Multinuclear Magnetic Resonance Studies on the Calcium(II) Binding Site in Trypsin, Chymotrypsin, and Subtilisin†

Foluso Adebodun and Frank Jordan*

Department of Chemistry, Rutgers, The State University of New Jersey, Newark, New Jersey 07102

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ABSTRACT: Two different nuclear magnetic resonance experiments were conducted to elucidate the properties of the Ca(II) binding locus on serine proteases in solution. Trypsin, α-chymotrypsin, and subtilisin were inactivated with diisopropyl fluorophosphate, and the distance of the phosphorus from Gd(III) in place of Ca(II) was determined from the lanthanide-induced relaxation on the ^{31}P resonance. The distances found (between 20 and 21 Å) were in excellent agreement with those reported in the X-ray crystallographic structures of trypsin and subtilisin, demonstrating that the method has wide applicability to systems for which no X-ray structure is available. Subsequently, the ^{113}Cd spectra [in place of Ca(II)] were examined in the presence of the native enzymes. At ambient temperatures only a single ^{113}Cd resonance could be observed, presumably representing the weighted average of the variously weakly bound ions and the free ion. At 280 K for trypsin and chymotrypsin, and at 268 K for subtilisin there was observed a resonance at ca. 65–70 ppm higher field than the previous averaged resonance that could be attributed to tightly bound Cd. The chemical shift of the resonance was consistent with its assignment to an octahedral environment around Cd with oxygen ligands.

It has been known for a number of years that serine proteases possess a Ca(II) binding site with dissociation constants of 1 mM or smaller (Delaage & Lazdunski, 1967; Abbott et al., 1975a,b; Epstein et al., 1977). The role of Ca(II) is still somewhat controversial. While there appears to be general agreement that Ca(II) protects the enzymes from autolysis (Gorini, 1951; Bier & Nord, 1951), there are some reports claiming that at least some of the enzymes are also activated by Ca(II) (Sipos & Merkel, 1970). Ca ions also assist in the conversion of the zymogen to the active enzyme (McDonald & Kunitz, 1941) and in the activation of several components of both the blood coagulation (Shore, 1987; Sinha et al., 1987) and complement cascades (Busby & Ingham, 1987). With the ready availability of mutants for a number of interesting and important serine proteases, the location and function of the Ca(II) ions take on renewed importance. There is a very recent report locating the ion binding site in subtilisins (McPhalen & James, 1988), and there are earlier ones on trypsin (Bode & Schwager, 1975a,b) and on trypsinogen (Kossiakoff et al., 1977) in the solid state. Defining the binding locus in solution has lagged behind. The technique of oligonucleotide-directed site-specific mutagenesis has been applied to pinpointing the location of the Ca site in solution, especially on subtilisins. It would appear that Asp41 is the high-affinity site (Pantoliano et al., 1988). We have been interested for the past few years in the application of nuclear magnetic resonance (NMR) techniques to this problem and here report two different approaches, each providing different types of information. One technique takes advantage of the essentially isomorphous replacement that Gd(III) in place of Ca(II) provides in many proteins (Darnall & Birnbaum, 1973; Matthews & Weaver, 1974; Shimomura & Johnson, 1973; Reuben, 1979). Because of the paramagnetic relaxation caused by the Gd(III) ion, its distance from a spin $^1/_2$ nucleus can be determined. Earlier studies have demonstrated that Gd(III) binds at the same site as Ca(II) in trypsin (Epstein et al., 1974), competes with Ca(II) for the same single metal ion binding site (Abbott et al., 1975a), and stabilizes the enzyme in the same manner as does Ca(II) (Gomez et al., 1974). In this study the three title enzymes were first inactivated with diisopropyl fluorophosphate, yielding the diisopropylphosphoryl or DIP derivatives. This methodology has been accepted for many years as yielding selective labeling of the active-center Ser. For purposes of the NMR experiments, the method introduces a single P atom, whose relaxation behavior under the influence of the paramagnetic ion can then be monitored readily. The advantage of the approach here employed is that the covalently bonded P atom is a "stationary" target, i.e., is not subject to exchange broadening, thereby simplifying both the theory and the experiment. In addition, the "dead" enzyme is stable for long time periods required by the relaxation measurements. One must still be on guard and perform the experiments at a high enough pH to avoid possible aging, i.e., conversion to the monoisopropyl (MIP) enzyme, a process known to require a protonated His at the active center (Van der Drift, 1983; Van der Drift et al., 1985).

While the paramagnetic relaxation allowed us to obtain distances between the ion and the P atom in solution, it was not very informative as to the number of tight binding sites. To approach the latter question, we performed ^{113}Cd NMR experiments that not only demonstrated the existence of tight binding sites but also gave an indication of the type of ligands and coordination around those sites.

Earlier solution studies on trypsin by a proton NMR relaxation method (Abbott et al., 1975a,b) gave results that were very different from those observed by X-ray, not only sug-

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gesting multiple binding sites but also raising the possibility of different protein conformations in solution and in the crystals. The present results also demonstrate that the potential of the paramagnetic relaxation effects to determine distances is greatly improved when the nucleus in question is covalently affixed to the protein.

Materials and Methods

Materials. Subtilisin Carlsberg, porcine trypsin, *N*-benzoyl-L-tyrosine ethyl ester, and *N*-benzoyl-L-arginine ethyl ester were purchased from Sigma. α-Chymotrypsin was from Worthington. Sephadex G-25 fine was from Pharmacia. Ultrapure HCl, Gd sesquioxide and La sesquioxide (both 99.9+% pure) were from Alfa. Diisopropyl fluorophosphate and *p*-nitrophenyl acetate were from Aldrich. ^{113}CdO (91.7 atom % ^{113}Cd) was purchased from Oak Ridge National Laboratories.

