Proton and Oxygen-17 Magnetic Resonance Relaxation in *Rhus* Laccase Solutions: Proton Exchange with Type 2 Copper(II) Ligands†

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ABSTRACT: Longitudinal (T_1^{-1}) and transverse (T_2^{-1}) nuclear magnetic resonance (NMR) relaxation rates of ^1H and ^{17}O of water molecules were measured in solutions of oxidized *Rhus vernicifera* laccase in order to investigate the structural and dynamic aspects of water coordination to its type 2 Cu(II) site. The magnitude and negative temperature dependence of the paramagnetically induced proton T_1^{-1} and T_2^{-1} showed that the proton fast-exchange mechanism is operative. The field dependence of $T_{1,pmg}^{-1}$ gave an apparent correlation time of 2.7 ns for the 9–60-MHz range. $T_{1,pmg}^{-1}$ is pH dependent, giving two dissociation steps with pK = 6.2 and 8.6 which are attributed to the hydrolysis of a H_2O molecule and a protein hydroxyl group coordinated to the type 2 Cu(II), respectively. Relaxation rates of ^{17}O were not paramagnetically enhanced. Neither T_1^{-1} nor T_2^{-1} values of both ^1H and ^{17}O were significantly affected by the binding of fluoride or azide ions to type 2 Cu(II). Our data lead to the following interpretation: The water oxygen equatorially bound to type 2 copper does not exchange on the NMR time scale. The paramagnetic relaxation via the fast exchange mechanism originates from proton transfer between the Cu(II)-bound water molecule or hydroxyl group and the buffered solution, acid–base catalyzed by a protein residue. The relative inertness of the water oxygen is consistent with the structural concept of a cavity which accommodates the laccase type 2 (and type 3) copper sites, the active centers involved in O_2 reduction, and is connected to the bulk solvent via an orifice which in the native oxidized enzyme is only penetrable by protons. A tentative model of the type 2 Cu coordination structure is presented.

Laccase from *Rhus vernicifera* (EC 1.10.3.4), an extracellular oxidoreductase which catalyzes the reduction of O_2 to water by various substrates, contains four copper ions forming three different redox sites: the intense blue type 1 site, detectable by electron paramagnetic resonance (EPR), the type 3 site consisting of a pair of Cu(II) ions which are antiferromagnetically coupled in the native-oxidized enzyme, and the solvent-accessible, EPR active type 2 site (Fee, 1975). Recent findings indicate that the type 2 copper is part of the O_2 reduction system since at least one of the water molecules formed in the reduction of O_2 by the fully reduced enzyme ends up coordinated to it (Bränden & Deinum, 1977a). Furthermore, the type 2 Cu(II) is the binding site for a number of anions known to inhibit the enzymatic activity of laccase (Peisach & Levine, 1965). In particular, it exhibits an unusually high affinity for fluoride ions (Bränden et al., 1973). This site has also been proposed to function as the primary electron acceptor in the reduction of laccase by hydroquinone and related compounds, reacting via an inner sphere mechanism (Holwerda et al., 1976). A common element of all these functions attributed to the type 2 site is the displacement of a ligand, presumably a water ligand, from the inner coordination sphere.

Although laccase contains four copper ions per molecule, their particular properties (Fee, 1975) allow one to assign the major part of the paramagnetic relaxation effect to the type 2 Cu(II). The two antiferromagnetically coupled Cu(II) ions in the type 3 site are ineffective. The type 1 Cu(II), though paramagnetic, is embedded inside the protein as indicated by various experiments using physicochemical techniques. For single type 1 proteins (plastocyanin, azurin) this has also been shown directly by crystallographic structure determinations (Colman et al., 1978; Adman et al., 1978). It explains why these proteins are inefficient in enhancing the ^1H magnetic relaxation rates, except for a minor long-range outer-sphere effect (Blumberg & Peisach, 1966; Boden et al., 1974; Koenig & Brown, 1973). Since the comparatively low reactivity of the type 1 Cu(II) suggests that it may even be less accessible to external ligands than in the single copper proteins mentioned above (Holwerda et al., 1976), its outer-sphere ^1H magnetic relaxation effect should be of similar magnitude or even less important. Hence, the only conceivable origin of significant paramagnetic enhancement of magnetic relaxation rates in native oxidized laccase solutions is the type 2 Cu(II) ion.

This report provides evidence for the equatorial coordination of one water molecule and one protein hydroxyl group to the type 2 Cu(II). The oxygen atom of this water molecule is highly inert, compared with the water exchange from the first coordination sphere of inorganic Cu(II) complexes. This and the unusually slow formation of type 2 Cu(II)–anion complexes suggest that except for protons, ligand access to and release from the inner coordination sphere of the type 2 site is strongly impeded in the oxidized state, a property which is probably of relevance for the catalytic mechanism of the enzyme (Bränden & Deinum, 1977a).

Experimental Procedures

Laccase was prepared from the acetone extract of *Rhus vernicifera* lacquer supplied by Saito and Co. Ltd., Tokyo, Japan, according to the modified procedure of Reinhammar (1970) (Pecht et al., 1978). The purity and activity were determined by checking the optical, EPR, and enzymatic properties of the protein sample. The A_{280}/A_{614} ratio was 14.6. The protein concentration was determined from the absorbance at 615 nm (ϵ = 5700 M^{-1} cm^{-1}). Vacuum dialysis in ultrathimbles (Schleicher & Schull, Dassel, West Germany) was used to concentrate the protein solution up to 1 mM (~110 mg/mL).

Laccase solutions enriched with ^{17}O (~15%) were prepared by mixing concentrated protein samples in $^{16}OH_2$ buffer with appropriate amounts of 40% $^{17}OH_2$ (Isotope Separation Plant, The Weizmann Institute of Science, Rehovot). Potassium

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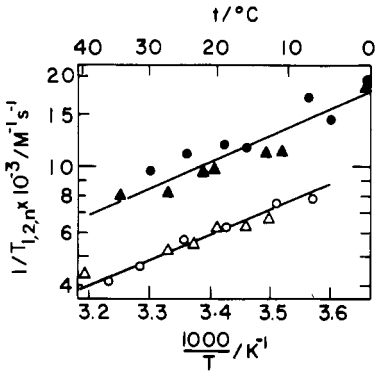


FIGURE 1: Molar longitudinal (○, △) and transverse (●, ▲) proton magnetic relaxation rates in solutions of *Rhus* laccase as functions of the temperature. The rates are corrected for solvent and impurity contributions. The Larmor frequency was 16 MHz and the enzyme 0.9 mM at pH 6.7 in 0.05 M potassium phosphate. Circles refer to native laccase, triangles to the laccase–F^- complex produced by incubating the former with 0.11 M KF.

