toluamidine, 0.05 M Mes buffer at pH 6.0, and an ionic strength adjusted to 0.3 with NaCl. After equilibration at 25° for 15-18 hr, metal ion concentrations were determined by EDTA titrations using either Xylenol Orange (for lanthanides) or fluorescein (for calcium) as the indicator.

All solutions used in nuclear magnetic resonance (NMR)

[2] Abbreviations used are: Tos-ArgOMe, tosyl-L-arginine methyl ester; PAB, p-aminobenzamidine; PTA, p-toluamidine, ATA, acetamidine; DSS, 2,2-dimethyl-2-silapentanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; BzArgOEt, benzoyl-L-arginine ethyl ester; AcTyrOEt, N-acetyltyrosine ethyl ester.

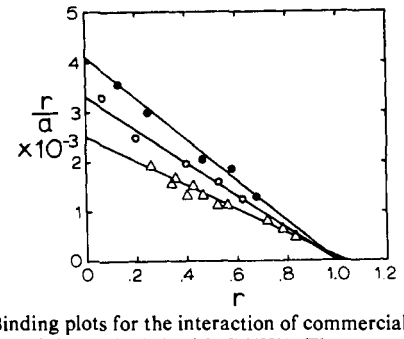

FIGURE 1: Binding plots for the interaction of commercial trypsin (△), α-trypsin (○) and β-trypsin (●) with Gd(III). The commercial trypsin concentration was 1.80×10^{-4} *M* and the Gd(III) concentration range was 5×10^{-3}–3×10^{-4} *M*. The α-trypsin and β-trypsin concentrations were 1.71×10^{-4} *M* and the Gd(III) concentration range was 1×10^{-3}–1×10^{-4} *M*. All solutions contained 0.05 *M* Mes (pH 6.0) and 0.2 *M* PTA. The ionic strength was adjusted to 0.3 with NaCl. The equilibration time was 18 hr and the temperature was 25°. The symbol *r* is defined as moles of bound metal ion per mole of protein, and *a* is defined as the free metal ion concentration.

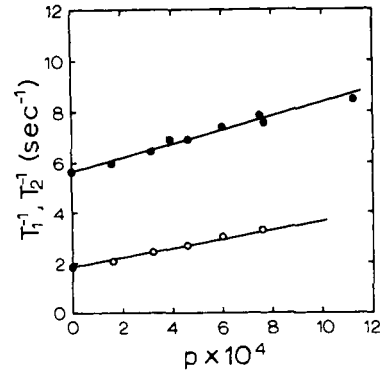

FIGURE 2: Relaxation rates, T_1^{-1} (○) and T_2^{-1} (●), for methyl resonance of *p*-toluamidine as a function of *P*, the fraction of total inhibitor in the ternary β-trypsin-gadolinium(III)-inhibitor complex at 100 MHz and 25°. Solutions contained 0.2 *M* *p*-toluamidine in 0.05 *M* Mes buffer at pH 6. β-Trypsin and gadolinium(III) concentrations varied from 2×10^{-4} to 1×10^{-3} *M*. No change in relaxation rates was observed due to free gadolinium(III).

measurements contained 0.20 *M* inhibitor (PTA or ATA) and 0.05 *M* Mes buffer. Gadolinium(III) concentrations were varied from 0.1 to 1.0 m*M*, and trypsin concentrations were varied from 0.1 to 0.7 m*M*.

All relaxation times were measured using a JEOL PS-100 high-resolution nuclear magnetic resonance spectrometer. Spin–lattice relaxation times, T_1, were obtained by the direct method using a saturating pulse and observing the recovery to equilibrium. The sweep circuit on the spectrometer was modified to allow faster sweep times and the output was displayed on an external storage oscilloscope. The relaxation time measured was independent of the sweep time used (generally 0.3 sec with a sweep width of 30 Hz). The external scope sweep was synchronized to start at the same time that the saturating rf pulse was removed and the results were averaged over at least six pulse sequences. Spin–spin relaxation times, T_2, were measured by line width techniques (Van Geet and Hume, 1965). Saturation of peaks was avoided by keeping the radio frequency low enough so that no broadening resulted when using longer sweep times. Operating at 100 MHz a sweep width of 54 Hz was used, and the water resonance peak provided the internal lock signal. The temperature of the NMR probe was 25 ± 1°.

Results

The effect of temperature upon the relaxation rates of diamagnetic and paramagnetic species was measured over the range from 5 to 40°. Since the relaxation rate decreased with increasing temperature, conditions of fast exchange exist in the system, i.e., $\tau_m \ll T_{2m}$, and eq 4 is valid.