Preparation of DIP–Enzymes (Jordan et al., 1985). The enzyme solutions were prepared by dissolving 200 mg of subtilisin in 3 mL of 0.01 M Tris, pH 8.0, or 100 mg of α-chymotrypsin or trypsin in 2 mL of 0.08 M Tris, pH 7.8. Each enzyme solution was treated with 20 μL of 0.5 M diisopropyl fluorophosphate (dissolved in 2-propanol). The extent of inactivation was monitored by periodic assay of the activity until at least 99.9% inactivation had been achieved. Typical incubation times at room temperature were about 6 h. The inactivated enzyme was purified on a Sephadex G-25 fine column that had been preequilibrated with 0.01 M Tris, pH 7.8. The protein was eluted with the same buffer. The purified protein was freeze-dried and stored in a freezer. All samples used for the NMR experiments showed the presence of only a single band on 10% polyacrylamide in the presence of sodium dodecyl phosphate. In order to minimize the interference from adventitious metal ions, all buffers were extracted with 8-hydroxyquinoline dissolved in CCl_4, and all glassware was presoaked in concentrated HNO_3 for at least 12 h.

Enzyme Assays. The activity of subtilisin was assayed by using *p*-nitrophenyl acetate as substrate and monitoring the release of *p*-nitrophenoxide at 400 nm. The activity of α-chymotrypsin was assayed by monitoring the increase in absorbance at 256 nm resulting from the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester as substrate (Hummel, 1959). The activity of trypsin was assayed by monitoring the hydrolysis of *N*-benzoyl-L-arginine ethyl ester at 253 nm (Schwert & Takenaka, 1955). The protein concentration was determined by measuring the absorbance at 280 nm [subtilisin according to Ottensen and Svendsen (1970); other enzymes according to Walsh and Wilcox (1970)].

Preparation of Lanthanide Solutions. $GdCl_3$ and $LaCl_3$ solutions were prepared by dissolving Gd_2O_3 or La_2O_3 in ultrapure HCl and evaporating to dryness. The concentration of stock solutions was determined by titrating with EDTA using arsenazo as indicator (Fritz et al., 1958).

Preparation of $^{113}Cd(II)$ Solutions. $^{113}Cd(ClO_4)_2$ was prepared by dissolving ^{113}CdO in perchloric acid and then adjusting the pH to 6.6 with NaOH. Some $^{113}CdCl_2$ solutions were prepared by dissolving 98% ^{113}Cd metal in HCl, followed by neutralization and evaporation to dryness.

pH Measurements. All pH measurements were done on a Radiometer pH m62 meter.

NMR Measurements. For most measurements an IBM WP-200 SY multinuclear instrument was employed.

^{31}P data were collected at 81.026 MHz in 10-mm sample tubes. Broad-band two-level decoupling was used to minimize the dielectric heating. The longitudinal relaxation time T_1 was measured by the fast inversion–recovery method (Levy et al., 1975). The data were fitted to the equation

$$Y = A - B \exp(-t/T_1) \quad (1)$$

where Y is the peak intensity, A is the normalized intensity of the largest peak, B is a constant related to the nuclear flip angle, and t represents variable delays. The transverse relaxation time T_2 was determined by the Meiboom–Gill modification (Meiboom & Gill, 1958) of the Carr–Purcell method (Carr & Purcell, 1954). The T_2 data were fitted to the equation

$$Y = B \exp(-t/T_2) \quad (2)$$

All T_2 experiments were performed on nonspinning samples to minimize diffusion effects.

A typical sample contained 2.1 mM DIP–enzyme in a 10-mm sample tube. All samples contained 10% D_2O to provide a lock signal. The concentration of lanthanide ion varied between 0.8 and 1.8 mM. The probe temperature was 22 ± 1 °C. Chemical shifts were recorded relative to external 85% H_3PO_4, with upfield shifts taken as negative. For T_1 experiments 1600 transients/τ value and for T_2 experiments 1200 transients/τ value were used. For the fast inversion–recovery T_1 a 1-s recycle time and for the Carr–Purcell–Meiboom–Gill T_2 determination a 10-s recycle time were employed. Total time for T_1 and T_2 experiments on the same sample required ca. 48 h (20 and 28, respectively). In each experiment four dummy scans initiated the process. All relaxation experiments were performed at pH 7.8.

^{113}Cd data were collected at 44.4 MHz in 10-mm sample tubes on 2-mL sample volumes containing 10% D_2O for a lock signal. Low-temperature measurements were performed with a Bruker VT-100 unit. Acquisition times of usually 0.1 s, along with a 90° pulse and a 1-s recycle time, were used. Chemical shifts were recorded relative to external 0.1 M $Cd(ClO_4)_2$, with an upfield shift taken as negative.

Molecular modeling was performed on an Evans and Sutherland PS 330 computer equipped with Mendyl/Sybil software from Tripos Associates, St. Louis, MO. The crystallographic coordinates were accessed from the Brookhaven Protein Databank with specific structures referenced under Results and Discussion.

Table I: Relaxation Times in Monoisopropylphosphoryl (MIP) and Diisopropylphosphoryl (DIP) Enzymes[a]

enzyme	T_1 (s)		T_2 (s)	
	DIP	MIP	DIP	MIP
subtilisin	1.95	1.75	0.03	0.056
α-chymotrypsin	1.64	1.50	0.045	0.100
trypsin	1.62	1.38	0.077	0.234

[a] Determined at pH 7.8, 22 °C. Uncertainties in T_1 and T_2 measurements were ± 3% and ±6%, respectively.