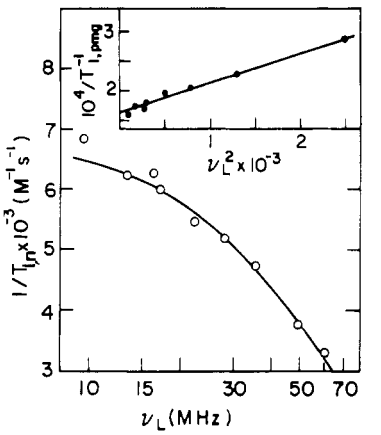


FIGURE 2: Molar longitudinal proton magnetic relaxation rate as a function of the Larmor frequency ν_L. The temperature was 20.1 °C and the enzyme 0.9 mM at pH 6.7 in 0.05 M potassium phosphate. The line was calculated by using the linear best fit values τ_c = 2.66 ns and $T_{1,n}^{-1}(\nu_L \rightarrow 0)$ = 6600 M^{-1} s^{-1}. (Insert) Plot of $1/T_{1,pmg}^{-1}$ vs. ν_L^2.

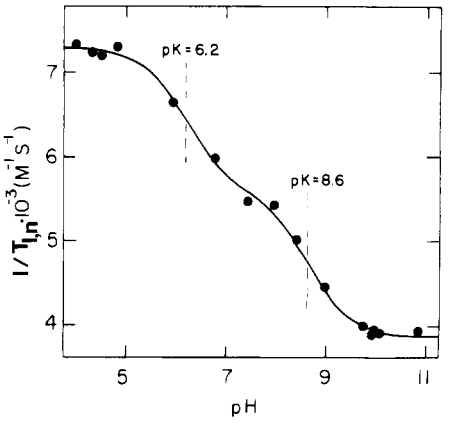


FIGURE 3: Molar longitudinal proton magnetic relaxation rate as a function of pH. The Larmor frequency was 15.6 MHz, the temperature 20 °C, and the enzyme 0.5 mM in 0.05 M potassium phosphate over the pH range 5–9 (for further details cf. Experimental Procedures). The line corresponds to the best fit assuming two ionization equilibria.

phosphate buffer was prepared from doubly distilled water. All reagents used were of analytical grade.

The samples for the measurement of the pH–T_1 profile were prepared by mixing different amounts of a pH 5 and a pH 9 solution of equal laccase concentration (pH range 5–9) or by adding dilute acetic acid (pH range 4–5) or potassium phosphate (pH range 9–11) and correcting for dilution. pH values were measured at 20 °C with a Metrohm Model E388 pH meter equipped with a combination glass electrode and carefully calibrated with a series of standard buffers. The pH readings were taken inside the sample tube, in most cases before and after the T_1 experiment.

Relaxation measurements were conducted on a Bruker BKR 322S pulsed NMR spectrometer equipped with a modified Varian 4012A high-resolution magnet and a Varian 3508 flux stabilizer. T_1 was measured at frequencies from 8 to 60 mHz by the standard method of the π–τ–$\pi/2$ pulse sequence, and T_2 was measured by using the Meiboom–Gill pulse sequence. The ^{17}O measurements were made at 8.19 MHz, and the sequences were accumulated until a satisfactory signal to noise ratio was obtained. The static magnetic field homogeneity and stability were checked by measuring proton and ^{17}O longitudinal and transverse relaxation rates of water samples with controlled pH. These rates agreed well with data from the literature [cf. Glasel (1972)].

Results and Interpretation

Proton Magnetic Relaxation

Data. The longitudinal and transverse solvent proton magnetic relaxation rates, T_1^{-1} and T_2^{-1}, respectively, in solutions of native laccase were measured as a function of temperature at a proton Larmor frequency of 16 MHz. The contribution of the enzyme to the ^1H NMR relaxation rates was calculated by subtracting blank values measured in the protein-free filtrate obtained upon concentration of the protein solution in ultrathimbles. The resulting data, normalized to 1 M laccase concentration, are presented in Figure 1, $T_{1,n}^{-1}$ denoting the molar longitudinal and $T_{2,n}^{-1}$ the molar transverse magnetic relaxation rates. The slopes of linear regression lines through the $T_{1,n}^{-1}$ and $T_{2,n}^{-1}$ values yield temperature coefficients of −3.75 ± 0.25 and −4.2 ± 0.5 kcal/mol, respectively. The field dependence of $T_{1,n}^{-1}$ was investigated over the range 9.5–60 MHz at 20 °C and pH 6.7 (Figure 2). A pronounced relaxation dispersion is observed, similar to extensively studied field dependencies in several other copper proteins (Koenig & Brown, 1973). We have also measured the pH profile of the longitudinal ^1H NMR relaxation rates between pH 4 and 11 at 15.6 MHz and 20 °C (Figure 3). $T_{1,n}^{-1}$ is found to be strongly pH dependent, decreasing from a constant level at pH <5 to a ~50% lower level at pH >10.

Analysis and Interpretation. The laccase-induced ^1H NMR relaxation rates ($T_{1,2,n}^{-1}$) comprise paramagnetic ($T_{1,2,pmg}^{-1}$) and diamagnetic terms ($T_{1,2,dia}^{-1}$), the latter due to the effects of protein molecule–solvent interaction. $T_{1,dia}^{-1}$, obtained by reducing the laccase copper sites with excess ascorbate, was 900 M^{-1} s^{-1} at 20 °C, 15.6 MHz, and pH 5.2. This is very close to the corresponding value [calculated from data reported by Koenig & Brown (1973)] for reduced ceruloplasmin, a related multicopper protein of comparable molecular weight (Fee, 1975). $T_{1,dia}^{-1}$ values for other temperatures (7–40 °C) or fields (60 MHz) were derived from the measured $T_{1,dia}^{-1}$ value by using empirical equations for proton relaxation enhancement by diamagnetic proteins (Hallenga & Koenig, 1976), getting the required parameters by interpolation of Hallenga and Koenig's extensive data. The $T_{1,dia}^{-1}$ values thus obtained do not exceed 15% of T_1^{-1} at pH <6.8 and were used to calculate $T_{1,pmg}^{-1}$. The diamagnetic correction barely affects the temperature dependence; the slope of the Arrhenius plot of $T_{1,pmg}^{-1}$ is positive and corresponds to an apparent activation energy E_a' = −3.55 ± 0.30 kcal/mol.

The negative temperature coefficient of $T_{1,pmg}^{-1}$ excludes "slow exchange" of water as the dominant ^1H NMR relaxation mechanism in the range of temperatures investigated. Thus the paramagnetic enhancement is due either to fast exchange or to an outer-sphere effect (Dwek, 1973). However, a rough quantitative estimation taking into account both the type 1 and type 2 Cu(II) contributions only gives $T_{1,os}^{-1} < 700\ M^{-1}\ s^{-1}$. Thus we conclude that the ^1H NMR relaxation is controlled by the fast exchange of solvent protons between the site of magnetic interaction with the copper ion and the bulk of the solvent (Luz & Meiboom, 1964).

