Characterization of Enzyme-Bound Ligand Dynamics by Solid-State NMR in the Presence of Ligand Exchange: L-Phenylalanine on Carboxypeptidase A

Huiming Zhang and Robert G. Bryant
Department of Chemistry, University of Virginia, Charlottesville, Virginia USA 22901

ABSTRACT Deuterium NMR spectra were obtained for L-phenylalanine-d5, deuterated on the phenyl ring, in cross-linked polycrystalline samples of carboxypeptidase A containing different amounts of water. The deuterium powder pattern line shapes are simulated by extension of the theory to include both a local reorientational motion of the bound L-phenylalanine phenyl ring and exchange of the L-phenylalanine with an intracrystalline isotropic environment. The spectral simulations are consistent with the phenyl ring of the phenylalanine executing π-flips in the bound environment at rates that vary from 3×10^4 Hz at 6% water content to 1×10^5 Hz at 21% water content. At all water contents studied, the ligand exchanges with an essentially isotropic environment in the crystal with a rate constant of $\sim 2.5 \times 10^{-3}$ Hz. Although the dissociation constant for the L-phenylalanine is only 18 mM, the spectral simulations that reproduce the experimental line shape well do not require significant wobble of the phenyl ring rotation axis, which is consistent with the binding interactions identified by x-ray crystallography.

INTRODUCTION

The structures reported for enzymes or even enzyme-substrate complexes do not generally provide a picture of how the catalytic event occurs, because the necessary catalytic groups are not arranged properly (Mangani and Orioli, 1992a, b). Motion within the enzyme-ligand complex in addition to structural alterations on binding are very likely. Although molecular dynamics simulations provide a stimulating picture of short time structural fluctuations, it is difficult with present technology to extend these simulations to times on the order of the turnover times of most enzymes (Karplus, 1990). X-ray methods provide a superposition of static structures that may be examined for a characterization of rms structural fluctuations, but the time scale of the motions is not clearly revealed. However, nuclear magnetic resonance (NMR) spectroscopy, in particular solid-state NMR spectroscopy, may provide a useful and direct observation of local structural reorientation of protein segments or bound small molecules (Griffin, 1981; Torchia, 1984; Speiss, 1985; Opella et al., 1987; Griffin et al., 1988). There has been considerable effort to characterize protein dynamics using solid-state NMR. The difficulty of selecting a particular residue may now be overcome with elegant site-specific labeling methods. An alternative approach to characterizing the relative motion of the enzyme and the ligand is to examine the motion of the small molecule when bound to the enzyme. The difficulty or opportunity in this case is that the enzyme-bound ligand is not necessarily covalently linked to the protein and may therefore exchange with another environment including an isotropic or unbound environment. In this case, chemical exchange averaging of the line shape must be considered in detail.

We examine carboxypeptidase A because it is structurally and mechanistically well characterized by a considerable variety of methods (Hartsuck and Lipscomb, 1971; Rees et al., 1981a, b, 1983; Vallee et al., 1983; Christianson and Lipscomb, 1985, 1986; Williams and Auld, 1986; Auld and Vallee, 1987; Bicknell et al., 1988; Bertini et al., 1988a, b; Luchinat et al., 1988; Christianson et al., 1989; Christianson and Lipscomb, 1989; Martinell et al., 1989; Bal et al., 1990; Bertini et al., 1990a, b; Kim and Lipscomb, 1990, 1991; Mangani and Orioli, 1992a, b; Zhang et al., 1992). The active site contains a number of interesting features including a zinc ion, unique water molecules, and a hydrophobic binding domain. We examine the small molecule L-phenylalanine because it binds weakly, which is an advantage for our present purposes; the deuterium label provides a simple solid-state NMR spectrum; the weak binding permits testing of the effects of the exchange on the observable anisotropic spectrum. The problem addressed in an earlier manuscript (H. Zhang and R. G. Bryant, submitted for publication) is that, when the labile ligand exchanges between a bound and anisotropic environment, the active site of carboxypeptidase A in this case, and an isotropic environment, the effects of the exchange averaging process on the observed spectrum may depend on the nature of the long-range order of the bound environment. The solid-state spectrum with the information carried by the preservation of chemical shift and nuclear electric quadrupole or dipole anisotropies may be realized if the reorientational motion of the protein is slow compared with the size of the couplings investigated. These constraints may be achieved in the dry state, which may be criticized for lack of water that is key in generating the native structure and undoubtedly native structural fluctuations. A

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Address reprint requests to Robert G. Bryant, Department of Chemistry, University of Virginia, McCormick Road, Charlottesville, VA 22901. Tel.: 804-924-1494; Fax: 804-924-3710; E-mail: RGB4G@VIRGINIA.EDU.
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cross-linked gel is also adequate; however, in the case where a labile system is studied, the apparent motional averaging of the spectrum caused by the exchange of the bound ligand may suppress the usual anisotropic information contained in the NMR spectrum if the exchanging ligand samples too many different orientations of the enzyme in the unordered gel. We simplified this situation by studying the crystalline or semicrystalline environment where the long-range order of the protein orientations is maintained over the sampling dimensions permitted by the exchange of the ligand or the ligand with the enzyme.

MATERIALS AND METHODS

Carboxypeptidase A (Allan type, from Sigma Chemical Co., St. Louis, MO) was crystallized and cross-linked by the method described elsewhere (Christianson et al., 1989). The carboxypeptidase-L-Phe-d5 complex was prepared by soaking cross-linked carboxypeptidase A crystals in 20 mM L-Phe-d5 (Cambridge Isotope laboratories, Woburn, MA), 20 mM Tris buffer at pH 7.4 for 1 week. These crystals were washed with 20 mM Tris buffer, pH = 7.4, three times, the supernate discarded, and the wet crystal dried in vacuo for 30 min. Then the sample was placed in a jar for a week over a saturated salt solution, which was used to control the humidity of the moist environment. The salts used were calcium chloride hexahydrate, ammonium sulfate, and copper sulfate pentahydrate. The binding for L-phenylalanine is reported to be 18 mM (Christianson et al., 1989). If this equilibrium is not significantly altered by the change in conditions, the concentration of bound ligand should be ~50%.

