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Environment of the Heme in Myoglobins. NMRD and EPR Spectroscopy of Val68X (X = Asn, Asp, and Glu) Mutants of Human Myoglobin

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Abstract: Analyses of the temperature dependences and shapes of nuclear magnetic relaxation dispersion (NMRD) signals of site-directed mutants of human myoglobin indicate that a water molecule is bound to the sixth coordination site of the ferric heme in proteins in which the valine at position 68 is changed to either aspartate (Val68Asp) or asparagine (Val68Asn). Both NMRD data and electron paramagetic resonance (EPR) spectra show that carboxylate is axially ligated to Fe(III) in the Val68Glu mutant. The EPR spectra of the Val68Asp and Val68Asn derivatives show much smaller rhombic splittings than the spectrum of the Val68Glu protein.

Introduction

Heme proteins play key functional roles as electron carriers and oxygen reductants in biological systems.[1] Several enzymatic transformations also are carried out by these proteins, including hydrogen peroxide disproportionation (catalase),[2] stereo- and substrate-specific peroxidation (cytochrome *c* peroxidase)[3] and hydroxylation (cytochrome P-450),[4] and oxygen transport (myoglobin and hemoglobin).[5] Although there are small differences in the covalent structure of the porphyrin ring of the heme as it occurs in these natural systems (e.g., cytochromes *a*, *b*, and *c*),[6] the surrounding protein medium is thought to be mainly responsible for the differences in heme activities.[7]

In a previous study, we measured the temperature dependences of the reduction potentials ($E^{\circ\prime}$) of wild-type and three site-directed mutants of human myoglobin.[8] The decreased $E^{\circ\prime}$ values (200 mV for Val68Glu and Val68Asp mutants and 80 mV for the Val68Asn protein) were explained in terms of electrostatic effects, pH effects, protein conformational changes, and specific bonding changes at the heme site (Figure 1). We have now extended this study to include NMRD and EPR experiments to probe whether a water molecule is present in the Val68Asn and Val68Asp proteins and if so, whether it is replaced by the Glu carboxylate in the Val68Glu mutant.[8]

In NMRD, the spin–lattice relaxation time (T_1^{-1}) of the bulk water protons is measured as a function of magnetic field strength in the presence of a solute that facilitates relaxation. Metalloproteins relax water protons by a variety of mechanisms. The dominant mechanisms of relaxation can be deduced from the

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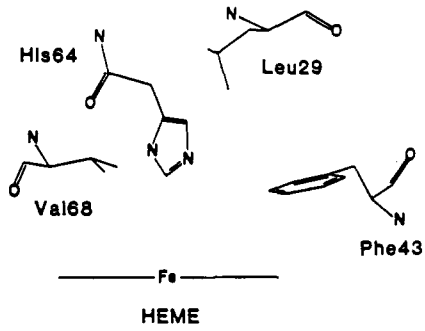

Figure 1. A view of the heme pocket in native myoglobin.[14]

NMRD profile, thereby determining how water molecules interact with the metal center in the protein. Davidson and co-workers were the first to carry out experiments of this sort on hemoglobin and myoglobin,[9] and many other metalloproteins have been studied subsequently.[10-13] Here we report the results of NMRD and EPR experiments that have enabled us to define more precisely the environment of the heme in Val68X (X = Asn, Asp, and Glu) mutants of human myoglobin.

Experimental Section

Materials and Methods. Protein samples were prepared as previously described[14] and chromatographed on a size exclusion (Sephadex, G-25) column prior to use. They were then concentrated to between 2 and 4 mM using ultrafiltration (Centricon, YM10). All samples were in μ 0.1 M, pH 7.0 sodium phosphate buffer (reagent grade, Baker). A 3-fold excess of NaCN (reagent grade, Baker) was added to convert native myoglobin to cyanometmyoglobin. Due to scarcity of sample, the volume of 100 μL was placed inside a 4-mm-diameter tube that was then placed inside the 10-mm tube normally used for these experiments. A copper coil was subsequently wound around the smaller tube. It was found that the data were reproducible with a standard deviation of ±2% using this modification. The relaxation rates were determined directly by observing the exponential change in the magnetization of solvent water protons in response to changes in the applied magnetic field. The measurements were performed using a field-cycling relaxometer in the proton Larmor frequency range of 0.01–50.0 MHz.[15]