The effect of any bulk diamagnetic or paramagnetic contribution on line widths was examined. With up to 1.0 m*M* trypsin in the sample no broadening was observed for the *p*-toluamidine resonances in both the presence and absence of La(III). Likewise it was determined that Gd(III) produced no change in the inhibitor proton relaxation rates in the absence of trypsin. This was not the case for another inhibitor, *p*-aminobenzamidine, where bulk paramagnetic effects were observed, presumably due to weak interaction between the amino group and the Gd(III) ion.

At pH 6 in 0.05 *M* Mes buffer and an ionic strength of 0.3, the *p*-toluamidine–trypsin dissociation constant (K_I) was determined from Lineweaver–Burk plots to be 2.95×10^{-5} *M*. This compares favorably with the values of 2.65×10^{-5} *M* determined for *p*-toluamidine by Mares-Guia (1968) and 3.0×10^{-5} by Markwardt et al. (1968). Figure 1 shows results of gadolinium–trypsin binding experiments with commercial trypsin which contains a mixture of α-trypsin, β-trypsin, and "inert" protein. The results are consistent with a single binding site for Gd(III) with a $K_a = 2.5 \times 10^3\ M^{-1}$. Figure 1 also shows results of equilibrium dialysis of pure α-trypsin and β-trypsin. Again a single binding site is found for both proteins and binding constants of 3.3×10^3 and $4.1 \times 10^3\ M^{-1}$ for pure α-trypsin and β-trypsin, respectively.

Binding constants of calcium to β-trypsin at pH 6.0 (Mes buffer) and 8.0 (Tris buffer), ionic strength 0.3, were also determined by the same technique. A value of 260 M^{-1} was obtained at pH 6.0 and a value of 3700 M^{-1} was obtained at pH 8.0. All equilibrium constants were obtained under the same solution conditions used for the NMR measurements.

Experiments reported earlier (Abbott et al., 1975) with commercial trypsin (containing α-trypsin, β-trypsin, and "inert proteins") showed a linear dependence of $1/T_1$ and $1/T_2$ as a function of *P*, the fraction of *p*-toluamidine in the ternary complex of trypsin–PTA–gadolinium(III). To calculate values for *P*, it was assumed that all trypsin was bound with *p*-toluamidine in a binary complex. Under the experimental conditions stated earlier and a $K_I = 2.95 \times 10^{-5}$ *M*, less than 0.05% of the total trypsin was uninhibited. The fraction of *p*-toluamidine in the ternary complex was then calculated using the enzyme–metal binding constant. We have used here the same procedure for calculating *P* values for both α-trypsin and β-trypsin.

The dependence of T_1^{-1} and T_2^{-1} for the methyl protons of *p*-toluamidine as a function of *P* for β-trypsin is shown in Figure 2. Using eq 4, T_{1m} was found to be 5.9×10^{-4} sec and T_{2m} was determined to be 3.6×10^{-4} sec. The ratio $T_{1m}/T_{2m} = 1.6$ results in a correlation time, τ_c, of 1.4×10^{-9} sec (eq 2) at 100 MHz. From the Solomon–Bloembergen equations, *r* was then calculated to be 9.5 Å (±0.5 Å) for the distance from the methyl protons of *p*-toluamidine to the gadolinium ion in β-trypsin.

The variation of T_1^{-1} and T_2^{-1} vs. *P* for α-trypsin exhibited the same linearity as the data for β-trypsin with T_{1m} and T_{2m} determined to be 7.3×10^{-4} and 3.1×10^{-4} sec,

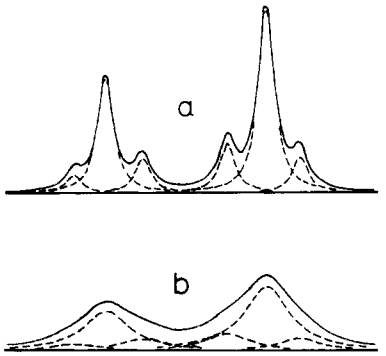

FIGURE 3: A typical computer analysis of the low-field (ortho protons) multiplet of the AA'XX' spectrum of *p*-toluamidine in this case with (a) $T_2 = 0.32$ sec and (b) $T_2 = 0.11$ sec. Individual components of the multiplet were assumed to have the same relaxation rate and to be of Lorentzian form. The observed spectrum was fitted using a nonlinear multiple regression computer program.

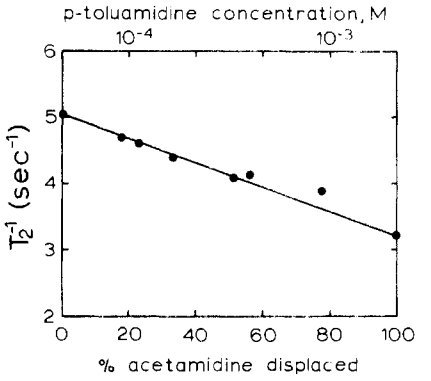

FIGURE 4: Effect of *p*-toluamidine on the transverse relaxation rate, T_2^{-1}, of 0.2 *M* acetamidine in the presence of 1.4×10^{-4} *M* trypsin-gadolinium(III) complex. *p*-Toluamidine concentrations varied from 10^{-4} to 10^{-3} *M* at pH 6. Percent acetamidine displaced by *p*-toluamidine was calculated using the inhibition constants for the two inhibitors. The line was extrapolated to T_2^{-1} for free acetamidine to correspond to full displacement.

respectively, resulting in a value of τ_c of 2.1×10^{-9} sec (eq 2) and a value for r of 9.7 Å (±0.5 Å) for the methyl protons (of PTA) metal distance in α-trypsin.