Under fast-exchange conditions $T_{1,pmg}^{-1}$ is composed of two terms corresponding to two modes of interaction between the electron spin and the nuclear spin of protons bound to, or near, the paramagnetic center: the dipolar and the scalar interaction terms. The contribution of the latter is negligible as shown by order of magnitude considerations which were based on the hyperfine splitting constant of protons in water molecules bound to Cu_{aq}^{2+} [0.035 G; Luz & Shulman (1965)] and on an upper limit for the correlation time of the scalar term ($\tau_e < 1 \times 10^{-8}$ s). The field dependence of $T_{1,pmg}^{-1}$ is therefore that of the dipolar term. It is characterized by τ_c, the effective correlation time of the modulation of the local magnetic field at the relaxing nucleus. The correlation time controlling the field dependence in the 10–60-MHz range was obtained from a plot of $1/T_{1,pmg}^{-1}$ vs. ν_I^2 (Figure 2, insert), under the implicit assumption that it is constant in the frequency range considered, a point to be discussed below (Dwek, 1973). The best fit value for τ_c thus obtained is $(2.66 \pm 0.15) \times 10^{-9}$ s, corresponding to an inflection point in the dispersion curve at ~60 MHz (Figure 2).

Knowing τ_c, we can obtain E_a, the activation energy of $1/\tau_c$. The apparent activation energy of $T_{1,pmg}^{-1}$, E_a', was measured at 16 MHz, a field where $2\pi\nu_I\tau_c = \omega_I\tau_c < 1$. Thus the sign of E_a must be opposite to the sign of E_a', i.e., it is positive. Moreover, E_a can be calculated from E_a' to a good approximation. It can easily be shown that

$$E_a' = E_a(\omega_I^2\tau_c^2 - 1)/(\omega_I^2\tau_c^2 + 1) \quad (1)$$

Obviously, E_a' is intrinsically temperature dependent, and in the general case, the Arrhenius plot should not produce a straight, but rather a curved, line. However, when $|\omega_I^2\tau_c^2 - 1| \gg 0$, i.e., when ω_I is far enough from the inflection point of the dispersion curve, the Arrhenius plot will be fairly linear, and E_a is obtained from the above equation by using a τ_c value measured at a temperature equal or close to the median value of all temperature points measured. Our data are consistent with this equation: $\omega_I^2\tau_c^2(20\ °C) = 0.077 \ll 1$ indeed leads to a straight line in the Arrhenius plot ($r^2 = 0.99$). From E_a', τ_c, and ν_I one calculates $E_a = +4.15\ kcal/mol$.

τ_c is given by $1/\tau_c = 1/\tau_R + 1/\tau_M + 1/\tau_s$, where τ_R is one-third the rotational relaxation time of the protein to which the paramagnetic center is bound, τ_s is the electron spin relaxation time, and τ_M is the mean residence time of the relaxing nuclei at, or near, the paramagnetic site. The question is which one of these terms is dominant. In the present case it is certainly not rotational diffusion, as it can easily be shown that the laccase molecule (M_r 110000) is too large for a rotational diffusion rate of such a magnitude. Therefore, τ_c is determined either by τ_M or by τ_s.

The measured $1/\tau_c$ value, $3.7 \times 10^8\ s^{-1}$, is within the range of rate constants for water exchange in cupric complexes (Hague, 1977). Indeed, superoxide dismutase, a protein with a Cu(II) site related to the type 2 Cu(II) (Vänngård, 1972), displays in the 10–60-MHz range a relaxation dispersion quite similar to that of laccase, controlled by a correlation time which was interpreted as the exchange lifetime of the water bound at the cupric site (Fee & Gaber, 1973). However, this is unlikely in the case of laccase, as appears from ^{17}O data reported below. Another possibility, $1/\tau_M$ being the exchange rate of protons of water molecules coordinated to type 2 Cu(II), could also be excluded. Although the pH dependence of $T_{1,pmg}^{-1}$ suggests that such an exchange takes place (cf. Discussion), it cannot account for a first-order rate of $3.7 \times 10^8\ s^{-1}$. Such a rate is certainly much too high for proton exchange with the bulk solvent at close to neutral pH (Eigen & Kruse, 1963), and proton transfer involving buffer species does not determine $T_{1,pmg}^{-1}$, since the latter was not affected when the buffer concentration was varied from 0.05 to 0.005 M (15.6 MHz, 20 °C).

It is a priori difficult to assess the importance of τ_s, because a detailed physical model for the electron spin relaxation of protein-bound Cu(II) is not yet available. Frequency dependence of τ_c is usually taken as evidence for the dominance of τ_s, because it is the only component of τ_c which can be frequency sensitive. But in the present case this criterion is not conclusive. The linearity ($r^2 = 0.993$) of the $1/T_{1,pmg}^{-1}$ vs. ν_I^2 plot (Figure 2, insert) indicates that τ_c is practically frequency independent in the range from 10 to 60 MHz. Still, if τ_c is indeed dominated by τ_s, it is safe to assume an underlying frequency dependence which, however, will be manifested only when low-field data would become available (Koenig & Brown, 1973). The room temperature EPR spectrum at X band (field corresponding to a proton Larmor frequency of ~14 MHz) looks much like the spectrum in the frozen state, although it is less well resolved (unpublished data). We therefore expect that the line widths used in the simulation of low-temperature spectra (unpublished results) are rough estimates or at least lower limits for the line widths at room temperature. On the basis of this premise and assuming a Gaussian line shape (Vänngård, 1972), we estimate a full line width of ~50 G for the type 2 Cu(II), leading to $\tau_{2,s} \gtrsim 2$ ns. Only a limiting value can be given owing to the unknown line broadening contribution of unresolved ligand hyperfine splitting. Now, since $\tau_s = \tau_{1,s} > \tau_{2,s}$, this value is a lower limit for τ_s ($\tau_{1,s}$ and $\tau_{2,s}$ are the longitudinal and transverse electron spin relaxation times, respectively). Thus the EPR data are consistent with $\tau_c = \tau_s$.

The longitudinal electron spin relaxation time has been measured in many inorganic Cu(II) systems, both in crystalline environment and in solution. It is interesting that both homogeneously and inhomogeneously coordinated Cu(II), with ligands such as water, alcohols, amines, or dipyridine, assume $\tau_{1,s}$ values within the relatively narrow range of 1–3 ns, irrespective of the phase (complex in solution or Cu(II)-doped crystal) and irrespective of the specific relaxation mechanism proposed in each case (Lewis et al., 1966a,b; Noack & Gordon, 1968; Eisenstadt & Friedman, 1968; Poupko & Luz, 1972). This gains particular significance in view of the fact that protein bound Cu(II) is in a phase somewhere in between solution and solid state. Furthermore, the ^1H NMR relaxation dispersion in laccase is similar to that found in other Cu(II) proteins where τ_c was identified with τ_s on the basis of data including also the 0–10-MHz range [e.g., copper(II) carbonic anhydrase, τ_s = ~1 ns; Koenig & Brown (1973)].