Results and Discussion

Relaxation Experiments on DIP- and MIP–Enzymes. As a function of pH, the DIP–enzymes can be converted to the monoisopropylphosphoryl or MIP–enzymes by the process sometimes called "aging" (Van der Drift, 1983; Van der Drift et al., 1985). The DIP and MIP derivatives give rise to ^{31}P chemical shifts that are readily differentiated even at 81 MHz. Prior to the determination of the effect of the paramagnetic ion on the phosphorus relaxation, T_1 and T_2 were determined for the DIP and MIP derivatives of all three serine proteases at the same temperature and all at pH 7.8 (Table I). The relaxation times for the DIP and MIP derivatives were determined on partially aged samples in the same NMR tube; i.e., all experimental conditions were identical for the MIP and

DIP derivatives of the same enzyme. Also, the molecular weights of the MIP and DIP derivatives are very nearly the same. These factors enable us to draw some conclusions about the relative mobilities at the active centers in the two types of derivatives.

The relaxation times are proportional to the correlation time τ_c, which in turn has contributions from the rotational correlation time τ_r, the electron spin relaxation time τ_s, and the lifetime of the nucleus in the bound state τ_m, related to the exchange rate. The smallest of these correlation times dominates τ_c (Dwek, 1973; James, 1975). Under the conditions of our experiments, i.e., a diamagnetic system and a nonexchanging phosphorus nucleus, the electron spin relaxation time and the exchange can be assumed to make negligible contributions to τ_c. The rotational correlation time τ_r therefore dominates or is essentially equal to τ_c. The relaxation times can therefore be interpreted in terms of the mobility at a particular site. From the relationship of relaxation times and correlation time τ_c, and the observed T_1/T_2 ratios (Bloembergen & Purcell, 1948; Gutowsky, 1975), one can deduce that the condition $\omega_I\tau_c \geq 1$ holds. This condition predicts higher mobility for the environment exhibiting lower T_1 and higher T_2. Referring to Table I, this condition holds uniformly for the MIP derivatives (compared to the DIP ones) for all three enzymes examined. It may well be true for other serine proteases as well. The lower mobility in the DIP derivatives may be due to steric hindrance to rotation of the more bulky diisopropylphosphoryl group. The results also suggest that the $-+-$ charge distribution in the MIP active-center triad (the MIP is a phosphodiester with a negative charge that very much stabilizes the adjacent $HisH^+$; Adebodun & Jordan, 1989) does not constrain the mobility compared to the $-+$ or -0 charge distribution found in the DIP analogues and in the native enzyme. Given that the MIP analogue is a better structural analogue of the oxyanionic tetrahedral intermediate, and preceding transition state, than the DIP analogue (which at least in charge distribution is a ground-state mimic), one would predict that there is mobility in both the rate-limiting transition state and the subsequent oxyanionic tetrahedral intermediate.

Elsewhere it was reported (Adebodun & Jordan, 1989) that the pK_a's of the active-center His in the MIP derivatives are 9.7 for trypsin, 10.3 for α-chymotrypsin, and 11.4 for subtilisin. Employing the reasoning from above, one would be tempted to speculate that, among the MIP derivatives examined, the phosphorus enjoys the greatest mobility at the MIP–trypsin active center, followed by the MIP–α-chymotrypsin and the MIP–subtilisin. Furthermore, the greater mobility in these putative "transition-state analogues" is associated with lower pK at the His, and on the relevant reaction pathway perhaps a less "tight" oxyanionic intermediate, and preceding transition state.

Experiments on Gd(III)–DIP–Enzymes. (1) Theoretical Framework. The paramagnetic contributions to the relaxation times T_1 and T_2 of a spin $^1/_2$ nucleus bound near a paramagnetic ion are described by the Solomon–Bloembergen equations (Solomon, 1955; Bloembergen, 1957) and elaborated for the case in point by several key contributors (Mildvan & Cohn, 1970; Dwek, 1973; James, 1975; Inagaki & Miyazawa, 1981):

$$\frac{1}{T_{1,M}} = \frac{2}{15}\frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right) + \frac{2}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\left(\frac{\tau_e}{1+\omega_S^2\tau_e^2}\right) \quad (3)$$

and

$$\frac{1}{T_{2,M}} = \frac{1}{15}\frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6}\left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_S^2\tau_c^2}\right) + \frac{1}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\left(\frac{\tau_e}{1+\omega_S^2\tau_e^2} + \tau_e\right) \quad (4)$$

where γ_i is the magnetogyric ratio of the nucleus, S is the electron spin quantum number, g is the electronic Lande g factor, β is the Bohr magneton, ω_I and ω_S are the Larmor angular precession frequencies for the nuclear and the electron spin, respectively, r is the distance between the nucleus and the paramagnetic ion, A/h is the electron-nuclear hyperfine coupling constant, and τ_c and τ_e are the correlation times modulating the dipolar and scalar interactions, respectively. The first term in eq 3 and 4 represents the dipolar contribution that is a function of the distance between the ion and the nucleus. The second term represents the scalar contribution. Since the nucleus of interest in this study (^{31}P) is not directly bonded to the paramagnetic ion, the scalar contribution is assumed negligible. The correlation times in eq 3 and 4 are given by

$$1/\tau_c = 1/\tau_s + 1/\tau_r + 1/\tau_m \quad (5)$$

$$1/\tau_e = 1/\tau_s + 1/\tau_m \quad (6)$$

where τ_s is the electron spin relaxation time, τ_r is the rotational correlation time of the bound paramagnetic ion, and τ_m is the lifetime of the paramagnetic ion in the complex. Under the conditions of fast exchange of the ion between the free and bound forms, the observed relaxation enhancement $(1/T_p)$ is related to the relaxation in the first coordination sphere of the paramagnetic ion by the equation

$$1/T_{ip} = (1/T_i)_a - (1-f/T_i)_b = f/(T_{im}+\tau_m) + (1/T_i)_{os} \qquad (i = 1 \text{ or } 2) \quad (7)$$