EPR spectra were typically recorded on 0.2-mL samples containing the protein at a concentration of 1 mM in a 4-mm-diameter quartz tube. All oxygen was removed from the sample by cycling it on a vacuum ($\sim 10^{-3}$ Torr)/argon dual-manifold line. All samples were lyophilized and then rehydrated in μ 0.01 M sodium phosphate buffer, pH 7.0. The $^{17}OH_2$ was 37.5% atom-pure, normalized $^{17}OH_2$ (MSD, Inc.).

The EPR spectra were recorded on a Varian E-line Century Series X-band spectrometer equipped with an Oxford Instruments cryostat (Model no. C1167A). Liquid helium was pumped through the system as a heat exchanger. At temperatures above 50 K electric heating of the sample was required. Temperatures were monitored with a thermocouple placed inside the cryostat and double-checked with a thermocouple placed inside a dummy EPR tube so that the temperature measurement error was typically less than 0.05 K. Further, at each temperature a power dependence study was performed so that the maximum power that did not saturate the signal could be chosen.

Equations. The experimental longitudinal relaxation rate T_1^{-1} is given by three terms (eq 1):[16,17]

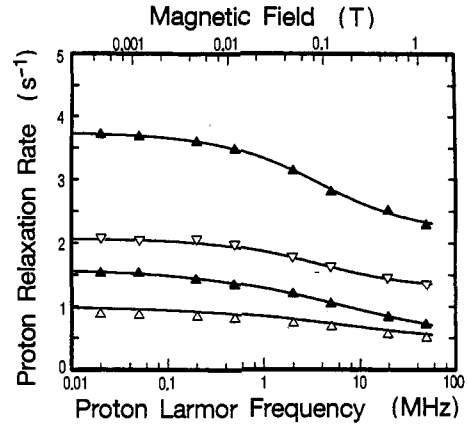

Figure 2. NMRD profiles for metaquomyoglobin (solid triangles) and cyanometmyoglobin (open triangles). The lower curves are for 2.1 mM protein; the higher curves are for 4.2 mM protein.

$$T_1^{-1} = T_{1B}^{-1} + T_{1D}^{-1} + T_{1P}^{-1} \quad (1)$$

where T_{1B}^{-1} is the background relaxation rate of the aqueous system including the buffer ($\approx 0.3\ s^{-1}$), T_{1D}^{-1} is the diamagnetic contribution to relaxation from the protein, and T_{1p}^{-1} is the paramagnetic contribution due to the unpaired electrons–proton interaction. The derivation of T_{1D}^{-1} has proved to be a very difficult problem. At first T_{1D}^{-1} was thought to be field dependent with a dispersion frequency determined by the rotational relaxation time of the solute molecules.[18] However, later work has shown that up to half of the diamagnetic relaxation can arise from cross relaxation between protein and solvent protons.[19] Since a paramagnetic center in the protein would alter the protein proton relaxation rates through contact and dipolar interactions, there is no assurance that the two contributions T_{1D}^{-1} and T_{1P}^{-1} can be entirely separated.[20] With the present theory, we have no way of quantifying this potential correction. Instead, we know that for a similar system, hemoglobin, all of the $S = 0$ and $S = 1/2$ heme derivatives tested (oxy, carbonmonoxy, deoxy, azidomet, and cyanomet) have essentially the same relaxation profile.[20] Using this profile (at appropriate temperature, concentration, and pH) as the diamagnetic correction allowed the paramagnetic $S = 5/2$ data for hemoglobin to be fit well with existing dipole relaxation theory;[20] therefore, we used the same procedure for myoglobin, with cyanometmyoglobin specifically as the diamagnetic control. The diamagnetic correction was linear within the concentration ranges we used (Figure 2).