In conclusion, the correlation time τ_c is most probably dominated by τ_s. The limits for τ_M are given by $\tau_c = 2.7 \times 10^{-9} \ll \tau_M \ll n/(111T_{1,pmg}^{-1}) = \sim n(2 \times 10^{-6})$ s, where n is the number of exchanging protons at the paramagnetic site.

The transverse relaxation rates are fully consistent with the results and conclusions derived from the $T_{1,n}^{-1}$ values. The

difference between $T_{2,n}^{-1}$ and $T_{1,n}^{-1}$, ~4500 M^{-1} s^{-1} at 20 °C (Figure 1), is due to the theoretically predicted difference between $T_{2,pmg}^{-1}$ and $T_{1,pmg}^{-1}$ at $\omega_s^2\tau_c^2 \gg 1$ (Dwek, 1973), ~1300 M^{-1} s^{-1}, to the contribution of outer-sphere relaxation, approximately 400–700 M^{-1} s^{-1}, and to a diamagnetic enhancement of the transverse relaxation rate which is probably much more efficient than that of the longitudinal relaxation rate (Grösch & Noack, 1976). The slope of the Arrhenius plot of $T_{2,n}^{-1}$ is positive (Figure 1), as expected for the case of fast exchange and positive true activation energy. Moreover, by deriving for $T_{2,pmg}^{-1}$ an equation analogous to eq 1, one can show that the ratio of the apparent activation energies for $T_{2,pmg}^{-1}$ and $T_{1,pmg}^{-1}$ should be 1.12 under our conditions ($\omega_I^2\tau_c^2 = 0.077$). This is in agreement with the ratio of the observed temperature coefficients and thus is consistent with an activation energy of $T_{2,dia}^{-1}$ of ~4 kcal/mol and an insignificant contribution of the scalar interaction to $T_{2,pmg}^{-1}$.

In order to get further insight into the molecular aspects of the fast exchange controlled ^1H NMR relaxation, we investigated its dependence on chemical parameters like pH and exogenous ligands of the type 2 Cu(II) ion. The magnitude of the pH effect on $T_{1,n}^{-1}$ implies a decrease in $T_{1,pmg}^{-1}$. The pH profile clearly reveals the presence of two ionization equilibria. By use of a standard nonlinear least-squares procedure, it was fitted to the equation

$$T_1^{-1} - T_{1,pH>10}^{-1} = [T_{1,A}^{-1}/(1 + 10^{pH-pK_A})] + [T_{1,B}^{-1}/(1 + 10^{pH-pK_B})]$$

(where the subscripts A and B refer to the first and the second dissociation steps, respectively), yielding pK values of 6.2 and 8.6, respectively (Figure 3). $T_{1,A}^{-1}$ and $T_{1,B}^{-1}$ were equal, within error range, showing that the protons dissociating with pK 6.2 and 8.6, respectively, contribute equally to the paramagnetic enhancement.

In a further experiment, we examined the ^1H NMR relaxation rates of the type 2 Cu(II)–fluoride complex, the full formation (100% occupancy) of which was confirmed spectrophotometrically.[1] F^- is believed to replace bound H_2O (but see Discussion), although it is not clear whether in *Rhus* laccase this ion can occupy two coordination positions like in fungal laccase (Bränden et al., 1973). An effect on the ^1H NMR relaxation rates could be expected, if the binding of F^- significantly affects the position or the number of protons exchanging from the vicinity of the Cu(II) ion. However, $T_{1,n}^{-1}$, obtained as described before and shown in Figure 1, barely differs from the data for the native enzyme. The same holds for $T_{2,n}^{-1}$, if one takes into account the larger error range. N_3^- is supposed to bind to type 2 Cu(II) at high concentrations (Morpurgo et al., 1974; Holwerda et al., 1976). Thus transverse ^1H NMR relaxation rates were measured in the presence of 0.2 M N_3^-, a concentration estimated to give at least partial saturation of type 2 Cu(II) at pH 7.0. Within experimental error range, the values obtained were essentially the same as those for the native protein.

^{17}O Magnetic Relaxation

In order to investigate the exchange behavior of the water oxygen coordinated to the type 2 Cu(II), we measured the longitudinal (T_1^{-1}) and transverse (T_2^{-1}) relaxation rates of ^{17}O magnetic resonance in $^{17}OH_2$ enriched laccase solutions. Experimental T_1^{-1} and T_2^{-1} values measured at pH 6.5 and 8.19 MHz are presented in Figure 4 as functions of the temperature, without any correction.

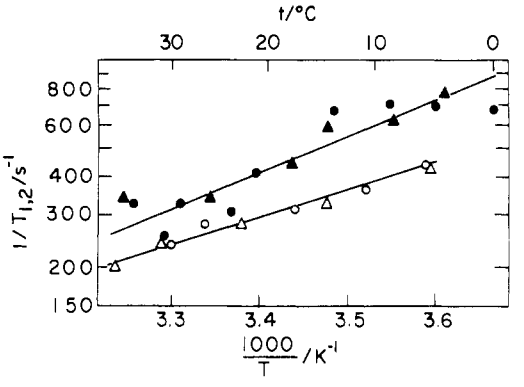


FIGURE 4: Longitudinal (○, △) and transverse (●, ▲) ^{17}O magnetic relaxation rates in solutions of *Rhus* laccase as functions of the temperature. The Larmor frequency was 8.19 MHz and the enzyme 0.4 mM at pH 6.5 in 0.0125 M potassium phosphate. The solutions contained ~15% $^{17}OH_2$. Circles refer to native laccase, triangles to the laccase–F^- complex.

Solvent, protein, and the paramagnetic Cu(II) ions are a priori expected to contribute to the ^{17}O magnetic relaxation rates, the latter two via water exchange with the bulk solvent (Connick & Stover, 1961; Swift & Connick, 1962; Koenig et al., 1975). The solvent component of T_1^{-1} was ~170 s^{-1} at 20 °C and under the conditions used, in good agreement with the literature values (Hindman et al., 1971). Few data about protein-induced ^{17}O magnetic relaxation have as yet been published. However, T_1^{-1} of ^{17}O should hardly be affected by paramagnetic species at concentrations as low as in the present experiment (0.4 mM). Hence, subtracting the solvent component from the measured value of T_1^{-1} yields the protein contribution, ~130 s^{-1} (20 °C). This value is fully consistent with the value for the diamagnetic part of the longitudinal proton relaxation rate given before. Using again the treatment described by Hallenga & Koenig (1976), but this time making the calculation for 8.2 MHz and an inflection frequency of 1.9 MHz and normalizing with respect to the pure water relaxation rates of ^1H (0.28 s^{-1}) and ^{17}O (170 s^{-1}), we obtain a protein-induced ^{17}O relaxation rate of 130 s^{-1} at the protein concentration used. The close agreement supports the implicit assumption that the enhanced relaxation of proton and ^{17}O resonances is due to the same molecular processes (Koenig et al., 1975). The temperature dependence of the ^{17}O relaxation rate induced by the interaction with the diamagnetic protein matrix was obtained from an Arrhenius plot of the differences between the individual T_1^{-1} values and the solvent contributions calculated for a given temperature according to Hindman et al. (1971). The apparent activation energy at 8.2 MHz is −3.2 ± 0.3 kcal/mol, a value which is typical of processes controlled by rotational or translational diffusion, and is consistent with data from other proteins of comparable size and collected at the same field (Hallenga & Koenig, 1976; Grösch & Noack, 1976).