In these equations $(1/T_i)_a$ and $(1/T_i)_b$ are experimentally observed relaxation rates in the presence and absence, respectively, of paramagnetic ion; T_{im} is the relaxation time in the first coordination sphere of the paramagnetic ion (*note*: while in general this definition would hold, at the distances estimated from our experiments, one can only speak of the "sphere of influence" of the paramagnetic ion); f is the mole fraction of the bound ligand; τ_m is the residence time of the nucleus in the first coordination sphere; and $(1/T_i)_{os}$ is the outer-sphere contribution to the relaxation rate. Under conditions of fast exchange (i.e., $\tau_m \ll T_m$) and negligible outer-sphere relaxation, eq 5 reduces to

$$1/T_{im} = 1/fT_{ip} \quad (8)$$

If the condition $\omega_i\tau_c \geq 1$ holds, or if τ_c values longer than 8×10^{-10} s/rad determine the relaxation of the nucleus under investigation (i.e., values within a frequency-independent region), τ_c can be evaluated from the ratio T_{1m}/T_{2m}, assuming that the contribution of the hyperfine terms in eq 3 and 4 is negligible (Dwek, 1973; Abbott et al., 1975a,b).

(2) Measurements of Relaxation Enhancements. The possibility that $1/T_{1p}$ is dominated by outer-sphere relaxation rather than by T_{1m} can be ruled out if $T_{1p} > (7/6)\ T_{2p}$ (Mildvan & Cohn, 1970). These ratios for the three cases studied here were much greater than 7/6 (Table II). Therefore, outer-sphere relaxation is unlikely to dominate $1/T_{1p}$. The dependence of relaxation rate enhancement $1/T_{1p}$

Table II: Paramagnetic Contributions to Relaxation Rates and Estimated P···Gd(III) Distances in DIP–Enzymes

enzyme	$1/T_{1,M}$[a] (s^{-1})	$1/T_{2,M}$[a] (s^{-1})	τ_c[b] (ns/rad)	distance[c] (Å)
subtilisin	0.738	42.83	18.1	20.4 ± 1
α-CHT	0.788	29.20	14.4	21.0 ± 1
trypsin	1.128	20.82	10.0	20.9 ± 1

[a] Estimated error is ±3% on T_1 and ±6% on T_2 based on standard deviation on relaxation time measurements. [b] Maximum error is 4.6%. [c] With all uncertainties such as the magnitude of the binding constant, the uncertainty in distance is not larger than ±1 Å. As an example, a 10% error on $1/T_{1,M}$ would lead to 0.4-Å uncertainty in distance, and even a 50% error in the $1/T_{1,M}$ would lead to no more than a maximum error of ±2.5 Å on the distance estimate.

on Gd(III) concentration is essentially stoichiometric. The plots of relaxation rate enhancements $1/T_{1p}$ and $1/T_{2p}$ against the molar ratio of [Gd(III)]/[enzyme] were all linear with slopes for $1/T_{1p}$ of 0.154, 0.123, and 0.376 for DIP–subtilisin, DIP–chymotrypsin, and DIP–trypsin, respectively. For $1/T_{2p}$ the slopes were 8.12, 5.19, and 5.22 for DIP–subtilisin, DIP–chymotrypsin, and DIP–trypsin, respectively. All lines passed through the origin. These data suggest that the bulk paramagnetic effect of unbound Gd(III) is negligible and the contribution from the outer-sphere relaxation of the bound Gd(III) can be neglected. Since the condition $T_1/T_2 \gg 7/6$ holds, $1/T_{1p}$ may be considered not to be limited by exchange. When La(III) was used as a diamagnetic control at 1.8 mM concentration, it did not produce any relaxation enhancement. Using the binding constant of 2500 M^{-1} for the assocation of Gd(III) to the enzyme (Abbott et al., 1975a,b), T_{1m} and T_{2m} were calculated for each enzyme and are presented in Table II. Also presented in Table II are the τ_c values. From eq 1 and the values of $1/T_{1m}$, τ_c, ω_I (5.09×10^8 rad/s), $S(^7/_2)$, and the electronic g factor (2.0), the P to Gd(III) distances were calculated to be 20.9 ± 1 Å for DIP–trypsin, 21.0 ± 1 Å for DIP–α-chymotrypsin, and 20.4 ± 1 Å for DIP–subtilisin. Before the addition of the paramagnetic ion, T_1 and T_2 were measured several times in the DIP–enzymes to establish the uncertainty in the determinations. During the time required for the relaxation time measurements it was sometimes found that the DIP–subtilisin underwent hydrolysis (aging) to the monoisopropyl analogue. For this reason, on this derivative alone data were collected for predominantly aged and predominantly unaged (albeit fewer data point) samples. The distances calculated for the monoisopropyl- and the diisopropylsubtilisin were identical within experimental error. On DIP–chymotrypsin and DIP–trypsin there was no need to perform such dual experiments.

While the distances here estimated are within the upper limit of the distances accessible from relaxation measurements, the distance up to 21 Å is accessible if accurate relaxation times are used. The relatively small uncertainties of 3% for T_1 and 6% for T_2 give us confidence in the distance estimates.

(3) Modeling of the Gd(III) onto the DIP–Enzymes. The participation of carboxylic acid side chains in the Ca(II) binding site is strongly supported by the results of numerous experiments, including X-ray crystallography. Chemical modification of 13 of 14 carboxylates in α-chymotrypsin and 10 of 11 in trypsinogen resulted in the loss of Ca(II) binding ability, and consequently in a loss of protection from autolysis (Abita & Lazdunski, 1969). The pH dependence of Ca(II) binding was also suggestive of the contribution of some carboxylate ligands (Epstein et al., 1974). A survey of Ca(II) binding sites in proteins by Delaage and Lazdunski (1967), Epstein et al. (1977), Kretsinger (1976), Argos (1977), Serpersu et al. (1987), and Vyas et al. (1987) suggests a binding site that consists of at least two carboxylate side chains.