The water contents were determined experimentally by measuring the weight loss of hydrated protein after drying under mechanical vacuum in a drying pistol for 24 h using isopropanol (b.p. 81°C) as refluxing solvent.

All NMR experiments were performed with a Varian Unity-Plus NMR spectrometer (Varian Associates, Inc., Palo Alto, CA) operating at 76.7 MHz for deuterium with a home-built ^{2}H probe that employed a 7-mm solenoidal coil positioned perpendicular to the field that had a typical 90° pulse width of 2.8 μs using the 300-W rf amplifier. The solid-echo sequence used an echo delay of 35 μs and recycle delay times between 0.5 s and 2 s (Davis et al., 1976). Longer delays did not improve the spectrum.

The numerical simulation routine employed was modified from the programs SPOWDER (Wittebort et al., 1987) and the numerical routine for two-site exchange in heterogeneous phases from the program QEXCH (Zhang and Bryant, submitted). If we turn off reorientation motion, this program is equivalent to QEXCH; if we turn off the chemical exchange effects in addition, it is equivalent to SPOWDER. 2048 points were used in the simulation with a line broadening of 1 kHz.

Theory

Deuterium spectra for rotationally immobilized materials in the presence of local internal motions have been discussed in detail (Spiess, 1985, Wittebort et al., 1987). Here we use the approach of Wittebort et al. (1987). In a completely dry sample, only the local reorientation of ligand makes a contribution to the dynamical averaging of the spectrum. For an N-site jump process, the equation of motion in the Zeeman rotating frame for the transverse magnetization, M, (Wittebort et al., 1987) is

$$\dot{M}_i = \sum_{j=1}^{N} (i\omega_i\delta_{ij} + R_{ij})M_j \tag{1}$$

where the frequency of site i, ω_i, is orientation dependent and given by

$$\omega_i = \pm\frac{3}{4}\frac{e^2qQ}{\hbar}\sum_{m'=-2}^{2} D^{(2)}_{m'm}(\alpha\beta\gamma)V^{(2)}_m(\varphi\theta\phi) \tag{2}$$

where φ, θ, and ϕ are the Euler angles that define the orientation of the unique component of the electric field gradient (EFG) tensor in a molecular frame. Thus, $V^{(2)}_m(\varphi\theta\phi)$ are irreducible nuclear electric quadrupole tensor components in the molecular frame. The angles α, β, and γ specify the orientation of molecular frame with respect to the lab frame. $D^{(2)}_{m'm}(\alpha\beta\gamma)$ are the elements of the Wiger rotation matrix. R_{ij} are the elements of the exchange matrix,

$$\begin{aligned} R_{ij} &= \tau_c^{-1}(P_i/P_{i\pm1})^{1/2} && j = i \pm 1, \\ R_{ij} &= -(R_{i,i+1} + R_{i,i-1}) && j = i, \\ R_{ij} &= 0 && \text{otherwise} \end{aligned} \tag{3}$$

These elements satisfy the conditon of microscopic reversibility:

$$R_{ij}P_j = R_{ji}P_i \tag{4}$$

where P_i are equilibrium populations.

In the case in which a single site of bound ligand exchanges with the free ligand (Zhang and Bryant, submitted), the resonance frequency of bound ligand is defined by Eq. 2. The frequency of free ligand is set to 0 for convenience, and using the two-site model, the elements of exchange matrix are

$$R_{12} = k_{off} \qquad R_{21} = k_{off}\frac{C_{ES}}{C_S} \qquad R_{11} = -R_{12} \qquad R_{22} = -R_{21} \tag{5}$$

where C_{ES} is the concentration of total bound ligand, and C_S is the concentration of free ligand. Eq. 5 includes the condition of microscopic reversibility that is required by the ligand-binding equilibrium.

$$E + S = ES \tag{6}$$

$$K = \frac{k_{on}}{k_{off}} = \frac{C_{ES}}{C_E \cdot C_S} \tag{7}$$

To consider the reorientation of bound ligand and the exchange between bound and free ligand, we assume that Eqs. 5 and 6 are applicable to all possible orientations and sites of the bound ligand. That is, the requirement that each bound orientation simultaneously satisfy the condition of microscopic reversibility with the isotropic environment and other bound orientations has been handled by the simplifying assumption that the bound site populations are equal. Mathematically, this condition permits definition of a diagonal unitary matrix that can symmetrize the exchange matrix **R**. If the dimension of the exchange matrix is N, then there are $N - 1$ sites for bound ligand; the Nth site is the unbound ligand. Therefore, the exchange density matrix **R** has the generalized form:

$$\begin{aligned} R_{ij} &= k_1 && j = i \pm 1, \quad i,j \neq N, \\ R_{iN} &= k_2, && i \neq N \\ R_{Ni} &= k_2\frac{C_{ES}}{(N-1)C_S}, && i \neq N \\ R_{ii} &= -\sum_{j=i+1}^{N} R_{ij} - R_{i,i-1}, && i \neq N \\ R_{NN} &= -\sum_{i=1}^{N-1} R_{Ni} \\ R_{ij} &= 0 && \text{otherwise.} \end{aligned} \tag{8}$$

where k_1 is the reorientational rate, k_2 or k_{off} is the exchange rate, C_{ES} is the total concentration of bound ligand, and C_S is the concentration of free ligand. **R** may be symmetrized with the diagonal matrix **U**, where $U_{ii} = (P_i)^{-1/2}$, where P_i is the distribution population for the site i. Using the diagonalization routine of Wittebort et al. (1987), the eigenvalues of the matrix define the resonance frequencies after exchange. An echo sequence is generally used to collect solid deuterium spectra. Because the echo delay causes only negligible intensity loss and dephasing

for the isotropic part, the intensity scale factors are defined according to Eq. 3.19 in Wittebort et al. (1987).

$$I(\omega, \tau) = \int_0^{2\pi} \int_0^{\pi} I(\omega, \tau, \beta, \alpha) \sin \beta \, d\beta \, d\alpha \tag{9}$$

where

$$I(\omega, \tau, \beta, \alpha) = \mathrm{Re} \sum_{k=1}^{N} \frac{b_k}{\lambda_k - i\omega} \tag{10}$$

and

$$b_k = a_k \sum_m a_m^* \left(\sum_n X_n^{(k)} X_n^{(m)*} \right) \exp[(\lambda_m^{\pm} \lambda_k)]\tau \tag{11}$$

$$a_k = \sum_i (P_i)^{1/2} (X_i)^{(k)} \tag{12}$$

where λ_i and X_i are the eigenvalues and eigenvectors of the exchange density matrix $\mathbf{i\omega + R}$ (Wittebort et al., 1987).