T_2^{-1} exceeds T_1^{-1} by approximately 30–60% throughout the temperature range measured. Under the conditions of fast exchange between bound sites and the bulk solvent and motional narrowing in the latter, the T_1 process of a quadrupolar nucleus such as ^{17}O can always be analyzed as a single exponential relaxation (Bull et al., 1979). The transverse relaxation, however, is multiexponential, and the observed T_2 values are in fact approximate mean relaxation times. This makes a quantitative interpretation much more complex.

[1] At pH 7.0 and 20 °C the formation of the type 2 Cu(II)–fluoride complex, monitored at 320 nm, was found to follow a biphasic time course. The slow phase is a true first-order process with k_{obsd} = (2.1 ± 0.4) × 10^{-3} s^{-1} (three experiments with 0.02–0.10 M F^-). The total absorption change corresponds to $\Delta\epsilon_{320}$ = 700 M^{-1} cm^{-1}, closely similar to the pH 5.5 value given by Bränden et al. (1973).

Instead, the ^{17}O relaxation rates can be compared with the respective values measured in other solutions and proteins. Namely, the observed values of T_2^{-1} are much longer than found in protein-free medium and consistent with a decrease of the average H_2O mobility due to exchange between the bulk solvent and the hydration shell of the protein (Hallenga & Koenig, 1976). It is also in agreement with the data of Koenig et al. (1975), the ratio $T_1^{-1}/T_2^{-1} = 0.72$ for laccase being very similar to their value of 0.70 for lysozyme. Still, it could not be excluded that the difference between T_2^{-1} and T_1^{-1} might at least in part be the result of paramagnetic enhancement by the type 2 Cu(II). As concluded from literature data (Lewis et al., 1966a; Poupko & Luz, 1972), this contribution might amount to $\sim 50\ s^{-1}$.

In an attempt to clarify this point, a set of measurements was carried out on ^{17}O enriched solutions of the laccase–fluoride complex. However, the presence of fluoride failed to give any significant effect (Figure 4). This could be interpreted to mean either that the exchange of ^{17}O between the bulk solvent and the first coordination sphere of type 2 Cu(II) is too slow to be observed in the native enzyme or that F^- does not displace coordinated water. In a separate study on ^{17}O-induced line broadening in the type 2 Cu(II) EPR spectrum (M. Goldberg, Y. Siderer, and I. Pecht, unpublished experiments) it became apparent that this exchange is extremely slow. The time dependence of the line broadening at pH 7.0 and ambient temperature indicates a mean residence time of more than 10 min.[2]

Discussion

Whereas the ligands coordinated to the type 2 copper ion do not confer on it unusual spectroscopic properties, as proposed to be the case for the type 1 site (Fee, 1975), it is by virtue of a particular protein structure surrounding it that the type 2 site exhibits some unique features in its chemical behavior. Although the spectroscopic and magnetic properties of the type 2 cupric ion are similar to those of "normal" Cu(II) complexes, in contrast to the type 1 and type 3 copper sites (Fee, 1975), the way it interacts with water and extrinsic ligands is rather unusual. We shall attempt to get a consistent picture, however limited, of the "anomalous" behavior and dynamics of protons and water molecules in the coordination sphere of type 2 Cu(II) and of its structural basis. This is of particular interest in view of the postulated role of the type 2 copper as primary electron acceptor (Holwerda et al., 1976) or as terminal electron donor in the O_2 reduction (Brändén & Deinum, 1978), respectively.

The salient experimental facts that any model must account for are as follows. (1) $T_{1,pmg}^{-1}$ at pH 4, 20 °C, and 16 MHz is 5700–6000 $M^{-1}\ s^{-1}$ (depending on the outer-sphere contribution to $T_{1,n}^{-1}$ which is difficult to estimate), indicating that the paramagnetically relaxed protons exchange from a locus lying close to the Cu(II) ion. (2) The pH profile of $T_{1,n}^{-1}$ is characterized by two deprotonation steps with pK = 6.2 and 8.6, each one accounting for approximately 29–33% of $T_{1,pmg}^{-1}$, and a $T_{1,n}^{-1}$(pH >10.5) value ~50% lower than $T_{1,n}^{-1}$(pH 4) and containing the remaining 35–40% of $T_{1,pmg}^{-1}$. (3) No paramagnetic enhancement of the ^{17}O relaxation rate is observed (pH 6.5, 20 °C). This is consistent with the results of EPR experiments in ^{17}O-enriched solutions which show that the exchange half-time of at least one coordinated water oxygen is >10 min at pH 7.0 (to be published) and >30 min at pH 4.4 (Brändén & Deinum, 1977a). (4) As concluded from $^{17}OH_2$-induced EPR line broadening, one water molecule is coordinated to the type 2 Cu(II) in an equatorial position (Deinum & Vänngård, 1975; Brändén & Deinum, 1977a). (5) The effect of type 2 Cu(II) coordinated F^- on $T_{1,pmg}^{-1}$, and probably also on $T_{2,pmg}^{-1}$, in negligibly small (pH 6.7). The same holds for the effect of azide. (6) The EPR spectrum of type 2 Cu(II) indicates axial microsymmetry of its ligation structure.

These results are best rationalized in terms of equatorial coordination of one water molecule and one protein hydroxyl group to the type 2 Cu(II). The fast exchange of their protons with the bulk solution is responsible for the paramagnetic enhancement of the ^1H NMR relaxation rates, and, also, in the case of the water molecule this exchange involves individual protons and not the whole molecule. The two ligands hydrolyze with pK = 6.2 and 8.6, leading to a hydroxo–alkoxo complex at high pH.

This interpretation is based on the following findings and considerations. The primary evidence for equatorial coordination of water is the $^{17}OH_2$-induced EPR line broadening (Deinum & Vänngård, 1975; Brändén & Deinum, 1977a; Goldberg, 1980). The pH–$T_{1,n}^{-1}$ profile and the magnitude of $T_{1,pmg}^{-1}$ indicate that the protons dissociating with pK = 6.2 and 8.6 exchange from *equivalent* sites lying *close* to the Cu(II) ion. These pK values of 6.2 and 8.6 do not seem to be compatible with the ionization of putative axially bound water molecules.[3] At first look, all this might suggest the presence of two equatorially coordinated water molecules, since it would account for the equivalence of the exchange sites and the magnitude of the pK values (see below). However, the magnitude of the $^{17}OH_2$-induced EPR line broadening indicates that only one H_2O is bound in an equatorial position, and any additional H_2O, if at all present, is bound in an axial position (Deinum & Vänngård, 1975; Brändén & Deinum, 1977a). The only conceivable ligand which under these conditions can provide a proton exchange site structurally equivalent to H_2O is a hydroxyl group. Proton exchange taking place independently from water oxygen exchange is implied by the observed inertness of the type 2 Cu(II) bound water oxygen.