Table III: Carboxylate to Phosphorus Distances[a] in the DIP–α-Chymotrypsin Complex

carboxyl residues	distance from P (Å)	carboxyl residues	distance from P (Å)
Glu20	20.75	Glu78	23.47
Glu21	23.17	Asp102	6.12
Asp35	13.63	Asp128	25.46
Glu49	28.20	Asp129	25.52
Asp64	12.73	Asp153	19.41
Glu70	16.9	Asp178	22.01
Asp72	21.72	Asp194	9.17

[a] Measured from X-ray coordinates of Tsukada and Blow (1985).

Table IV: Carboxylate to Phosphorus Distances[a] in the DIP–Trypsin Complex

carboxyl residues	distances from P (Å)	carboxyl residues	distances from P (Å)
Glu70	19.09	Asp153	18.30
Asp71	20.51	Asp165	21.39
Glu77	23.61	Glu186	25.40
Glu80	23.62	Asp189	11.42
Asp102	5.80	Asp194	9.17

[a] Measured from X-ray coordinates of Bode et al. (1975).

A model of the DIP–α-chymotrypsin complex was constructed by using the coordinates of Tsukada and Blow (1985). The distance between the active-site phosphorus atom and each carboxyl group in the enzyme was measured (Table III). The measured intercarboxylate distances narrowed the possible Ca(II) binding locus to one. This region, approximately 20 Å from the phosphorus atom of the inhibitor, is unique in the sense that it is the only one found in this structure with more than one carboxylate clustered. Possible ligands, with their distances measured in angstroms from the P atom in parentheses, are Glu70 (16.9), Asp72 (21.7), Asp153 (19.4), and Glu78 (23.5). When Ca(II) is fitted at a distance of 2.4 Å from the ligands, the distance measured between this Ca(II) and the P atom is 20.3 Å; it compares well with the 21-Å value derived from the relaxation measurements in solution.

By use of the X-ray coordinates of Bode et al. (1983) a model of trypsin with DIP attached at Ser195 was constructed, and the distance between each carboxylate and the P atom was measured (Table IV). The measurement of intercarboxylate distances [2 × 2.4 or 4.8 Å was used as a limit on the basis of a 2.4-Å oxygen to Ca(II) distance; for a very recent compilation, see Carrell et al. (1988)] narrowed the choice of clustered carboxylates to one. This region is also approximately 20 Å from the active-center P atom. The likely ligands, with their distances from the active-center P measured in angstroms in parentheses, are Glu70 (19.1), Asp71 (20.5), Glu77 (23.6), and Glu80 (23.6). Placing the Ca(II) in this cluster at 2.4 Å from the carboxylates gave a Ca(II) to P distance of 20.3 Å, compared to the 20.9 Å deduced from the NMR experiments. The X-ray studies by Bode and Schwager (1975) listed side chains 70, 72, 77, and 80 as Ca(II) binding ligands.

To construct the DIP–subtilisin model, the coordinates of Drenth et al. (Drenth et al., 1971, 1972; Hol, 1971) and Kraut's group (Alden et al., 1971) were used. Both groups determined the structure of the enzyme isolated from the BPN′ strain. Table V lists the distances measured between the P at the active center and all carboxylates. There is only one cluster of carboxylates found. Possible ligands, with their distances from the P atom measured in angstroms in parentheses, are Asp36 (18.0), Asp41 (18.5), Glu54 (18.3), and

Table V: Carboxylate to Phosphorus Distances[a] in the DIP–Subtilisin Complex

carboxyl residues	distances from P (Å)	carboxyl residues	distances from P (Å)
Asp32	7.25	Asp120	22.84
Asp36	17.95	Asp140	24.32
Asp41	18.50	Glu156	15.98
Glu54	18.33	Asp181	14.79
Asp60	12.45	Glu195	18.82
Asp61	18.37	Asp197	15.97
Asp98	13.72	Asp259	22.80
Glu112	24.50		

[a] Measured from X-ray coordinates of Drenth et al. (1972).

Asp61 (18.4). The early X-ray study by Hol et al. identified two metal ion binding sites: one of them tighter near Asp41 and the other in the proximity of Asp197. While the former is indeed about 20 Å from the P atom, the latter is only 16 Å away and is inconsistent with the NMR results. More recent higher resolution X-ray studies on subtilisin have also confirmed Asp41 to be directly complexed to the Ca(II) and also suggested that the other ligands around Ca(II) are either main-chain or side-chain amide carbonyls (Bode et al., 1987; Bott et al., 1988; McPhalen & James, 1988). A measurement of intercarboxylate distances indicates that only Asp61 and Glu54 would be close enough to each other to act as ligands simultaneously. When the Ca(II) is fitted into the DIP–subtilisin model with two oxygens from Asp41 and carbonyl oxygens from Leu75, Val81, and Asn77 and a Ca(II) to O bond length of 2.4 Å, the distance between this Ca(II) and the P atom on Ser195 is 20.3 Å, again in excellent agreement with the NMR data. It is indeed perhaps more than coincidental that the most likely Ca(II) binding sites are in essentially identical places on chymotrypsin and trypsin. What is equally interesting is the apparent conservation of Ca(II) to active-center distance in all three enzymes, including a bacterial protease. Asp41 is highly conserved on all subtilisins whose primary sequence has been determined.