RESULTS

The ^{2}H NMR spectra of L-Phe-d5 in cross-linked carboxypeptidase A crystals are shown in Fig. 1 for samples of different water content, and the corresponding simulated spectra are shown in Fig. 2. Spectrum a of Fig. 1 demonstrates that this sample with 6% water content (110 water molecules per enzyme) is dominated completely by motionally restricted features. There are three pairs of maxima corresponding to splittings of ±64.3 kHz, ±15.6 kHz, and ±4.2 kHz. As will be shown later, this spectrum may be simulated using a model where the enzyme-bound L-phenylalanine ring undergoes rapid π-flips.

Raising the water content of the cross-linked crystals to 11% yields spectrum b of Fig. 1. An isotropic peak at the 0 frequency is now apparent. The narrow central resonance

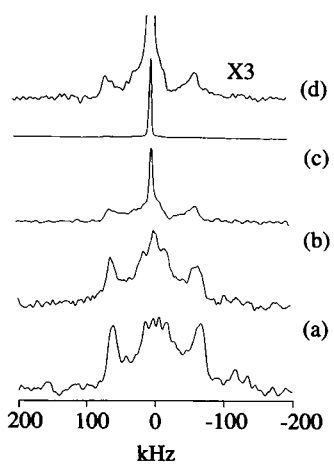

FIGURE 1 ^{2}H NMR spectra of carboxypeptidase A polycrystalline samples cross-linked with glutaraldehyde and soaked in a 20-mM solution of L-phe-d5 obtained at 11.7 T at different water contents. Spectra were obtained using ~200-mg samples, 60,000 to 128,000 transients, and a deuterium quadrupole-echo delay of 35 μs: (*a*) 6% water, 2-s recycle delay. (*b*) 11%, 2-s recycle delay, (*c*) 21%, 0.5-s recycle delay, (*d*) saturated crystals, 0.5-s recycle delay, and an expanded version of spectrum.

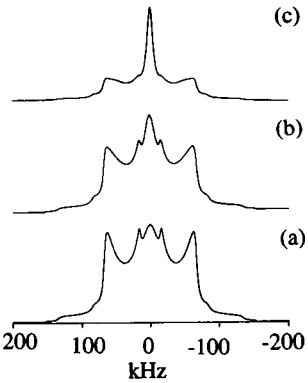

FIGURE 2 Simulation patterns for the L-phenylalanine-d5 spectra for different water contents obtained as summarized in Figs. 4–8 assuming a π-flip motion for bound ligand, a deuterium quadrupole coupling constant of 180 kHz, $\eta = 0.05$, line broadening of 1 kHz, and 2048 points in the spectral array. The exchange rate constant, $k_2 = 2.5 \times 10^3$ Hz, the concentrations, C_i, and π-flip rates are: (*a*) $C_{ES} = 0.9$, $C_S = 0.1$, $k_1 = 3 \times 10^4$ Hz, (*b*) $C_{ES} = 0.85$, $C_S = 0.15$, $k_1 = 8 \times 10^4$ Hz, (*c*) $C_{ES} = 0.7$, $C_S = 0.3$, $k_1 = 1.2 \times 10^5$ Hz. The rate constants in s^{-1} are 2π times larger than the values in Hz.

results from phenylalanine that apparently reorients or exchanges sufficiently rapidly for the central features of the anisotropy in the deuterium spectrum to be lost. The water content of the crystal under these conditions corresponds to ~210 water molecules per enzyme molecule. It is remarkable that this low water content is apparently sufficient to permit some nearly isotropic motion. Considerable motion of small molecules in protein crystals has been known for some time (Hsi et al., 1976). The presence of an apparently isotropic component in the spectrum raises the interesting question of exchange between the bound and isotropic environment, which has been discussed in more detail elsewhere (Zhang and Bryant, submitted for publication). Application of an exchange model taking into account the anisotropy of the bound-state spectrum demonstrates that the exchange process is not fast enough to supress the effects of the phenyl ring flip in the bound environment. Thus, the features at ±64.3 kHz, ±15.6 kHz.

When the water content is raised to 21%, or 400 water molecules per enzyme molecule, spectrum c of Fig. 1 results, showing a substantial increase in the sharp isotropic feature. This increase is consistent either with very considerable isotropic motion of some fraction of the bound L-phenylalanine or with increased dissociation of the L-phenylalanine. Attempts to prepare a 1:1 enzyme-phenylalanine complex in solution and precipitate the crystals rapidly by dialysis failed to produce a deuterium spectrum with a significant solid component spectral feature. This difficulty was also noted in the x-ray reports (Christianson et al., 1989). Although the isotropic feature is increasingly dominant, the maxima at ±64.3 kHz remain clearly observable; however, the pair at ±15.6 kHz is merged into the isotropic peak as shoulders. The loss of these ±15.6-kHz features is consistent with the chemical exchange averaging of the L-phenylalanine resonances between the bound and isotropic environments. We

note that this averaging occurs in spite of the fact that the cross-linked crystal preparation remains relatively dry. The exchange rate is not sufficiently rapid for the features at ±64.3 kHz to be eliminated.