Within experimental error (reflected in the variation in the τ_c values obtained), we find there is little or no difference in our distance measurements between α-trypsin and β-trypsin or a commercial mixture of the two forms of trypsin taking into account the difference in the metal-trypsin binding constants. Therefore most of the experiments that follow were done on the commercial mixture of α-trypsin and β-trypsin.

The proton resonance spectrum of *p*-toluamidine at 100 MHz consists of a singlet at 2.64 ppm due to the methyl protons (with DSS at 0) and a complex multiplet, that approximates an AA'XX' spectrum, centered at 7.78 ppm due to the aryl protons. The low-field half of the multiplet was assigned to the protons ortho to the amidine group following assignments made by Jardetzky and Wade-Jardetzky (1965) and by comparison with NMR spectra of other para-substituted benzamidines. The peaks at higher field assigned to the protons meta to the amidine group are somewhat broader and some of the fine structure has been lost presumably due to weak coupling with the methyl protons.

In order to assist in establishing a possible metal site, relaxation rates (T_2^{-1}) were measured for the aryl protons ortho to the amidine group. Theoretically a maximum of 12 components might be observed in the low-field half of the pseudo AA'XX' multiplet; however, only six peaks were observed. To calculate T_2 for these ortho protons, frequencies and relative intensities were assigned to the six observable peaks in the diamagnetic spectrum. Assuming that the components remain Lorentzian in form and that there was no preferential line broadening, the observed spectrum was considered to be a composite of six broadened resonances. A nonlinear multiple regression computer program was then used to calculate T_2 for the spectrum. (Simulated spectra for the unbroadened case and for a typical broadened case are shown in Figure 3.) The assumptions made appear reasonable since the relative intensities and separation of components in the observed spectra did not change significantly as the broadening increased.[3]

The relaxation rates (T_2^{-1}) of the ortho protons as a function of the fraction of bound *p*-toluamidine using the above procedure were found to be linear and T_{2m} was calculated to be 1.7×10^{-4} sec. Using the same correlation time (2.2×10^{-9} sec) reported earlier for the commercial trypsin mixture (Abbott et al., 1975), r is 8.8 Å for the ortho protons of *p*-toluamidine.

Acetamidine is also known to be a reversible competitive inhibitor of trypsin with $K_I = 2.55 \times 10^{-2}$ (Mares-Guia, 1968). As with *p*-toluamidine, T_1^{-1} and T_2^{-1} varied linearly as a function of P for acetamidine. T_{1m} is calculated to be 7.2×10^{-4} sec and T_{2m} is 3.7×10^{-4} sec. With $T_{1m}/T_{2m} = 1.9$, τ_c is calculated to be 1.65×10^{-9} sec. Taking into account the relatively low K_I for ATA (approximately 12% of the trypsin remains uninhibited), the distance between the methyl protons of acetamidine and the gadolinium ion is calculated to be 9.5 ± 0.5 Å.

There have been several reports of substrate activation of trypsin, particularly at high substrate concentrations (Trowbridge et al., 1963; Elmore et al., 1967; Nakata and Ishii, 1972). Although binding studies have shown that only one benzamidine molecule binds trypsin at low concentrations of benzamidine (East and Trowbridge, 1968), the possibility remained that under the conditions of our NMR experiments (where 0.2 *M* inhibitor is present) more than one inhibitor was binding trypsin. Our distance measurements under these conditions would then represent an average value from the metal ion to two or more inhibitors. To discount this possibility we took advantage of the large differences in inhibition constants for *p*-toluamidine and acetamidine. Low concentrations of *p*-toluamidine should displace acetamidine from the specificity site in trypsin. Therefore a solution containing 0.2 *M* acetamidine, 1.12×10^{-3} *M* trypsin, and 2.4×10^{-4} *M* Gd(III) was titrated with m*M* amounts of *p*-toluamidine and the NMR spectra of the acetamidine protons were observed. Results of this competition experiment (Figure 4) are consistent with the premise that only one inhibitor site is responsible for the observed relaxation rates since extrapolation of the acetamidine data to high PTA concentration yields the relaxation rate of free ATA.