While the results are in excellent agreement with X-ray data, they are at variance with previous relaxation enhancement and fluorescence energy transfer experiments that gave a distance of 10 Å between the active site and Ca(II) in trypsin (Abbott et al., 1975a,b; Darnall et al., 1976). On the basis of this distance the authors suggested that the binding site is composed of Asp194 and Ser190—these are approximately 10 Å from the active site. Those reports were subsequently criticized (Epstein & Reuben, 1977; Epstein et al., 1977), pointing to the fact that with the large amounts of Ca(II) present in the lanthanide studies the primary binding site may have been saturated with Ca(II) and the distances measured referred to weaker binding sites occupied by Gd(III). More recent solution ENDOR spectroscopic studies on Gd(III) ion attached to chymotrypsin indicated the presence of carboxylate ligands, consistent with X-ray results on trypsin (Yim & Makinen, 1986). The present approach offers one very distinct advantage over the previous studies: the distances are measured to a "nonexchanging target"; this leads to greatly reduced uncertainty in the distance calculations. A very recent publication compared paramagnetic ion induced shifts and relaxation as approaches to distance estimates in model systems and concluded that the relaxation measurements lead to more reliable values (Kemple et al., 1988).

^{113}Cd Experiments on the Native Enzymes. Cd(II) is a suitable structural analogue for Ca(II) in a number of Ca(II) binding proteins, including serine proteases (Abbott et al., 1975a,b; Epstein et al., 1977; Armitage & Otvos, 1982; Drakenberg et al., 1978; Forsen et al., 1979, 1980; Armitage & Boulanger, 1983; Anderson et al., 1983; Kingsley-Hickman et al., 1986). The ionic radii for Cd(II) and Ca(II) are 0.97 and 0.99 Å, respectively. When the exchange of the Cd(II) between the free and bound form(s) is slow on the NMR time scale, distinct Cd(II) resonances are observed. Because of the favorable spin of this ion ($^1/_2$), on enzymes the size of serine proteases here studied (24 000–28 000 daltons), relatively narrow line widths can be anticipated, making the observation of tightly bound ions feasible.

In order to bring the system into a slow-exchange regime, we performed variable-temperature ^{113}Cd magnetic resonance experiments at single conditions on the native enzymes in the presence of a large excess of Cd(II). Conditions were found that allowed us to observe bound Cd(II) signals for all three systems. Figure 1 presents a ^{113}Cd(II) spectrum for 2 mM trypsin, pH 6.6 at 280 K, in the presence of 7.5 mM ^{113}Cd(II). Figure 2 presents a spectrum for α-chymotrypsin employing

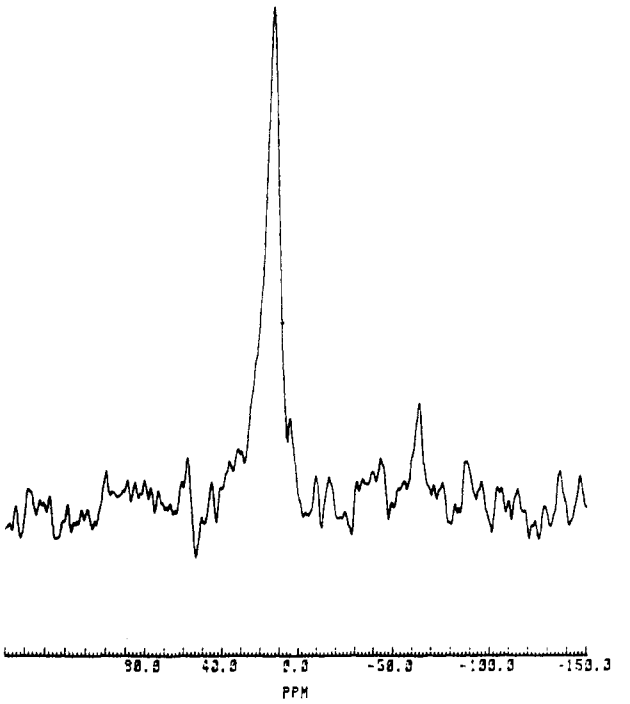

FIGURE 1: ^{113}Cd NMR spectrum of a solution containing 2.0 mM trypsin and 7.5 mM cadmium-113 ion at pH 6.6 and 280 K; 64 000 transients were accumulated.

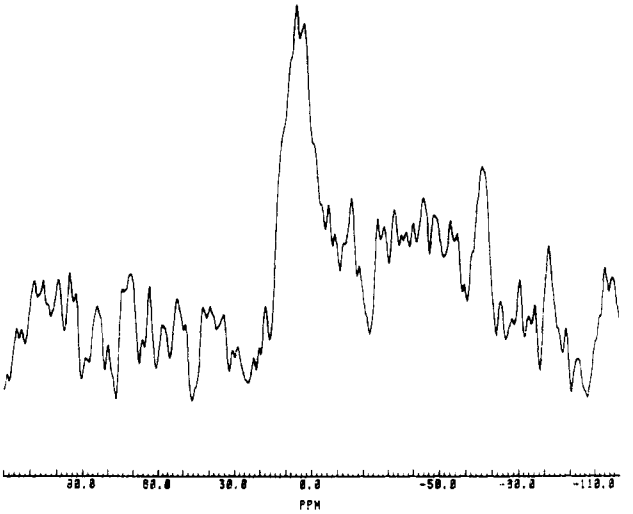

FIGURE 2: ^{113}Cd NMR spectrum of a solution containing 2.0 mM α-chymotrypsin and 7.5 mM cadmium-113 ion at pH 6.6 and 280 K; 48 000 transients were accumulated.