Increasing the water content further to saturation with solvent yields spectrum d of Fig. 1. The narrow feature has a substantially larger intensity, which may result from additional motion in the bound environment or a higher concentration of ligand in the isotropic environment. The increase in the isotropic component concentration is likely, since the spectrum preserves the features at ±64.3 kHz. Thus, what motions are present are insufficient to affect this broader component of the powder pattern. Qualitatively, the preservation of this 64.3-kHz splitting also requires that the exchange rate between the enzyme-bound site and the isotropic sites is insufficient to collapse the spectrum.

Simulations

The information available from the deuterium spectra is conveniently discussed from the perspective of numerical simulations shown in Fig. 2. To simulate these spectra we define Euler angles ($\theta\phi$) between the electric field gradient tensor principle axes and a molecular axis system. This transformation is obtained by rotating the irreducible quadrupolar tensor components from principal axis frame to an intermediate molecular frame:

$$V_m^{(2)}(\varphi\theta\phi) = \sum_{m'=-2}^{2} D_{m'm}^{(2)}(\varphi\theta\phi)V_{m'}^{(2)}. \qquad (13)$$

Here $V_{m'}^{(2)}$ is the irreducible tensor in the principal axis system, and $V_m^{(2)}$ $(\varphi\theta\phi)$ is expressed in the molecular frame. The unique component of the nuclear electric quadrupole coupling tensor of the deuteron, Vzz, is along the C—D bond, and the asymmetry parameter is 0.05 (Gall et al., 1981). We choose the molecular frame with the z axis along the rotation axis, i.e., along the C_β-phenyl ring bond. The y axis is perpendicular to the plane of the phenyl ring, and the x axis is in the ring plane. The possible motion of the ring is a rotation around the C_β—C_γ bond. Under this circumstance, the resonance frequencies of the 2, 3, 5, and 6 position deuterons are affected by the ring motion, but the 4 position deuteron is not. In the case of the first-order quadrupolar interaction where only the second-rank tensors are included, the four deuterons at o-, m-position are equivalent because of the spatial symmetry properties of the tensor components. If an echo sequence is used, there is substantial intensity loss for these four deuterons during the echo delay period if the rate of the ring flip is in the vicinity of the reciprocal of the echo delay time. In the same circumstance, the p-position deuteron spectrum is unaffected because the reorientation does not change the resonance frequency. However, if there is an exchange between the bound environments and an isotropic environment with a bound lifetime near the reciprocal of the echo delay time, all deuteron contributions suffer echo intensity losses.

Two typical limits of ring motion will be considered here first: 1) π-flip model ($n = 2$) where two discrete sites have Eular angles of 0°, 60°, and 0°), and 0°, 60°, and 180°; and 2) threefold rotation or continuous rotation. The effects of this motion on the second-rank tensor or the deuterium powder pattern line shape do not distinguish between rotational motions, which correspond to a three-site hop, $n = 3$, and a more continuous rotational motion, $n > 3$. Thus we use three sites with Eular angles of 0°, 60°, and 0°; 0°, 60°, and 120°; and 0°, 60°, and 240° to simulate this situation. The assumptions of the model used to compute these spectra are then that the phenyl ring of the labeled L-phenylalanine undergoes π-flip or continuous rotational jump motions when bound to the protein with a rate constant, k_1. The exchange of the phenylalanine between the anisotropic enzyme-bound site and the isotropic or free environment located between protein molecules in the cross-linked crystals is characterized by the rate constant k_2, or k_{off}.

A crucial point about this model is the assumption that the crystalline environment presents the exchanging ligand with a uniformly oriented arrangement of binding sites. That is, all bound environments within a single crystallite have the same enzyme orientation with respect to the molecular frame and thus the same Euler angles for the bound phenylalanine are appropriate for all bound ligands. If the simulation were conducted for a single crystal, the result would be a sharp line for each deuterium transition associated with that particular orientation of the crystal. However, we have a polycrystalline sample, and a powder pattern results from the superposition of crystallite spectra, each of which is averaged by the exchange processes described by k_1 and k_2. We assume that L-phenylalanine exchanges between one crystal and another in the polycrystalline sample do not occur on the time scale of the acquisition, which is a good assumption for the macroscopic crystals used and the lack of excess solvent between the crystals except in the saturated case of Fig. 1 spectrum d.

This approximation for a single orientation per crystallite would be fine except that carboxypeptidase A crystallizes in the space group $2P_1$, which has a twofold screw axis parallel to the b axis of the unit cell. The symmetry operation brings the position (x, y, z) to the second position $(-x, y + ½, -z)$ (Blundell and Johnson, 1976). Because the magnetic resonance experiment is invariant to translations, this operation essentially generates two magnetically inequivalent sites in the unit cell, (x, y, z) and $(-x, y, -z)$, which are related by the rotational transformation, R_y $(\theta = \pi)$. The two sites in the unit cell related by this symmetry operation have nearly the same principal axis components for the quadrupole-coupling tensor, but different direction cosines. As a result, chemical exchange between an isotropic position and both of these enzyme sites is essentially indistinguishable from exchange with only one of them as shown in Fig. 3. In this simulation, the 4 position of the ring only is used to examine the sensitivity of the spectrum to exchange averaging of the powder pattern between the two positions of the unit cell. The relationship between the two positions in the unit cell is shown by the axis system inset; the computed powder spectra for

