THE LOCATION OF THE LANTHANIDE ION BINDING SITE ON BOVINE TRYPSIN[1]

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Summary

Using the effect of a paramagnetic probe, Gd^{+3}, on the NMR relaxation time of inhibitor protons, the metal-inhibitor distances in a bovine trypsin ternary complex has been measured. The decrease due to Gd^{+3} in the spin-spin and spin-lattice relaxation times of the ortho and methyl protons of the inhibitor, p-toluamidine, has been measured at pH 6. The Solomon-Bloembergen equations were used to calculate distances of 8.8 ± 0.5 A and 10.0 ± 0.5 A from the metal ion to the ortho and methyl protons, respectively. From examination of the crystal structure of the enzyme it appears that the side chains of Asp 194 and Ser 190 are likely ligands for the metal ion.

Within the past few years the lanthanide ions have begun to be used as structure probes in biological systems (1-4). Their magnetic and spectroscopic properties lend themselves to a variety of investigations concerning the environment of the metal ion. Lanthanide ions have also been shown to be suitable replacements for calcium ions in several proteins (5-8).

Bovine trypsin, a proteolytic enzyme of molecular weight 24,000, has a single calcium ion binding site (9). The presence of calcium ion does not affect the activity of the enzyme, but the calcium ion does remarkably stabilize the enzyme toward autolysis and denaturation (9-11). The crystal structure of bovine trypsin in the absence of Ca^{+2} has been well characterized by Stroud and coworkers (12-14), however the calcium ion binding site is not known, although possible binding sites have been proposed. The following NMR experiments were done to assist in establishing the metal binding site in trypsin. By substitution of a paramagnetic metal ion (Gd^{+3}) for Ca^{+2} into the metal site of the p-toluamidine (PTA)-inhibited enzyme, the metal-inhibitor

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distance for the ternary complex can be calculated from the resulting decrease in the relaxation times of suitable nuclei on the inhibitor in accordance with the Solomon-Bloembergen equations.

THEORY

The longitudinal relaxation rate ($1/T_{1m}$) and the transverse relaxation rate ($1/T_{2m}$) for protons of a substrate (or inhibitor) in a ternary complex (with enzyme and metal) are given by (15-16):

$$\frac{1}{T_{1m}} = \frac{2}{15}\frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right) \qquad (1)$$

$$\frac{1}{T_{2m}} = \frac{1}{15}\frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6}\left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_s^2\tau_c^2}\right) \qquad (2)$$

These equations neglect the scalar (contact) contribution to the relaxation rates, since the metal ion is not bound directly to the inhibitor. Equations (1) and (2) represent the dipolar interaction between the electron spin of Gd^{+3} and the PTA nuclear spins which is modulated by a correlation time τ_c; r is the distance between the paramagnetic metal ion and the protons of the inhibitor (p-toluamidine) and the rest of the symbols have their usual meaning (17). T_{im}, (where i = 1 or 2 for longitudinal or transverse relaxation time, respectively) the relaxation time for a proton on the bound inhibitor under conditions of fast exchange as shown by temperature studies is given by:

$$\frac{1}{T_{i,obs}} = \frac{P}{T_{im}} + \frac{1-P}{T_{io}}$$

where $T_{i,obs}$ is the observed T_i, T_{io} is the relaxation time in the absence of a ternary complex and P is the fraction of inhibitor bound in the ternary complex. T_{1m} and T_{2m} were determined from Equation (3) for the trypsin-gadolinium-PTA complex, which were then substituted into Equations (1) and (2) to calculate r, the metal ion-proton distances.

EXPERIMENTAL

Twice crystallized, dialyzed and lyophilized bovine trypsin (Sigma Chemical Co., Type III) was again dialyzed at pH 3 and lyophilized. Gadolinium(III) chloride was prepared by adding 0.1 N HCl to an excess of Gd_2O_3 (Kerr-McGee Corp. and Molybdenum Corp.), filtering the solution and evaporating the filtrate to dryness. p-Toluamidine (PTA) was obtained from Columbia Organic Chemicals; MES buffer (2[N-Morpholine]-ethane sulfonic acid) was obtained from Sigma. All solutions used in NMR measurements contained 0.2 M PTA and 0.05 M MES buffer, pH 6. Gadolinium(III) concentrations varied from 0.1 to 1.0 mM, and trypsin concentrations varied from 0.1 to 0.7 mM.

All T_2's were measured on JEOL PS-100 high resolution NMR spectrometer using line width techniques (18). At 100 MHz a sweep width of 54 Hz was used and water protons were used as the internal lock. T_1's were obtained at the same frequency with the PS-100 by means of the direct method using a saturating pulse and observing the return to equilibrium. All experiments were done at 25 ± 1°C.

RESULTS

The variation of T_1 and T_2 for the methyl proton resonance of PTA as a function of ternary complex is shown in Figure 1. Our kinetic experiments showed PTA was a competitive inhibitor of trypsin with a $K_I = 2.95 \times 10^{-5}$ M. This agrees well with values of 3.00×10^{-5} and 2.65×10^{-5} determined previously (19-20). Equilibrium dialysis (under the same conditions as the NMR experiments showed a single binding site for Gd^{+3} on trypsin with a $K_a = 2.5 \times 10^3$ M^{-1}. Therefore under our conditions it was assumed that all trypsin was bound with inhibitor, and the amount of ternary complex present during the NMR experiment was governed by the association of Gd^{+3} with trypsin.