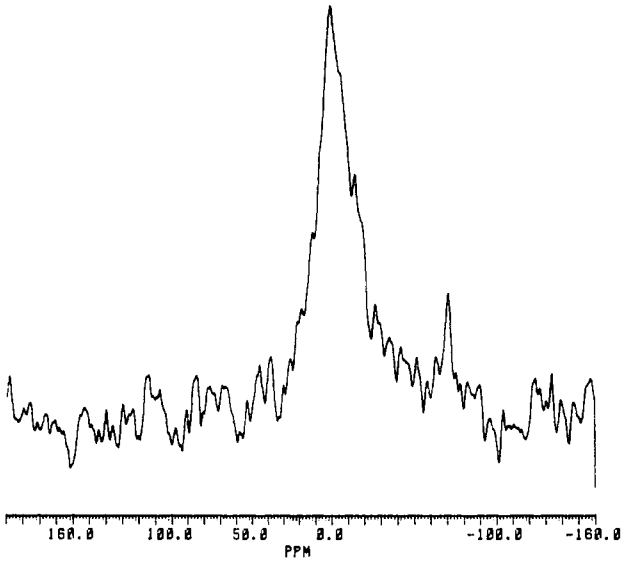

FIGURE 3: ^{113}Cd NMR spectrum of a solution containing 2.0 mM subtilisin and 7.5 mM cadmium-113 at pH 6.6 and 268 K; 64 000 transients were accumulated.

the same concentrations, temperature, and pH. Figure 3 presents a $^{113}Cd(II)$ spectrum for 2.0 mM subtilisin in the presence of 7.5 mM $^{113}Cd(II)$, pH 6.6 at 268 K, and also with 2 *m* $NaClO_4$ to suppress the freezing point.

To demonstrate that the smaller signal is indeed due to Cd occupying a Ca site, a direct competition experiment was performed. The spectrum of 2.0 mM DIP–trypsin was recorded at 278 K with excess (7.5 mM) ^{113}Cd and then rerecorded in the presence of 18 mM Ca(II). As Figure 4 indicates, the smaller signal (at −60 ppm) that we have attributed to tightly bound Cd in the above experiments is no longer visible in the presence of a large excess of Ca(II) ions. The chemical shift of the "free" Cd is different in this experiment because of the presence of Cl^- rather than ClO_4^- in the experiments depicted in Figures 1–3. This experiment also confirms several other points. The DIP–enzymes and the native enzymes have very similar Cd (and presumably Ca) binding sites. The relative ratios of bound and free Cd in Figures 1–3, compared to that in Figure 4, demonstrate that, during the long spectral acquisition time required for the Cd experiments, the native enzyme probably underwent significant autolysis.

Under the conditions shown there is a tightly bound Cd in each spectrum: at −67 ppm for trypsin, −66 for chymotrypsin, and −70 for subtilisin [all upfield from external 0.1 M $Cd(ClO_4)_2$]. There is also a broad larger signal due to the exchange of Cd(II) among a number of weak sites and the unbound form of free Cd(II). The chemical shift of the bound signal is characteristic of Cd bound to oxygen ligands (Haberkorn et al., 1976; Armitage & Boulanger, 1983) in an octahedral environment (Dean, 1981; Armitage & Boulanger, 1983).

Conclusions

The paramagnetic relaxation enhancements led to distances between Gd(III) and the active-center P atom in the DIP–enzymes that are in excellent agreement with crystallographic data, where available. The approach has clear utility to similar enzymes in which there is no flood of X-ray data yet (as seems to be the case for subtilisins). The measurements on a covalently bound rather than a rapidly exchanging nucleus make the relaxation measurements easier to perform and, apparently, more reliable. Preparation of the DIP–enzymes is straightforward. The novelty of the Cd results is the ability to observe and characterize tight metal binding loci on these enzymes in solution—a task not easily achieved by any other current technique. The fact that a lower temperature was required to enable observation on subtilisin, than on trypsin and chymotrypsin, probably reflects the higher affinity in the latter two due to the presence of two carboxylate ligands compared to only one in subtilisin. The two techniques here explored should be of great utility in defining the Ca binding properties of mutant serine proteases, currently a topic of intense research both in industrial and in academic laboratories. The paramagnetic ion induced relaxation measurements at a covalently attached label to establish metal to active-center distances have applicability to a large number of metalloproteins.