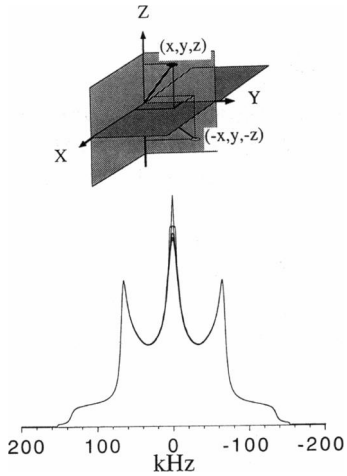

FIGURE 3 Superposition of nine simulated deuterium powder pattern spectra for the deuterium atom at position 4 of L-phenylalanine phenyl ring at the two crystallographically distinct sites for $2P_1$ symmetry in exchange with free ligand. The simulation parameters are: Qcc = 180 kHz, $\eta = 0.05$, a 1-kHz line broadening, $k_1 = 0$, $k_2 = 2.5 \times 10^3$ Hz, $C_{ES} = 0.8$, $C_S = 0.2$ where the orientations are $\alpha_1 = 10°$, $\beta_1 = 10°$; $\alpha_1 = 20°$, $\beta_1 = 20°$; $\alpha_1 = 30°$, $\beta_1 = 30°$; $\alpha_1 = 40°$ $\beta_1 = 40°$; $\alpha_1 = 50°$, $\beta_1 = 50°$; $\alpha_1 = 60°$, $\beta_1 = 60°$; $\alpha_1 = 70°$, $\beta_1 = 70°$; $\alpha_1 = 20°$, $\beta_1 = 45°$; $\alpha_1 = 20°$, $\beta_1 = 170°$. The corresponding symmetrically related sites have $\alpha_2 = 180° - \alpha_1$, and $\beta_2 = 180° - \beta_1$.

nine different orientations are superimposed and demonstrate that only the most central portion of the spectrum is at all sensitive to the subtle difference between these two positions in the crystal. For the simulations that follow, we ignore this difference to simplify the computations.

The simulated spectra of Fig. 2 are good approximations to the experimental spectra; however, a number of simulations are necessary to arrive at these results. Thus, we examine several of these to provide the background leading to Fig. 2.

Fig. 4 shows the input spectra for a complete simulation of the exchange averaged powder patterns for the bound environment only. Fig. 4 *a* shows the effects of just the π-flip of the bound spectrum for positions 2, 3, 5, and 6 of the phenyl ring where equal probability is assumed for each position. Other assumptions may provide the same spectral shape (Hirschinger and English, 1989); however we take this simplest case of the π-flip with equal probability as a useful but not unique model. This assumption appears to be justified in the present case more than in the glassy polymer case because the environment is crystalline so that each bound anisotropic environment should be the same, and a distribution of flip angles is not anticipated based on a distribution of locally different steric environments. Fig. 4 *b* shows the sum of the Fig. 4 *a* spectra with that for the 4 position of the ring that is unaffected by the π-flip. The rate constant that is permitted to change is k_1, or the ring flip rate. No exchange with free environment is permitted. The exchange process described by k_2 will average the spectra of Fig. 4 *b* with an isotropic line.

The spectra shown in Fig. 5 were simulated for the bound environment where the phenyl ring of L-phenylalanine ex-

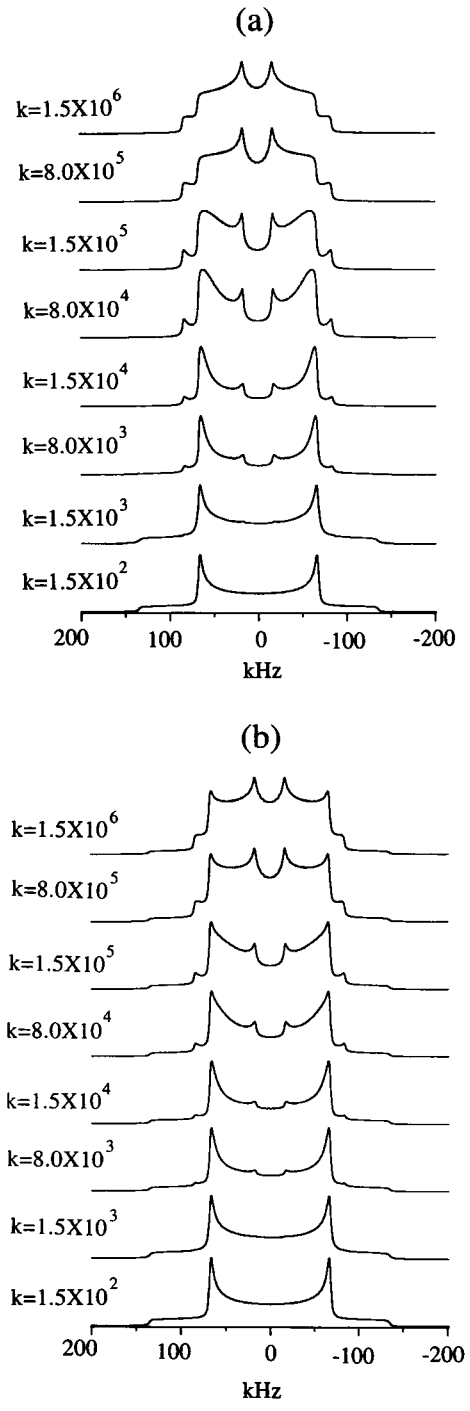

FIGURE 4 Simulation of deuterium powder pattern for π-flip motion of L-phenylalanine bound to carboxypeptidase A, with a deuterium quadrupole coupling constant of 180 kHz, $\eta = 0.05$, line broadening of 1 kHz, and 2048 points in the simulation. $\Omega_1 = (0°, 60°, 0°)$, $\Omega_2 = (0°, 60°, 180°)$, $P_1 = P_2 = 0.5$. (*a*) Spectra for ring positions 2, 3, 5, and 6. (*b*) Spectra for all ring positions. No exchange with an isotropic phase is assumed.

ecutes rotational jumps on a threefold or higher rotational grid. No exchange of the ligand with the isotropic environment was permitted. Fig. 5 *a* spectra are for positions 2, 3, 5, and 6, and Fig. 5 *b* spectra are the sum of Fig. 5 *a* spectra and the spectra for position 4. These spectra are essentially