Experiments with various concentrations (0.05–0.25 *M*) of *p*-toluamidine produced slightly larger values of T_{1m}^{-1} and T_{2m}^{-1}'s for the methyl protons. T_{1m}^{-1} for these solutions increased at a somewhat faster rate than T_{2m}^{-1}. The

[3] Private communications with Drs. A. Allerhand, Ian Smith, and R. Vold.

net effect was a change in the calculated correlation time although only very minute changes in the metal-inhibitor distances resulted. Increasing the ionic strength by addition of KCl produced the same effect. With constant ionic strength the relaxation rates for bound inhibitor remained constant while varying the inhibitor concentration. The fact that the same distance is obtained over a variety of inhibitor concentrations is also consistent with a single inhibitor binding site to trypsin.

In order to determine if the effect of gadolinium(III) upon the relaxation rates is due to occupation of the calcium site in trypsin, NMR competition experiments between these two metal ions were performed. It was found that calcium did displace gadolinium(III) from trypsin, and a calcium binding constant of 210 M^{-1} was determined from these experiments. This value for the calcium binding agrees well with our value of 260 M^{-1} obtained using equilibrium dialysis under the same conditions.[4]

Discussion

Having determined values for the correlation times leads one to consider which of the terms in eq 1 dominates τ_c. From fluorescence polarization data, τ_r was found to be 1.29×10^{-8} sec for trypsin (Yguerabide et al., 1970) which is approximately twice the value obtained from Stoke's law and several times greater than the correlation times observed experimentally. If τ_m is assumed to be $>10^{-8}$ sec as would be expected, τ_c must be dominated by τ_s in this system. This is not unreasonable considering the electron spin resonance data reported by Reuben (1971b) since the exact nature of crystal field perturbations upon the electron relaxation rate of gadolinium(III) when bound to macromolecules is not known. If τ_s is indeed the dominant factor determining the value of τ_c, then we would expect τ_c for other gadolinium–protein systems to be similar to the values of τ_c ($1.4\text{–}2.2 \times 10^{-9}$ sec) which we have obtained. The values we obtained for correlation times compare favorably with the τ_c of 2.6×10^{-9} sec found for the staphylococcal nuclease–thymidine diphosphate–gadolinium complex obtained by Furie et al., (1974) at 220 MHz.

Our initial experiments (Abbott et al., 1975; Darnall et al., 1975) were completed on preparations of trypsin that contained considerable amounts of both α-trypsin and β-trypsin. Since there could have been some differences in the behavior of the ternary complexes containing α-trypsin and β-trypsin, we repeated these experiments using the separated components. Our results reported here show that there is no significant difference in distance between the inhibitor and Gd(III) for α-trypsin and β-trypsin. This confirms the data of Villanueva and Herskovits (1971) who showed by solvent perturbation spectroscopy that the conformations of benzamidine-inhibited α-trypsin and β-trypsin are the same.

The fact that the distance of approximately 10 Å that we measure from the methyl protons of *p*-toluamidine is larger than that from the ortho protons (8.8 Å) indicates that the metal ion is located above or below the phenyl ring as well as behind the amidine function. This also fits with the distance of 9.5 Å measured from the methyl protons of acetamidine to the gadolinium ion. At first glance one might expect the distance from the methyl protons of acetamidine to be more nearly the same as the distance from the ortho protons of *p*-toluamidine. But the association constant for acetamidine is three orders of magnitude less than that for *p*-toluamidine, so the amidine function of acetamidine might not be expected to approach as close to Asp-189 in the specificity pocket as *p*-toluamidine. In addition the lack of the aromatic ring in acetamidine may result in a slightly different fit of the substrate in the binding pockets resulting in the somewhat larger distance than expected. The distances of approximately 10 Å we measure are also in reasonable agreement with the 11–13.5-Å distance measured between Mn(II) and benzylamine for bovine trypsin (Cohen-Addad and Leyssieux, 1973). Although the report by Cohen-Addad and Leyssieux (1973) is only an abstract and few experimental details are given, the manganese ion is presumably binding at the calcium site. At any rate manganese ion has been reported to substitute for the calcium ion in porcine trypsin (Epstein et al., 1974).

The NMR experiments indicate that calcium ion will displace the gadolinium ion from trypsin. This in itself would argue strongly that gadolinium is occupying the single calcium ion binding site in trypsin. Our equilibrium dialysis experiments also support this since only single calcium ion and single gadolinium ion binding sites were observed. This would tend to rule out the possibility that gadolinium ion is binding at a nonspecific site on trypsin. We do observe, however, that at high gadolinium concentrations (~0.01 M) there exists at least one secondary metal binding site.