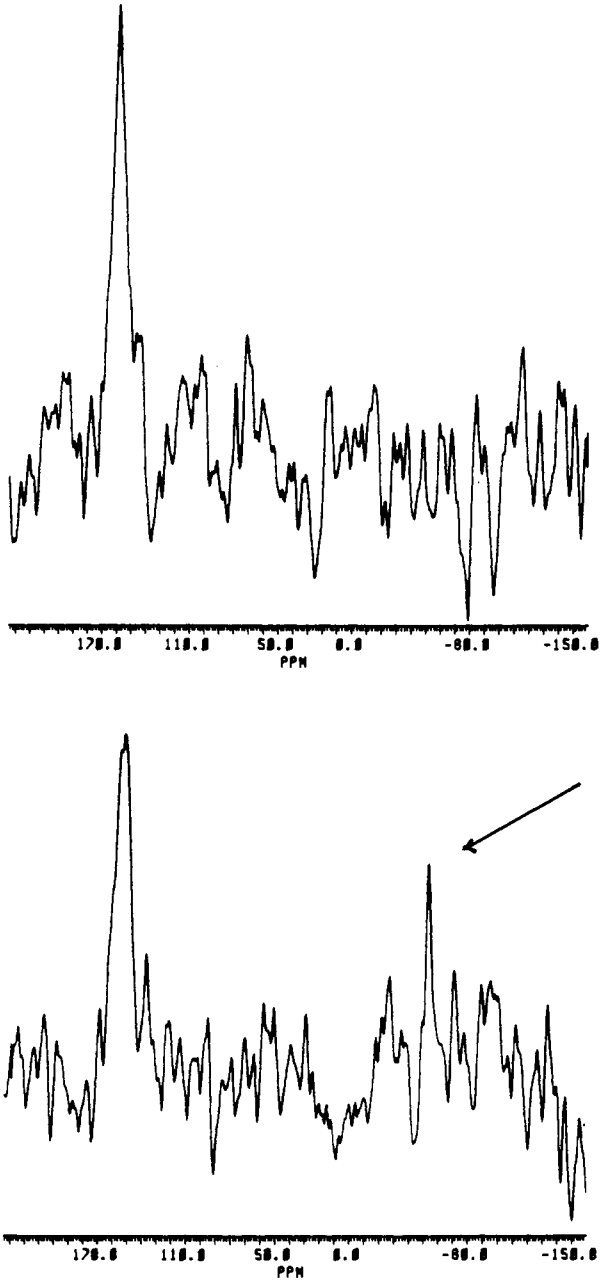

FIGURE 4: ^{113}Cd NMR spectrum of a solution containing 2.0 mM DIP–trypsin and 7.5 mM cadmium-113 at pH 7.6 and 278 K before (bottom spectrum) and after (top spectrum) addition of 18 mM Ca(II); 24 000 scans were accumulated for each spectrum. The arrow in the bottom spectrum points to the tightly bound Cd signal.

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Differences in the Binding of Aromatic Substrates to Horseradish Peroxidase Revealed by Fluorescence Line Narrowing†

Judit Fidy,*,‡ K.-G. Paul, and Jane M. Vanderkooi

Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6059, and Department of Physiological Chemistry, University of Umea, Umea, Sweden

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ABSTRACT: The heme in horseradish peroxidase (HRP) isoenzyme C was replaced by mesoporphyrin (MP), and the binding effect of the aromatic substrates benzo- and naphthohydroxamic acid (BHA, NHA), resorcinol (RE), isomeric resorcylic acids (α-, β-, γ-RE), and hydroquinone (HQ) was studied at pH 5 by conventional and laser-excited fluorescence spectroscopy on the basis of the signal of the porphyrin. Under laser excitation at cryogenic temperatures site selection was demonstrated, and the fluorescence line narrowing data were used to characterize the HRP/substrate complexes by the inhomogeneous distribution function for the $S_0 \leftarrow S_1$ ($0 \leftarrow 0$) transition energy and the vibrational energies in the S_1 electronic state. A comparison with ground-state vibrational energies for MP in chloroform/ether showed a downward shift in vibrational energies for S_1 by $\approx$20 cm^{-1}. The association characteristics of the substrates were in accordance with previous literature data indicating NHA to be of the strongest binding affinity. For BHA, spectral evidence was obtained for a second type of binding site where hydrophobic interactions with the porphyrin ring may be possible. The effect of the RE's was similar to each other, but only β-RE showed saturation. Complexation in every case caused the strong reduction of the splitting in the $0 \leftarrow 0$ transition energy for the tautomeric forms of MP and an increase in the $0 \leftarrow 0$ energy by 100–200 cm^{-1} depending on the substrate. The substrate binding also affected the phonon coupling of vibronic transitions exciting into the $\Delta\nu$ = 927- and 976-cm^{-1} modes; in the latter case, the vibrational energy was also increased to 983 cm^{-1} for β-RE. In the same energy range, however, the transition into the $\Delta\nu$ = 958–960-cm^{-1} mode was not affected by binding. Both the magnitude of the energy shifting and the change in the strength of phonon coupling gave the same relation, BHA < NHA < HQ < RE's, indicating a common conformational origin. A reduction of the fluctuational freedom of the protein chain at room temperature within the heme pocket was suggested on the basis of the reduction of the width of the inhomogeneous distribution of $0 \leftarrow 0$ energies (from 60–70 to $\approx$30 cm^{-1} in case of HRP/HQ) upon substrate binding. Ways to relate the transition energy splitting and shifting effects to conformational changes are discussed by invoking the Jahn–Teller effect.

Horseradish peroxidase C2 is a heme glycoprotein, one of at least seven isoenzymes catalyzing the oxidation of indoleacetic acid and other aromatic compounds in plant roots by hydrogen peroxide. The reaction proceeds in several steps involving interactions with different hydrogen donors in the intermediate states of the enzyme. Experimental results indicate that the native enzyme forms a 1:1 complex with aromatic donor molecules and that different substrates bind at the same site within the heme crevice (Schoenbaum, 1973; Leigh et al., 1975; Schejter et al., 1976; Paul & Ohlsson, 1978; Morishima & Ogawa, 1979). In these works it was also suggested that the binding of the aromatic donors involves hydrophobic interaction with aromatic residues of the protein and that the donor is at a distance of 5.8–11.2 Å from the center of the heme. However, the binding site and the nature and geometry of the binding process have not yet been identified. The most recent studies, performed by NMR spectroscopic techniques and computer simulation procedures, revealed that the binding site involves a tyrosine residue in the vicinity of the porphyrin peripheral 8-methyl (Sakurada et al., 1986) and 7-propionate (Thanabal et al., 1987) groups; however, these results somewhat contradict the model based on resonance Raman data (Oertling & Babcock, 1988).

Although a series of results showed somewhat uniform behavior in the binding of a great number of ligands, difference absorption measurements indicated different types of changes in the Soret band of the heme due to binding of various substrates. To help understand the reason for this, two categories were suggested (Sakurada et al., 1986), represented by resorcinol and 2-methoxy-4-methylphenol, respectively, but not all published results seem to fit into this picture [e.g., see Schoenbaum (1973)].

In recent years, we have successfully applied the high-resolution laser spectroscopic technique, fluorescence line narrowing, for small monomeric heme proteins, and experimental evidence was presented and discussed for the fulfillment of the

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* Address correspondence to this author.
‡ On leave from the Institute of Biophysics, Semmelweis Medical University, H-1088, Puskin utca 9, Budapest, Hungary.

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