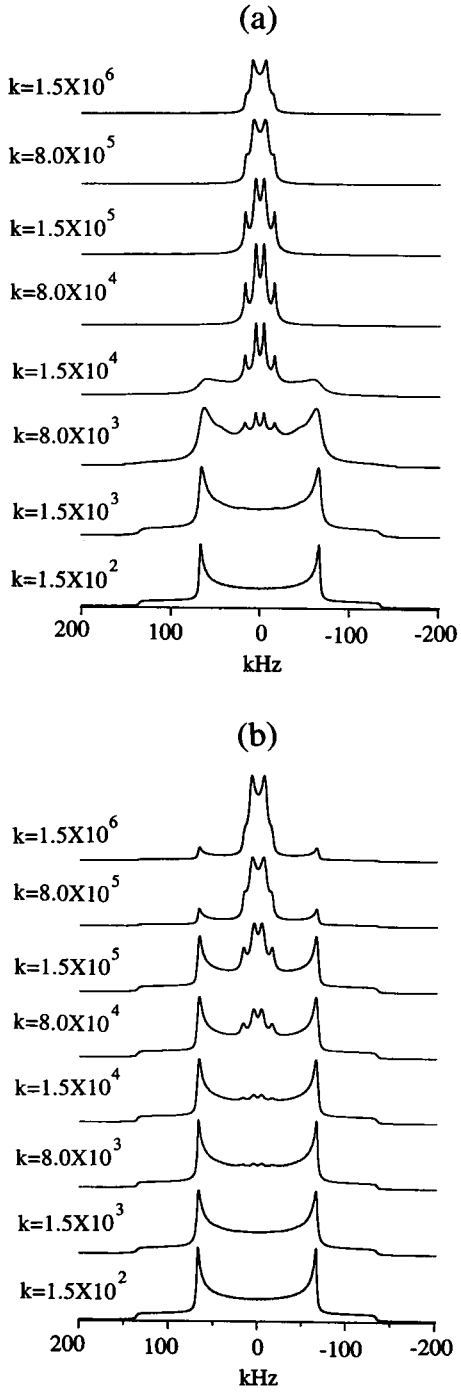

FIGURE 5 Simulation of deuterium powder pattern for continuous rotation of the phenyl ring of L-phenylalanine bound to carboxypeptidase A assuming a deuterium quadrupole coupling constant of 180 kHz, $\eta = 0.05$, line broadening of 1 kHz, and 2048 points in the simulation. $\Omega_1 = (0°, 60°, 0°)$, $\Omega_2 = (0°, 60°, 180°)$, $\Omega_3 = (0°, 60°, 240°)$ $P_1 = P_2 = P_3 = \frac{1}{3}$. (*a*) Spectra for ring positions 2, 3, 5, and 6. (*b*) Spectra for all ring positions. No exchange with an isotropic phase is assumed.

those for a continuous rotational rather than a twofold flip model and do not agree with the line shapes of the experimental spectra in Fig. 1.

Fig. 6 shows the results of simulations for position 4 of the phenyl ring in exchange with isotropic environments in the crystal. The simulation is elementary in that, given that the 4 position is independent of the π-flip motion, these spectra represent the effects of what is essentially a two-site exchange process where one of the sites is represented by a powder pattern. Several features are important. 1) The spectra for the rapid exchange limit shown at the top are a simple function of the ligand concentration; as the free ligand concentration increases, the powder pattern spectrum narrows, so that the concentration behaves essentially as a scale factor for the spectral width. 2) In the slow exchange limit shown at the bottom, the spectrum is a simple superposition of the bound and isotropic spectra. In the case where the isotropic position concentration is high, the features of the bound spectrum are difficult to discern because of the dynamic range problem created by the intense isotropic peak. 3) In the intermediate exchange regime, the line shape is a function of the exchange rate constant as well as the concentration. Even over the relatively narrow concentration range shown here, a concentration change may shift the appearance of the spectrum by an amount that corresponds to an order of magnitude change in the exchange rate constant. Based on the sensitivity of these patterns to the exchange rate constant k_2, this constant must be close to 10^3 Hz.

The effects of both the π-flip and the chemical exchange are summarized in Fig. 7 for different choices of concentration of the free and bound ligand. Fig. 7 *a* summarizes the contributions for positions 2, 3, 5, and 6, while *b* summarizes the effects on position 4; the sum of all deuteron positions is shown in Fig. 7 *c*. For these simulations the exchange rate constant was set at 2.5×10^3 Hz. These simulated spectra then provide the summary spectra of Fig. 2, which reproduce the simulations that reproduce the experimental spectra well.

The assumption that the exchange rate constant between the isotropic and anisotropic environments is fixed while the fraction of free ligand varies with water content may be questioned. However, the simulations shown in Fig. 8 demonstrate that the line shape is very sensitive to the choices of the rate constant k_2 and the concentration of free ligand. In addition, the parameters that affect the main spectral features are not strongly covariant. Therefore, the parameters used to compute the spectra shown in Fig. 2 are reasonable approximations to the rate constants k_1 and k_2 within the primary assumption of the model, i.e., that the flip angles are not widely distributed with unequal probabilities.

The computed spectra of Fig. 2 are obtained without the introduction of bound environment motion in addition to the aromatic ring flip. That is, the calculations do not include any wobble of the ring rotation axis in the bound environment; however, the signal-to-noise ratios in the experimental spectra make it impossible to exclude motions of this axis in the range of 6–8°. The x-ray diffraction results show that in the native enzyme, the Zn ion is coordinated to His-196, His-69, Glu-72, and water-571. This water molecule (2 Å from Zn) is believed to be dynamically disordered and moving between the water-571 site and the water-576 site (3 Å from Zn) with the population of 0.5 for each. At 1.5 Å resolution this two-site model is hard to distinguish from a completely disordered model (Rees et al., 1983). On the other hand, the bond distance of water-571 to Oε1 of Glu-270 and