The postulated hydroxyl ligand is most probably of aliphatic character. The coordination of a tyrosine residue is less likely, because being a much stronger acid than water (pK = 10.1 vs. 15.7) it would be expected to deprotonate well below pH 6 while bound to Cu(II) in planar position [cf. Frost et al. (1958)]. Aliphatic hydroxyl groups, as present in serine and threonine residues, have rarely been suggested as ligands of Cu(II) in proteins, because their intrinsic binding affinity in

[2] It should be noted that these conclusions apply to H_2O exchange from (primarily equatorial) positions where the density of the electron hole is large enough for superhyperfine interaction between Cu(II) and ^{17}O to be significant. Since both paramagnetically enhanced ^{17}O relaxation and Cu(II) EPR line broadening result from this scalar interaction (Swift & Connick, 1962; Deinum & Vänngård, 1975), ^{17}O ligands exchanging from positions of negligible spin density would hardly give any measurable effect.

[3] Axial interaction with the Cu(II) center would be far too weak [cf. Bereman & Shields (1979)] to enhance the acid strength of H_2O molecules by respectively 9.5 and 7.1 pK units (from pK = 15.7 to pK = 6.2 and 8.6); at best it might lower the pK by up to ~4 units [cf. Terenzi et al. (1974)]. Even then, an unreasonably large effect of the protein environment would be required to give the pK values observed. In model complexes, the ionization of Cu(II)-bound water molecules and hydroxyl groups in the pK = 5–9 range is accompanied by spectral changes which imply in-plane coordination [cf. Hall et al. (1957), Murakami & Takagi (1965), Margerum et al. (1968), and Bai & Martell (1969), and references cited therein]; binding in the equatorial position is also strongly suggested by the effect of the number of nitrogen donors on the pK of the coordinated H_2O [two N, pK_1 = 6.3–7.7; three N, pK > 8.5; cf. Allison & Angelici (1971) and references cited therein and Hauer et al. (1971)].

aqueous solution is rather weak (Friedman & Plane, 1963). However, when suitably combined with a stronger ligand(s) in a bi- or polydentate chelate, the hydroxyl group is found to participate in complex formation (Hall et al., 1960; Murakami & Takagi, 1965; Bai & Martell, 1969, and references cited therein; Sigel & McCormick, 1970; Hughes et al., 1972; Dotson, 1972). Further important factors which, in addition to the chelate effect, will enhance the strength of hydroxyl coordination to Cu(II) in a protein binding site are a lower water activity and a relatively hydrophobic environment (compared to aqueous solution) at such a site. This presumably corresponds to the conditions prevailing at the type 2 site [see below; Desideri et al. (1979)]. Furthermore, histidine imidazole groups, the coordination of which to the type 2 Cu(II) was recently shown (Mondovi et al., 1977), tend to stabilize mixed coordination spheres with oxygen ligands (Sigel, 1973).

Each of the protons dissociating with pK = 6.2 and 8.6 contributes ~1750 M^{-1} s^{-1} to the paramagnetic ^1H NMR relaxation rate at 15.6 MHz (Figure 3). Now, under fast-exchange conditions $T_{1,pmg}^{-1}$ is related to the distance between the paramagnetic center and the site of proton exchange (Solomon, 1955; Bloembergen, 1957). Applying the usual equation (Dwek, 1973), assuming one fast exchanging proton, neglecting any contact interaction, and using the τ_c value determined from the 10–60-MHz dispersion data, one calculates from $T_{1,pmg}^{-1}$ = 1750 M^{-1} s^{-1} a Cu(II)–proton distance of 3.1 Å. Although this value is within the range of distances reported for other copper proteins (Gaber et al., 1972), it is certainly larger than expected for equatorially bound water or hydroxyl (approximately 2.6–2.7 Å). After considering a number of possible reasons for this difference, it seems that the most probable cause is an underlying (masked) frequency dependence of τ_c or τ_s (Koenig & Brown, 1973), which leads to deviations of calculated Cu(II)–proton distances toward higher values.[4] Taking this into account, the calculated Cu(II)–proton distance is reasonably consistent with our model.

The third component of $T_{1,pmg}^{-1}$ is attributed to the associative interchange of the proton which at high pH remains bound as the proton of the Cu(II)-coordinated hydroxy anion. Its value can in principle be estimated from T_1^{-1}(pH >10) by subtracting the diamagnetic and outer-sphere contributions to T_1^{-1}. The value thus obtained, 2200–2500 M^{-1} s^{-1}, is somewhat larger than the $T_{1,pmg}^{-1}$ contribution of the protons exchanging from the hydroxyl group or H_2O. One should not, however, attach too much importance to this difference, since the estimate for the outer-sphere contributions of type 1 and type 2 Cu(II) (400–700 M^{-1} s^{-1}) is rather tentative. For instance, the carbohydrate "sheet" of laccase which accounts for 45% of the molecular weight (Reinhammar, 1970) might lower the self-diffusion rate of water in the vicinity of these copper sites, thereby increasing the outer-sphere effect.

The two pK values 6.2 and 8.6, attributed to the ionization of a water molecule and an aliphatic hydroxyl group coordinated to the type 2 Cu(II), are reasonably consistent with pK values displayed by Cu(II) complexes containing these ligands in an equatorial position (Doran et al., 1964, and references cited therein; Perrin & Sharma, 1967; Sigel, 1968; Davies & Patel, 1968). Moreover, the difference between the two pK values, 2.4, agrees well with pK differences typically observed in $Cu^{II}N_2O_2$ systems where the oxygen ligands are water molecules and/or hydroxyl groups (Courtney et al., 1959; Gustafson & Martell, 1959; Hall et al., 1960; Brauner & Schwarzenbach, 1962). The lower pK is ~1 unit less than typically found, possibly due to the relatively hydrophobic environment of the type 2 site which should enhance the avidity of the charged Cu(II) ion for negatively charged ligands such as OH^-. Type 2 Cu(II) coordinated histidine residues (Mondovi et al., 1977) might also contribute, as unsaturated nitrogen donors, like the imidazole group, preferentially stabilize the coordination of negatively charged oxygen donor atoms (Murakami & Takagi, 1965; Sundberg & Martin, 1974). A lack of axial ligands to type 2 Cu(II) in the native enzyme would also lower the pK (Margerum et al., 1968).