The position of the calcium site on bovine trypsin has been speculated to be composed of two carboxyl groups from Asp-71, Glu-77, or Asp-153 (Abita and Lazdunski, 1969; Stroud et al., 1971). However, these side-chain carboxyl groups lie approximately 25 Å from the specificity site of trypsin where *p*-toluamidine would bind. Since there is chemical evidence that at least one carboxyl group is involved in the binding of the calcium ion (Abita and Lazdunski, 1969; Delaage and Lazdunski, 1967; Duke et al., 1952), we have searched for carboxyl side chains which are closer to the specificity site of trypsin. By constructing a model of trypsin using coordinates graciously supplied by Dr. Robert Stroud, we find that there are only three carboxyl groups within a sphere of 15 Å from the binding site of *p*-toluamidine, assuming *p*-toluamidine binds in the same manner as benzamidine (Kreiger et al., 1974). Of the carboxyl residues within 15 Å, one, Asp-189, is at the recognition site, a second, Asp-102, is part of the "charge-relay" network in the active site, and the third is the carboxyl group at Asp-194. The first two potential sites appear to be poor choices for metal binding sites since the metal ion alters neither the binding of synthetic substrates or inhibitors to trypsin nor the activity of trypsin toward synthetic substrates (at least under the low concentrations of metal ion in our experiments). The carboxyl group of Asp-189 can also be ruled out on the basis that if the metal ion and PTA were competing for the same site, we would see no change in the relaxation times of PTA when the metal ion binds, since a ternary complex would not be formed. The side chain of Asp-102 we have also discounted since binding of gadolinium or calcium ion at Asp-102 should inhibit enzyme activity as it does when silver ion binds Asp-102 (Chambers et al., 1974).

The third site, Asp-194, has in close proximity a hydroxyl group from Ser-190. Metal ion distances from a metal ion placed between the side chains of Asp-194 and Ser-190 to the ortho and methyl *p*-toluamidine protons are 8.3 ± 0.8

[4] It should be pointed out that in other systems no displacement of gadolinium ion (or any other lanthanide ion) by calcium ion may be due to a difference in binding constants, rather than a lack of competition for the same site.

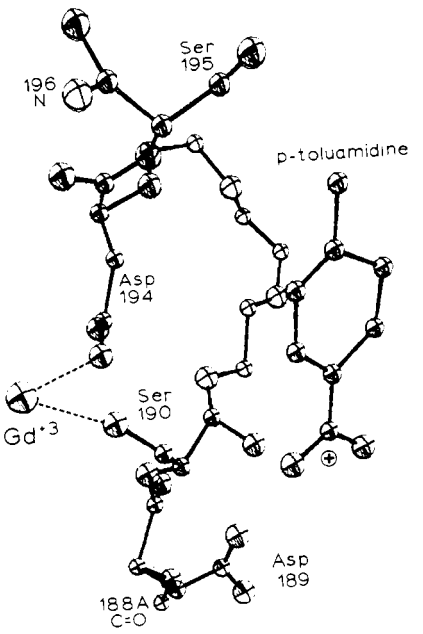

FIGURE 5: Illustration of residues 189–195 for bovine trypsin with *p*-toluamidine occupying the position described for benzamidine (Kreiger et al., 1974). Side chains and carbonyl oxygens have been omitted from the sequence 191–193 (unshaded peptide backbone). Gadolinium ion is shown at the proposed in close proximity to Ser-190 and Asp-194. The drawing was produced by the computer program ORTEP (Johnson, 1965).

and 9.8 ± 0.8 Å, respectively. The 1.6-Å range reflects the variability in the exact placement of the metal ion at this binding site, i.e., bidentate or monodentate with respect to the carboxyl group. It was because these distances agreed well with our experimentally measured distances, that we proposed the calcium binding site in trypsin to be composed of the carboxyl group of Asp-194 and the hydroxyl group of Ser-190 (Abbott et al., 1975; Darnall et al., 1975). Figure 5 shows a drawing of the proposed metal binding site as well as the PTA binding site in bovine trypsin as obtained from our distance measurements and the crystal structure coordinates.

The location of the metal ion binding site at Asp-194 and Ser-190 is consistent with a variety of supporting data. The binding constant for the gadolinium–trypsin association (K_a = 2.5–4.3 × 10^3) appears somewhat large for a hydroxycarboxylic acid ligand, e.g., the binding constant of Gd(III) to lactic acid is 780 (Moeller et al., 1965). However, inspection of the trypsin model shows that this site is somewhat buried in the interior of the protein and the pocket surrounding the metal ion is relatively hydrophobic, the adjacent side chains being donated by Ile-16, Ile-138, Trp-141, Leu-158, and Ala-160. One effect of the hydrophobic nature of this pocket might be to exclude a portion of the coordinated water from the metal ion when bound to trypsin. This would result in a larger than expected stability constant, as we observe. In addition, if water is indeed excluded from the coordination sphere of Gd(III), water proton longitudinal relaxation enhancement values should reflect this. For bovine trypsin we have observed a small enhancement of 1.5 for Gd(III) at 100 MHz (Sherry et al., 1973). Similarly, Epstein et al. (1974) observed a water proton relaxation enhancement of 1.4 for Gd(III) binding to porcine trypsin. Such low enhancement factors suggest either an extensively dehydrated metal ion or, alternatively, a hydrophobic site where the water molecules bound to the metal ion and near the metal ion are essentially "frozen" so their exchange with bulk water is too slow to affect the relaxation rate of bulk water protons. Both of these explanations are consistent with Asp-194 and Ser-190 as the binding site. The carboxyl groups at Asp-71, Glu-77, or Glu-153 are on the surface of trypsin, so that if these were the ligands for the metal ion, large longitudinal relaxation enhancement rates for water protons would be expected.