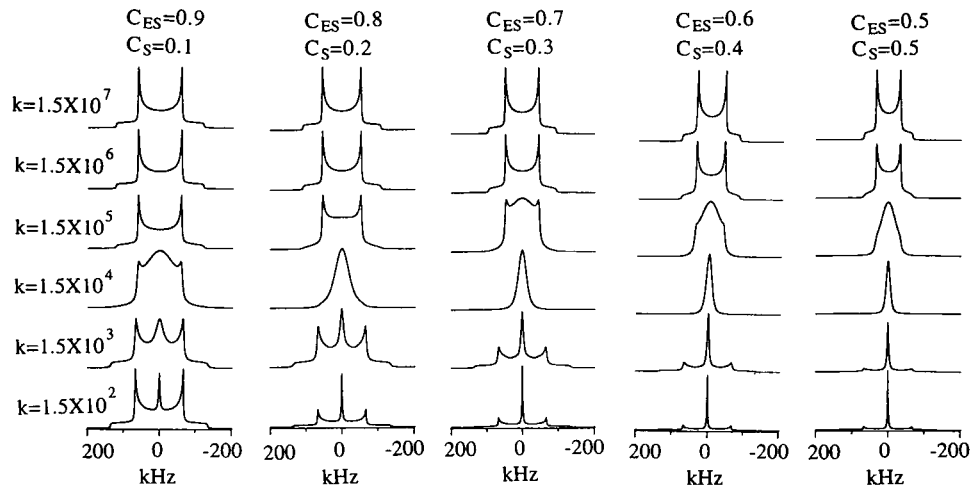

FIGURE 6 Simulation of the deuterium powder pattern in the presence of chemical exchange between the anisotropic environment and an isotropic environment for position 4 of the phenyl ring of L-phenylalanine bound to carboxypeptidase A as a function of the concentration of bound and free L-phenylalanine. Other parameters were as shown above.

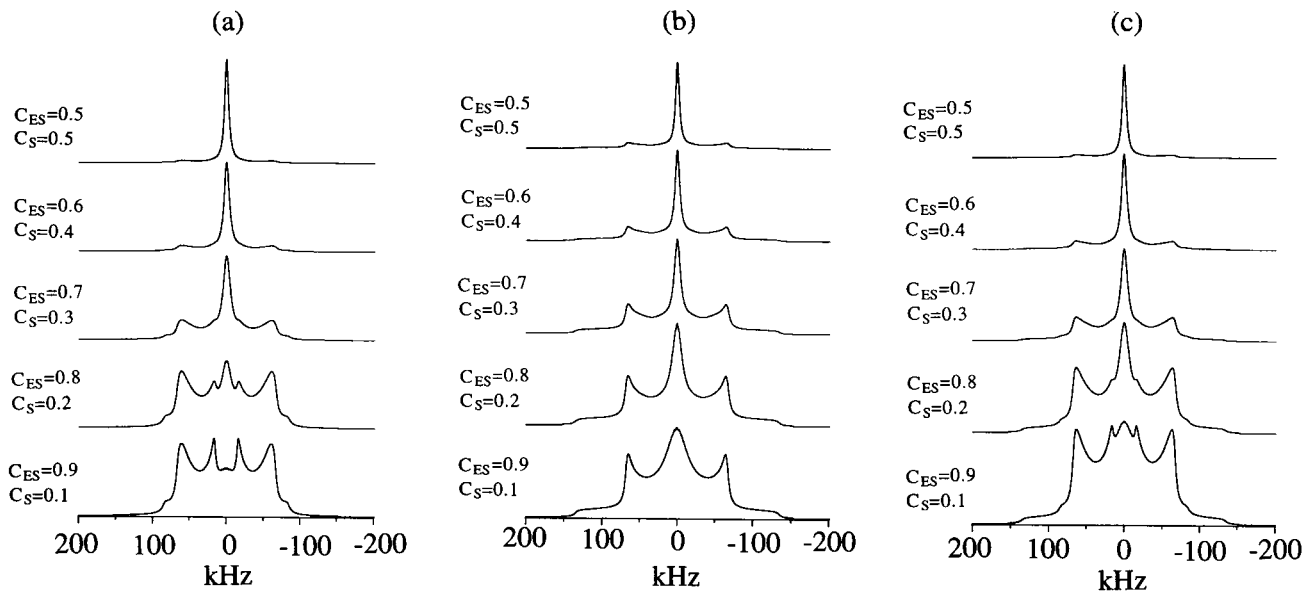

FIGURE 7 Simulation of deuterium NMR powder pattern for the phenyl ring of L-phenyl alanine bound to carboxypeptidase A that undergoes both π-flip motions and chemical exchange with an isotropic environment for different concentrations of bound and isotropic contributions assuming a deuterium quadrupole coupling constant of 180 kHz, $\eta = 0.05$, line broadening of 1 kHz, and 2048 points in the simulation. $\Omega_1 = (0°, 60°, 0°)$, $\Omega_2 = (0°, 60°, 180°)$, $P_1 = P_2 = 0.5$ $k_1 = 3 \times 10^4$ Hz, and $k_2 = 2.5 \times 10^3$ Hz. (*a*) Spectra for ring positions 2, 3, 5, and 6. (*b*) Spectra ring position 4. (*c*) Total spectrum for all ring deuterons.

water-576 to the carbonyl of Ser-197 are within the hydrogen bonding range. These bonds are presumably interrupted by the insertion of ligand (Rees et al., 1981a, b; 1983). The active site also includes Glu-270, Arg-127, Arg-145, and the hydrophobic pocket S1′, which favors ligands with a pendant phenyl ring. In models where the ligand is bound to the zinc ion, a five-coordinate transition state model is proposed based on the x-ray study that involves two oxygens from the carbonyl or phosphate binding to the Zn (Kim and Lipscomb, 1990, 1991). These two oxygens are hydrogen bonded to the Glu-270 and Arg-127 as well (Mangani and Orioli, 1992b). The L-, D-Phe, and D-Tyr are the exceptions to this binding model because they are not bonded to the metal ion but are stabilized by Glu-270 and Arg-145, where the $—NH_3^+$ hydrogen bonded to Glu-270 and $—COO^-$ make a salt link to Arg-145. In addition, the phenyl ring is extended into the hydrophobic pocket at the expense of displacing some of the water molecules (Mangani and Orioli, 1992a, b). This well constrained binding mode fixes the α-carbon of L-phe, which is consistent with the lack of wobbling apparent in spectrum a of Fig. 1.