At least two properties of laccase which are pH dependent may be related to the pK = 6.2 hydrolysis: (1) the transition from resolved to unresolved hyperfine splitting in the $g_\perp$ region of the type 2 Cu(II) EPR spectrum reported to occur around pH 6 (Bränden & Deinum, 1977b) and (2) the pH dependence of anion binding to the type 2 Cu(II) which is generally found to be strong at pH <6 yet weaker at pH >7.

The Cu(II) derivative of carbonic anhydrase which binds one water molecule in an approximately planar position (Lindskog et al., 1971; Taylor & Coleman, 1971) has a pK of 8.0 (Morpurgo et al., 1975), not far from the value for laccase. In contrast, the single water molecule coordinated to the Cu(II) site of superoxide dismutase hydrolyzes at much higher pH (Rotilio et al., 1972). Yet in both cases deprotonation was found to enhance $T_{1,pmg}^{-1}$ (Koenig & Brown, 1973; Terenzi et al., 1974), opposite to the direction of change in laccase (in all three cases the pH effect was measured at a field where $\omega_1\tau_c < 1$). This difference, however, is not in conflict with the laccase data and their interpretation. One possible reason for the increase of $T_{1,pmg}^{-1}$ as observed in superoxide dimutase and copper(II) carbonic anhydrase is the effect of deprotonation on the ligand field, as documented by pronounced EPR changes (Taylor & Coleman, 1971; Rotilio et al., 1972), which might affect either the expectation value of the proton–electron distance (Terenzi et al., 1974) or τ_s [only relevant as far as $R_{1,pmg}$ is dominated by it (Koenig & Brown, 1973; Gaber et al., 1972)].[5] No such effect is produced in laccase, as the EPR parameters of the type 2 Cu(II) remain practically unchanged up to pH 12, i.e., upon deprotonation of the protic ligands (Malmström et al., 1970). An alternative explanation for the $T_{1,pmg}^{-1}$ increase exists for the case that the correlation time is dominated by the exchange rate $1/\tau_M$ of Cu(II)-bound water (Fee & Gaber, 1973): substitution of H_2O by OH^- lowers the net exchange rate, thereby leading to an increase in $T_{1,pmg}^{-1}$.[5] The fact that this is not observed in laccase is consistent with the slow exchange of the type 2 Cu(II)-coordinated water molecule.

Keeping in mind the proposed coordination of equatorial water and hydroxyl group, the absence of a pronounced F^- effect on $T_{1,pmg}^{-1}$ and $T_{2,pmg}^{-1}$ throughout the temperature range measured (Figure 1) seems to exclude the displacement by F^- of the protic type 2 Cu(II) ligands responsible for the paramagnetic ^1H NMR relaxation. This suggests that F^- may bind in an axial position which in the native state is either free or occupied by a weak protein ligand. The coordination of

[4] In order to illustrate this point, we replotted the frequency-dependent $T_{1,pmg}^{-1}$ rates of superoxide dismutase [given by Fee & Gaber (1973)] according to $T_{1,pmg}$ vs. ν_L^2 and obtained an excellent straight line (r^2 = 0.998) for the 0 °C values in the 5–50-MHz range. The apparent correlation time, calculated from slope and intercept, is 4.07×10^{-9} s, leading to an apparent Cu(II)–proton distance of 3.2 Å. In contrast, the value obtained from $T_{1,pmg}^{-1}(\nu \to 0)$ and the correlation time at low field, 5×10^{-10} s, is 2.6 Å (Gaber et al., 1972).

[5] Note that in any case the intrinsic enhancement must be larger than the observed one, since the average number of protons present decreases from 2 to 1.

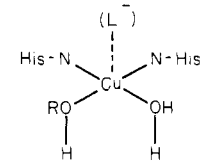
an axial water seems unlikely since it is hard to conceive that a metal ion inherently as labile as Cu(II) could be bound to a fluctuating protein matrix [cf. Munro et al. (1979)] as tightly as in the type 2 site of *Rhus* laccase and be as inert with respect to H_2O and F^- coordination as reported above, while having both equatorial and axial positions accessible to solvent molecules. This interpretation is strongly supported by the experiments with N_3^-, which also fails to show a significant effect on $T_{2,pmg}^{-1}$, and is consistent with conclusions drawn from the binding of different pseudohalide anions to type 2 Cu(II) (Desideri et al., 1979).

The inertness of the type 2 Cu(II) coordinated water oxygen is in marked contrast to the extremely fast rate of water exchange from the coordination sphere of small Cu(II) complexes (Eigen & Wilkins, 1965; Poupko & Luz, 1972; Hague, 1977). In fact, the water oxygen exchange proceeds up to 12 orders of magnitude more slowly, corresponding to an additional activation energy on the order of 16 kcal/mol. It is evident that neither stereoelectronic constraints like preventing exchange from an axial position (Eigen & Wilkins, 1965), nor interaction with neighboring groups, nor even extended hydrogen binding (Morpurgo et al., 1975) could suffice to stabilize Cu(II)-bound water oxygen to such an extent. The only efficient means is a steric barrier between the Cu(II)-coordinated water and the bulk. The same steric barrier acts also on extrinsic ligands, e.g., F^- or N_3^- ions. Although the former possess an outstanding capacity for hydrogen bonding, this cannot explain their extremely slow association rate. In fact, the rate-limiting first-order step is between $\sim 1 \times 10^{11}$ and $\sim 1 \times 10^{13}$ times slower than the rate of F^- association to $Cu(II)_{(aq)}$ divided by the outer-sphere complex formation constant (this paper, footnote 1; Bränden et al., 1973; Eisenstadt & Friedman, 1968; Hague, 1977). This amounts to an extra activation energy strikingly close to that of water oxygen exchange. The association of N_3^- is affected to a similar extent (Holwerda & Gray, 1974).

Indeed, the type 2 site has been proposed to be located in a cavity of the protein (Bränden & Deinum, 1977a). We suggest that this cavity is connected to the bulk through an orifice which in the oxidized laccase prevents the passage of whole water molecules and anions such as F^- but allows the unhindered transfer of protons. Thus, in the oxidized enzyme access to as well as dissociation from the type 2 copper ion requires a conformational change, a "widening of the orifice", with an activation energy on the order of 12–15 kcal/mol at ambient temperature. It is proposed that the slow monomolecular step in the binding of F^- and N_3^- to type 2 Cu(II) (Bränden et al., 1973; Holwerda & Gray, 1974) is related to this conformational rearrangement. This corresponds to the formation of an activated laccase intermediate prior to the association of the incoming anion (Fee, 1975). Water and extrinsic ligand exchange processes at the type 2 site are only efficient when the enzyme is reduced. Presumably the conformational state controlling the access to this site is coupled to the oxidation state of the enzyme or one of its copper sites.