Table I: Effect of Calcium Ion on the Autolysis of α-Trypsin.[a]

Substrate	$[Ca^{2+}]$ *M*	Activity (ΔOD/min) 0 hr	1 hr	3 hr	6 hr
BzArgOEt	0	0.22	0.11	0.04	0.03
	0.05	0.30	0.29	0.26	0.20
AcTyrOEt	0	0.024	0.019	0.017	0.015
	0.05	0.023	0.024	0.021	0.020

[a] α-Trypsin (0.80 mg/ml) was incubated in Tris (0.15 *M*, pH 8), μ = 0.3, 25°, for the times indicated. Activities were measured in 10^{-3} *M* substrate, 0.05 *M* Tris (pH 8), and 0.05 *M* Ca(II), 25°. Activities were followed by observing the increase in OD at 254 nm for BzArgOEt and the decrease in OD at 237 nm for AcTyrOEt.

If Asp-194 and Ser-190 do comprise the metal ion binding site, then it should be possible to explain how the calcium ion stabilizes trypsin toward autolysis. The first step in the autolysis of β-trypsin is the cleavage of the bond between Lys-145 and Ser-146 to produce α-trypsin (Schroeder and Shaw, 1968). Both α-trypsin and β-trypsin are active toward substrates with positively charged side chains, although their activities are not identical. Cleavage of the bond between Lys-188A and Asp-189 on α-trypsin produces ψ-trypsin which has a much lower activity for positively charged substrates (Smith and Shaw, 1969; Keil-Dlouha et al., 1971). The decrease in activity and loss in specificity for positively charged substrates for ψ-trypsin is easily attributed to changes in the vicinity of the recognition site of Asp-189 when the peptide bond between Asp-189 and Lys-188A is cleaved. Since the role of the calcium is to prevent autolysis, it seemed likely that calcium ion prevents the conversion of α-trypsin to ψ-trypsin. Table I presents evidence which indicates that calcium ion does indeed prevent the conversion of α-trypsin to ψ-trypsin. Smith and Shaw (1969) showed that α-trypsin and ψ-trypsin have very nearly the same activity with respect to AcTyrOEt whereas α-trypsin is much more active with respect to positively charged substrates (BzArgOEt) than is ψ-trypsin. Table I shows that after 6 hr the autolysis of α-trypsin in the absence of Ca(II) has decreased the BzArgOEt hydrolysis rate by a factor of 10 compared to α-trypsin with calcium ion. At the same time, the AcTyrOEt activity changes only slightly for the sample of α-trypsin without calcium ion as compared to the α-trypsin with calcium ion present. These results along with Smith and Shaw's (1969) data strongly suggest Ca(II) is inhibiting the conversion of α-trypsin to ψ-trypsin.

The crystal structure of β-trypsin in the absence of calcium ion shows the Asp-194 forms a salt bridge with the N-terminal Ile-16. If upon the binding of calcium ion between Ser-190 and Asp-194, this salt bridge is weakened or broken, one would expect the N-terminal chain to be shifted away from Asp-194. The first five amino acid residues of the N-terminal region of trypsin (Ile-16–Tyr-20) lie directly above the peptide bond which is cleaved in the conversion of α-trypsin to ψ-trypsin. If this N-terminal section moves

out slightly upon metal ion binding, the carbonyl function between Lys-188A and Asp-189 may well be protected so that further autolysis is prevented.

With porcine trypsin Epstein et al. (1974) have observed that the 545-nm fluorescence band of Tb(III) is enhanced by a factor of 10^5 when the metal ion is bound to the protein and the wavelength of excitation is near 295 nm. They conclude from this large fluorescence enhancement that the Tb(III) ion must be very near a tryptophan in the protein. We also observe a large fluorescence enhancement of Tb(III) fluorescence with the bovine enzyme upon excitation in the 280–295-nm region of the spectrum. In both the bovine and porcine enzymes there is a tryptophan located at residue 141. In the bovine enzyme this tryptophan lies 6–9 Å above our proposed binding site at Ser-190 and Asp-194. The close proximity of this tryptophan to this site would then account for the large fluorescence enhancement observed. The carboxyl groups located at residues 71, 77, and 153 are considerably further away (at least 15 Å) from any tryptophan residue. It is unlikely that energy transfer over a distance of 15 Å could account for the enhancement of fluorescence that is observed.