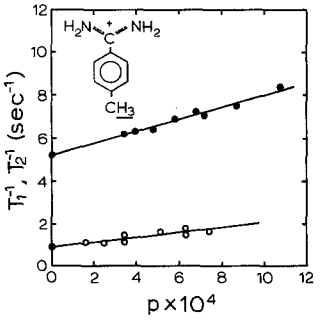

Figure 1. Relaxation rates of methyl protons, T_1^{-1}(O) and T_2^{-1}(●), vs. P, the fraction of p-toluamidine in ternary trypsin-Gd(III)-inhibitor complex.

By use of the Gd^{+3} binding constant and equation (3) T_{1m} and T_{2m} for the methyl protons were found to be 8.1×10^{-4} sec and 3.64×10^{-4} sec respectively. Due to the different analytical function for τ_c in the equations for T_{1m} and T_{2m} (17), τ_c may be determined by the ratio T_{1m}/T_{2m}. For $T_{1m}/T_{2m} = 2.22$, τ_c is calculated to be 2.0×10^{-9} sec.

The value of τ_c which we determined for the trypsin-PTA-Gd^{+3} system compares very favorably to the value of τ_c (2.6×10^{-9} sec) found for the Staphylococcal nuclease-thymidine diphosphate-Gd^{+3} system obtained by Furie et al. (4). Since the rotational relaxation time of trypsin is 1.29×10^{-8} sec (25), the values obtained for τ_c suggest that τ_s is the dominant factor in determining τ_c.

The variation of T_2 for the ortho protons of PTA in the ternary complex is shown in Figure 2. T_{2m} was found to be 1.5×10^{-4} sec from the low field portion of the well resolved AA'XX' multiplet.

Using equation (2) and the same τ_c, r was found to be 10.0 ± .5 A for the methyl group and 8.8 ± 0.5 A for the ortho protons of PTA.

Competition experiments between calcium ion and gadolinium ion show that both ions are competing for the same site on trypsin.

DISCUSSION

Various chemical evidence suggests that at least one carboxyl group is

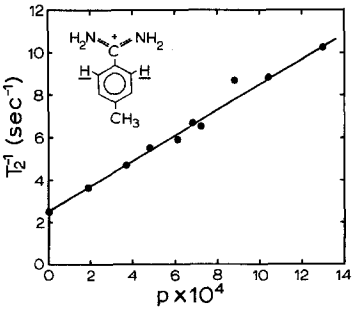

Figure 2. Relaxation rate, T_2^{-1}, of ortho protons vs. P, the fraction of p-toluamidine in ternary trypsin-Gd(III)-inhibitor complex.

involved in binding the calcium ion in trypsin (9,21,22). Stroud et al., (12) proposed that the calcium binding site was composed of the side chains of Asp 71 and Glu 77 or Asp 71 and Glu 153 (using the chymotrypsin numbering system). This was based primarily on the fact that these three amino acids have close structural analogues in chymotrypsin, which is also known to bind Ca^{+2} and that two of the carboxyls could act as a chelate for the metal ion.

The distances of 10.0 and 8.8 A from the metal ion to the methyl protons and the protons ortho to the amidine function of PTA, indicate that the metal ion is located above or below the plane of the aromatic ring and behind the amidine function. We have made a model of trypsin from coordinates kindly supplied by Dr. Stroud. After placing a model of p-toluamidine in the specificity pocket of trypsin in the same manner as benzamidine (14), we find only one site composed of a carboxyl group within our distance requirement (ignoring the specificity carboxyl, Asp 189). This site is composed of the side chains of Asp 194 and Ser 190 (using the chymotrypsin numbering system). Since the distances from PTA to Asp 71, Glu 77 or Glu 153 is on the order of 25 A, we feel that these carboxyls are much too far from the specificity site to be the calcium binding site.

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 (23). Both α- and β-trypsin are enzymatically active, but the cleavage of the bond between Lys 188A and Asp 189 on α-trypsin produces Ψ-trypsin which has much lower activity (24). Since a function of calcium ion is to prevent autolysis, it seems likely that calcium ion prevents the formation, of Ψ-trypsin from α-trypsin. The crystal structure of trypsin in the absence of calcium shows that the carboxyl group of Asp 194 forms an ionic bond with the N-terminal amino group of Ile 16. If upon binding calcium ion between Asp 194 and Ser 190, this salt bridge is weakened or even broken, one would expect the N-terminal chain to be slightly shifted away from Asp 194. The location of the first 5 amino acids in the N-terminal region of trypsin (Ile 16 - Tyr 20) lies directly above the peptide bond which

is cleaved in the conversion of α- to Ψ-trypsin. If this section of the molecule 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.

All of the work reported in this paper has been done on commercial trypsin which is largely a mixture of α- and β-trypsin. Preliminary experiments on pure α- and β-trypsin prepared according to Schroeder and Shaw (23) indicates there is no difference between the two forms which respect to metal ion binding or the NMR experiments. Additional work being done to determine if this proposed binding site is consistent with a large variety of experimental evidence will be reported later.

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