Postulating that protons exchange from an "inert" water oxygen and a protein hydroxyl group inside a cavity through an orifice to the bulk solution at a rate between 5×10^5 and 2×10^8 s^{-1} calls for some comment. It is obvious that such a process can only take place if promoted by efficient intramolecular acid–base catalysis. The protic ligands of the type 2 Cu(II), with their broad buffer range from pH 5 to 10, should be excellent proton donors or/and acceptors, respectively (Eigen & Kruse, 1963). The only special requirement is a suitable acid–base function which, positioned at the orifice, mediates the proton transfer between the cavity and the buffer (and OH^-) ions in the bulk. Transfer to and from buffer ions should then be diffusion controlled (Luz & Meiboom, 1965). Intramolecular proton transfer from an acid–base function located at the orifice of the cavity to the type 2 site might be of catalytic significance as this site seems to be involved in the reduction and protonation of an O^- intermediate to water. Circumstantial evidence for this suggestion is provided by the observation of rapid proton exchange between the bulk solvent and this intermediate (Bränden & Deinum, 1978).

On the basis of the results of this and other studies (Mondovi et al., 1977; Bränden & Deinum, 1977a), we tentatively propose structure I for the type 2 site in the native state. The



I

EPR spectrum of the type 2 Cu(II) implies an essentially planar arrangement of the equatorial ligands. One axial position is thought to be available to exogenous anionic ligands. The proposed N_2O_2 ligation is consistent with our ionization data (see above and footnote 3) and with the EPR parameters of the type 2 site (Peisach & Blumberg, 1974). It has also been proposed for the "nonblue" copper site of galactose oxidase (Bereman et al., 1977). Furthermore, it perfectly fits the redox potential of the type 2 (II/I) couple [+360 mV; Farver et al. (1978)]. Diimidazole diaquocopper has a potential of +348 mV, and, as a rule, one would expect the potential of a tetragonal N_2O_2 system to be $\sim$350 mV (Brill et al., 1964).

The participation of imidazole in the ligand sphere is of functional importance, as the coordination of O ligands such as H_2O is best stabilized by unsaturated N ligands (Sigel, 1973). The hydroxyl ligand, from a serine or threonine residue, might act as the proton donor in the reduction of O_2 to water which is known to involve the type 2 site (Bränden & Deinum, 1977). Both its p*K* (6.2 or 8.6) and its proximity to the actual electron transfer site make it a highly efficient potential proton donor to O_2 reduction intermediates.

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Carbon-13 Nuclear Magnetic Resonance Relaxation Studies of Internal Mobility of the Polypeptide Chain in Basic Pancreatic Trypsin Inhibitor and a Selectively Reduced Analogue†

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ABSTRACT: ^{13}C nuclear spin relaxation times and ^{13}C (^1H) nuclear Overhauser effects for the backbone α carbons, the protonated aromatic ring carbons, and the side-chain methyl carbons were measured in 25 mM solutions of the basic pancreatic trypsin inhibitor and a modified analogue obtained by reduction of the disulfide bond 14–38. The relaxation parameters for the methyl carbons could, on the basis of previous individual assignments, be correlated with specific locations in the molecular structure. Analysis in terms of a "wobbling in a cone" model, where isotropic overall rotational motion of the protein was assumed, showed that, in addition to the overall rotational motions of the molecule and the rotation of the methyl groups about the C–C bond, the relaxation data manifested librational motions of the polypeptide backbone and the amino acid side chains. The following parameters for the molecular mobility resulted from this analysis: for the overall rotational motions, $\tau_R = 4 \times 10^{-9}$ s; for the librational "wobbling" of the backbone α carbons, in a cone with $\theta_{max} = 20°$, $\tau_w = 1 \times 10^{-9}$ s; for the librational motions of individual aliphatic side chains in cones with θ_{max} varying between 30° and 60°, $\tau_w = 4 \times 10^{-10}$–$3 \times 10^{-9}$ s; for methyl rotation about the C–C bond, $\tau_F \lesssim 1 \times 10^{-11}$ s. From comparison of the two proteins, the molecular motions manifested in the ^{13}C relaxation parameters were found not to be correlated with the thermal stability of the globular conformation. This coincides with the behavior of aromatic ring flips and is different from that of the exchange rates for interior amide protons, which provides new information to further characterize the previously suggested hydrophobic cluster structure for globular proteins in solution.

The small globular protein BPTI,[1] which consists of a single polypeptide chain of 58 amino acid residues and has a molecular weight of 6500, has been used extensively for studies of fundamental aspects of protein conformation. A highly refined crystal structure at 1.5-Å resolution is available (Deisenhofer & Steigemann, 1975), and high-resolution NMR studies showed that the average spatial structure in aqueous solution corresponds very closely to that seen in single crystals (Richarz & Wüthrich, 1978; Wüthrich et al., 1978; Perkins & Wüthrich, 1978, 1979; Dubs et al., 1979). ^1H NMR was further used to investigate dynamic aspects of the globular form of BPTI and related proteins. These studies have so far mainly concentrated on measurements of the internal mobility of aromatic rings (Wagner et al., 1976), exchange of internal amide protons with the solvent (Wüthrich & Wagner, 1979; Richarz et al., 1979; Wagner & Wüthrich, 1979a), and thermal denaturation (Wagner & Wüthrich, 1978; Wüthrich et al., 1979a,b), i.e., relatively infrequent stochastic events, with characteristic times of $\gtrsim 1 \times 10^{-5}$ s, which consist of concerted motions involving sizable fractions of the protein structure (Hetzel et al., 1976; Wagner & Wüthrich, 1978, 1979b; Wüthrich & Wagner, 1978; Wüthrich et al., 1979b). In contrast, NMR relaxation studies (Doddrell et al., 1972; Wüthrich, 1976), X-ray techniques (Artymiuk et al., 1979; Frauenfelder et al., 1979; Huber, 1979), and molecular dynamics calculations (McCammon et al., 1977; Karplus & McCammon, 1979) have so far, for physical or practical reasons, provided exclusively information relating to much more frequent events, in the time range 1×10^{-8}–1×10^{-12} s. The present paper describes an attempt to correlate structural information obtained from measurements on largely different time scales. It reports on comparative ^{13}C NMR relaxation studies of BPTI and a chemically modified analogue, which were previously also investigated by the above-mentioned "slow" ^1H NMR experiments.

The strategy for the present investigation was influenced by previously recorded data on the ^{13}C NMR spectra of BPTI as well as by certain practical considerations. Since the relaxation parameters were markedly affected by higher BPTI concentrations (Wüthrich & Baumann, 1976), the present

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[1] Abbreviations used: BPTI, basic pancreatic trypsin inhibitor (Kunitz inhibitor, Trasylol, Bayer Wuppertal, Germany); RCAM-BPTI, basic pancreatic trypsin inhibitor obtained by reduction of the disulfide bond 14–38 with the cysteinyl residues protected by carboxamidomethylation; NMR, nuclear magnetic resonance; FT, Fourier transform; ppm, parts per million; Me_4Si, tetramethylsilane; TSP, 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate; NOE, nuclear Overhauser enhancement.

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