There have been reports that there are changes in the uv difference spectrum of both the bovine and porcine enzyme upon calcium binding (Sipos and Merkel, 1970; Matsushima et al., 1971).[5] These changes were thought to be indicative of changes in the environments of the tyrosine and tryptophan residues of the protein. Furthermore Matsushima et al. (1971) proposed that only one tryptophan was involved. Certainly the close proximity of Trp-141 to our proposed binding site could account for these changes. In addition Tyr-20 is located on the N-terminal chain which may very well change positions upon metal ion binding as we discussed above.

The amino acids Ser-190 and Asp-194 are conserved in bovine, porcine, sheep, and dogfish trypsin (Stroud et al., 1971; Hermodson et al., 1973; Titani et al., 1975) as well as in chymotrypsin. Asp-194 is conserved in *Streptomyces* trypsin while Ser-190 is replaced by a threonine, maintaining the hydroxyl group as a coordinating ligand (Olafson and Smillie, 1975). All of the above serine proteases are known to bind the calcium ion. The carboxyl groups of Asp-71, Glu-77, and Asp-153 which others have proposed as the calcium binding site in bovine trypsin are not conserved in all these proteins. Bovine chymotrypsin has carboxyl groups at positions 72, 78, and 153, but in the porcine trypsin, Asp-153 is replaced by serine and Asp-71 is replaced by a histidine residue. Dogfish trypsin, likewise, has Asp-171 substituted by a histidine residue. *Streptomyces* trypsin has Asp-71 replaced by alanine and Asp-153 replaced by tyrosine (Olafson et al., 1975). These amino acid substitutions alone would seem to mediate against the calcium binding site being composed of Asp-71, Glu-77, or Asp-153.

All of the above discussion has argued strongly that the calcium ion binding site is located at the side chains of Asp-194 and Ser-190 in all trypsins, as well as in chymotrypsin. We argue that the stabilization of trypsin to autolysis comes from the fact that the salt bridge between Ile-16 and Asp-194 is weakened or broken upon calcium ion binding. There has been evidence presented, however, that this particular salt bridge is necessary for the activity of both trypsin and chymotrypsins (Oppenheimer et al., 1966; Matthews et al., 1967; Hess, 1971; Robinson et al., 1973; Scrimger and Hofman, 1967; Fersht, 1972). Much of the evidence for the importance of the Asp-194–Ile-16 salt bridge has come from chemical modification experiments. Robinson et al. (1973) showed very elegantly that when the α-amino group of ϵ-guanidinated trypsin is modified by carbamylation, thiocarbamylation, or amidination, the enzyme becomes inactive toward specific ester and amide substrates. These experiments would tend to argue that if the calcium ion binds to Asp-194 and Ser-190 with a concomitant weakening or breaking of the salt bridge, then the enzyme should become inactive. The enzyme is of course still active when calcium ion binds. On the other hand, it may also be argued that the chemical modification of the Ile-16 produces other changes in the enzyme which account for the inactivation. And several investigators have questioned the necessity of the salt bridge for enzymatic activity in chymotrypsin. Valenzuela and Bender (1969, 1970) have argued strongly that the presence of the salt bridge is unnecessary for active δ-chymotrypsin. Argarwal et al. (1971) showed that N-terminal amidinated δ-chymotrypsin retains activity and Marini et al. (1975) showed that acetylation of the N-terminal isoleucine residue of δ-chymotrypsin results in an active enzyme.

In addition Osborne et al. (1972) have reported that a pH study of the binding of calcium ion to chymotrypsin indicates that the titration of the α-amino group (or His-40) results in the loss of calcium binding ability. Our own data for the binding of calcium to β-trypsin at pH 6.0 and 8.0 shows over a tenfold increase in the binding constant over that pH range. Both the data of Marini (1975) and Osborne et al. (1972) support our proposed binding site.

If the calcium ion binding site in trypsin is indeed that which we have suggested, several tests should confirm or deny this. For example, the α-amino group of trypsin should have a different chemical reactivity in the presence and absence of calcium ion. In addition, if the N-terminal group of trypsin were trifluoroacetylated, the effect of Gd(III) on the fluorine relaxation times should show the N-terminal group to be very near the gadolinium ion. These and other experiments are currently underway to confirm or deny our proposed calcium ion binding site at Asp-194 and Ser-190.

Added in Proof

Bode and Schwager have recently crystallized bovine β-trypsin containing a single calcium ion. Using x-ray diffraction, they have located a calcium ion bound at the side chain carboxyls of Glu-70 and Glu-80 and the carbonyl oxygens of Asn-72 and Val-75. Two other coordination sites of the calcium ion are occupied by water molecules, one of which is presumably hydrogen bonded to the carboxylic group of Glu-77. Even though the position of the calcium ion is known in this particular trypsin derivative, Bode and Schwager were unable to explain the stabilizing effect that the calcium ion has on the autolysis of trypsin.