The value of the rate constant k_1 and its dependence on the crystal water content is interesting. In a number of protein systems, the phenyl ring flip rates fall in the rapid exchange limit with respect to the deuterium quadrupole powder pattern spectrum, i.e., rapid compared with ~1 μs (Torchia, 1984). For phenylalanine bound in the active site of carboxypeptidase A, the ring flip dynamics are clearly in an intermediate exchange regime caused by intermolecular steric constraints of the active site. This result is consistent with the x-ray data, which place the phenyl ring in close proximity to atoms of the hydrophobic pocket. The simula-

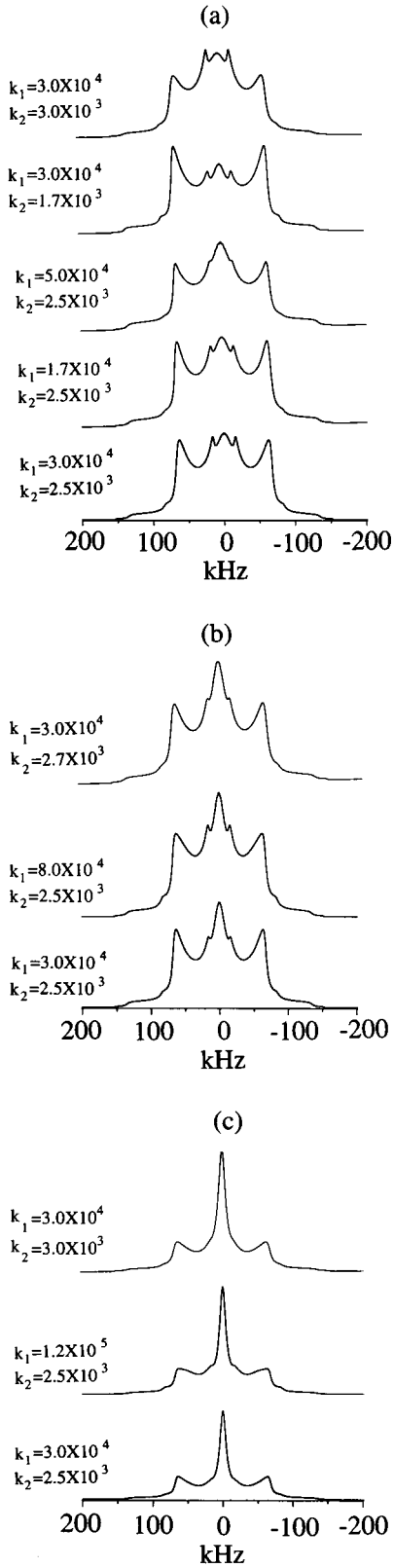

FIGURE 8 Simulations for various parameters to show the sensitivity of the computed spectrum to the model parameters k_1 and k_2 assuming a deuterium quadrupole coupling constant of 180 kHz, $\eta = 0.05$, line broadening of 1 kHz, and 2048 points in the simulation. $\Omega_1 = (0°, 60°, 0°)$, $\Omega_2 = (0°, 60°, 180°)$, $P_1 = P_2 = 0.5$ for different values of k_1 and k_2 in units of Hz. (*a*) $C_{ES} = 0.9$, $C_S = 0.1$. (*b*) $C_{ES} = 0.85$, $C_S = 0.15$. (*c*) $C_{ES} = 0.7$, $C_S = 0.3$.

tions of Fig. 2 show that the ring flip rate increases somewhat with increasing water content; however, in the range from 6–21% water, the flip rate only changes by about a factor of three corresponding to only 2.7 kJ/mol change in the potential barrier height. Thus, the changes in the protein structure caused by the increased water content of the crystal are not large. It is interesting to note that even in crystalline L-phenylalanine, apparently two different crystal forms provide different phenyl ring flip time scales, one fast and one slow (Rice et al., 1981; Torchia, 1984). For the present situation in which L-phenylalanine is bound in the active site region, the ring flip rate is in the sensitive intermediate range for all conditions studied. The increase in ring flip rates in protein systems with increasing solvation by water has also been observed by Klibanov and co-workers (Burke et al., 1992, 1993).

The value of k_2 is consistent with other reports of phenylalanine exchange kinetics. In solution phase experiments on the cobalt enzyme, Luchinat et al. (1988) report that the L-phenylalanine exchange rate lies in the fast exchange limit with respect to the paramagnetic transverse relaxation rate for the bound environment of 110 Hz. The value of 2.5×10^3 Hz obtained in the present work is an order of magnitude faster than required by this constraint. It is interesting that the slight shifts in the free ligand concentration required to fit the amplitude of the isotropic central portion of the spectra of Fig. 1 effectively cause a small change in the exchange rate between the bound and isotropic environments, but the rate constant k_2 remains quite tightly constrained by the line shape requirements. Thus, the dissociation rate for L-phenylalanine is not affected significantly by the water content of the crystal.

CONCLUSION

The deuterium NMR spectra of L-phenylalanine-d5 obtained on cross-linked polycrystalline samples of carboxypeptidase A demonstrate that in the active site environment the phenylalanine ring executes π-flip motions that are constrained by the active site environment to the range of 3×10^4 Hz to 1×10^5 Hz and for crystals in the water content range of 6 to 21%. At all water contents, the phenylalanine exchanges with an isotropic environment in the crystal with a rate constant of ~2.5 × 103 Hz, which does not depend on the water content over this range. The changes in water content cause relatively minor structural changes as gauged by the small changes in the kinetic parameters for the enzyme-bound ligand. The interpretation of the deuterium NMR spectra was made with the aid of line shape simulations for a polycrystalline sample in which a labile deuterium-bearing ligand may exchange with an oriented crystalline-bound environment in which a local reorientation is permitted.

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