It is difficult at the present time to explain the difference in results obtained by the two different techniques utilized by us and Bode and Schwager. The binding site proposed by us lies approximately 10 Å from the active site, whereas the site proposed by Bode and Schwager lies 20–25 Å away from the active site of the enzyme. Since our NMR are dependent upon r^{-6} (where r is the distance between the metal ion and the active site bound inhibitor), it is extremely unlikely that the observed effects could be due to a metal ion bound 20–25 Å from the inhibitor.

[5] Delaage and Lazdunski (1967) reported, however, that there was no uv difference spectrum obtained when Ca^{2+} bound state I′ of trypsinogen.

Another explanation to explain the differences observed by the two laboratories would be that the solution structure and crystal structures are different. Evidence that this may be the case comes from the fact that calcium ion is apparently bound much more strongly by the crystalline enzyme than by the solution enzyme. We showed that at pH 8.0 the binding constant for the single calcium ion–trypsin complex was 3700 M^{-1}. Bode and Schwager crystallized the enzyme from 2.4 M ammonium sulfate and, taking into account the solubility of $CaSO_4$, there should be only approximately 10^{-4} M Ca^{2+} in this solution. From the above binding constant, one can calculate that, under the conditions of crystallization, the enzyme should not be fully bound with calcium, yet the calcium site was found to be fully occupied under these conditions.

Preliminary fluorescence quenching experiments in our laboratory, utilizing praseodymium and holmium ions to quench the fluorescence of the competitive inhibitor proflavine, yield a distance of 11 Å, consistent with the NMR results.

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The Molecular Structure of a Dimer Composed of the Variable Portions of the Bence-Jones Protein REI Refined at 2.0-Å Resolution†

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ABSTRACT: The structure of the variable portions of a κ-type Bence-Jones protein REI forming a dimer has been determined by X-ray diffraction to a resolution of 2.0 Å. The structure has been refined using a constrained crystallographic refinement procedure. The final R value is 0.24 for 15,000 significantly measured reflections; the estimated standard deviation of atomic positions is 0.09 Å. A more objective assessment of the error in the atomic positions is possible by comparing the two independently refined monomers. The mean deviation of main-chain atoms of the two chains in internal segments is 0.22 Å, of main-chain dihedral angles 6.3° for these segments. The unrefined molecular structure of the V_{REI} dimer has been published (Epp, O., Colman, P., Fehlhammer, H., Bode, W., Schiffer, M., Huber, R., and Palm, W. (1974), *Eur. J. Biochem. 45,* 513). Now a detailed analysis is presented in terms of hydrogen bonds and conformational angles. Secondary structural elements (antiparallel β structure, reverse turns) are defined. A more precise atomic arrangement of the amino acid residues forming the contact region and the hapten binding site is given as well as the localization of solvent molecules. Two *cis*-prolines (Pro-8 and Pro-95) were detected. The intrachain disulfide bridge (Cys-23–Cys-88) occurs statistically in two alternative conformations. The structure suggests reasons for strong conservation of several amino acid residues. The knowledge of the refined molecular structure enables crystal structure analyses of related molecules to be made by Patterson search techniques. The calculated phases based on the refined structure are much improved compared to isomorphous phases. Therefore the effects of hapten binding on the molecular structure can be analyzed by the difference Fourier technique with more reliability. Hapten binding studies have been started.

Immunoglobulins are proteins with specific antibody activity. There exist several classes. The IgG class of immunoglobulins is composed of two light and two heavy chains. The Bence-Jones proteins excreted by patients with multiple myeloma into the urine have been shown to be free light chains. The Bence-Jones protein REI is a human immunoglobulin light chain of κ type. The purification, crystallization, and sequence analysis has been described (Palm, 1970; Palm and Hilschmann, 1973, 1975; Palm, 1974). The crystal structure of a dimer composed of the variable portions of this Bence-Jones protein at a resolution of 2.8 Å was reported (Epp et al., 1974). Data to a resolution of 2.0 Å have now been collected and the structure has been refined by constrained crystallographic refinement. The aim was to get a detailed insight into the conformation of this molecule (main chain, side chains, and bound solvent) and to obtain a model sufficiently accurate for its use in Patterson search techniques to determine the crystal structures of related molecules (Fehlhammer et al., 1975). As refined phases are considerably better than isomorphous phases (Watenpaugh et al., 1973; Deisenhofer and Steigemann, 1975; Huber et al., 1974), the quality of difference Fourier maps will be much improved; this will make it possible to determine the structure bound haptens and the subtle structural changes which might occur upon binding.

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