T_{1P}^{-1} also contains several contributions. One comes from the interaction between the proton and the unpaired electrons if there is a binding site for the proton that allows the interaction. This contribution is defined as T_{1M}^{-1}.[16,21] A second contribution comes from the exchange rate. If the exchange time between bulk water and the binding site is on the order of T_{1M}^{-1}, then eq 2 holds:[22]

$$(fT_{1p})^{-1} = (T_{1M} + \tau_M)^{-1} \quad (2)$$

where f is the molar fraction of interacting protons; for methemoglobin, the proton of the bound water exchanges slowly and τ_M determines T_{1p}.[20,23,24] Contributions to T_{1M}^{-1} may in principle come from several mechanisms;[21] however, it is generally assumed that the dominant mechanism is the dipolar coupling between the proton magnetic moment and the electron magnetic moment, even when the water molecule is directly coordinated to the metal ion.[25] The equation for the dipolar contribution to the nuclear relaxation is due to Solomon (eq 3):[26]

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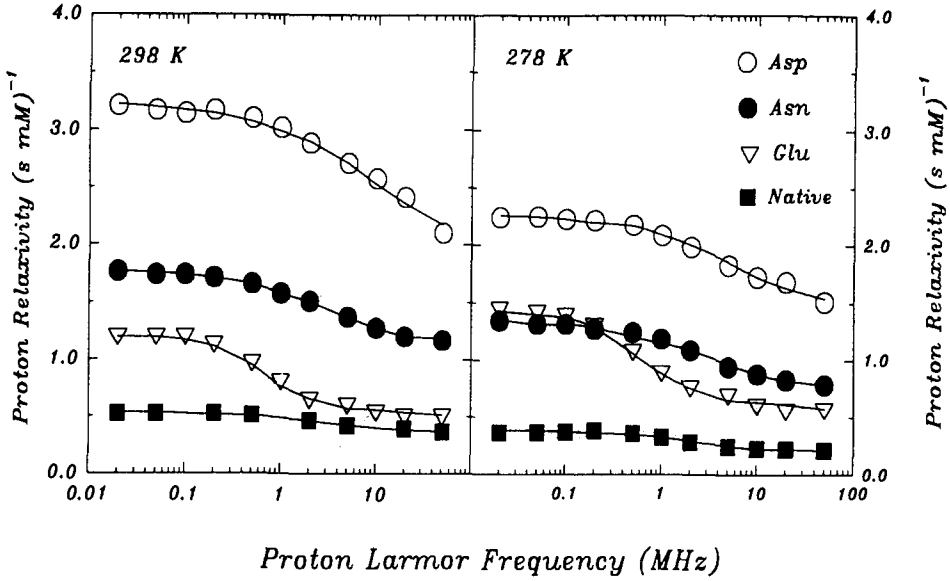

Figure 3. NMRD profiles for native and three Val68X mutant proteins at two different temperatures. The solid lines are the best-fit curves (including an outer-sphere contribution plus a slowly exchanging water) for the native, Asp, and Asn derivatives. In the case of the Glu mutant, the Solomon equation (eq 3) including ZFS was used with a **D** value larger than $\hbar\tau_c^{-1}$ (the parameter[28] that indicates the angle between the average Fe–H vector and the z axis of ZFS was found to be between 40° and 50°).

$$T_{1M}^{-1} = \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \gamma N^2 \mu_B^2 g_e^2 S(S+1)\langle r^{-6}\rangle \left[\frac{3\tau_c}{1+\omega_I^2 \tau_c^2} + \frac{7\tau_c}{1+\omega_S^2 \tau_c^2}\right] \quad (3)$$

where ω_I and ω_S are the nuclear and electron Larmor frequencies, respectively, and r is the proton–metal center distance. The Solomon equation holds as long as the splitting of the S manifold at zero magnetic field is smaller than the Zeeman energy. This may not be the case for high-spin iron(III), as the zero field splitting (ZFS) can be as large as 50 cm^{-1}.[27] The effect of the ZFS on nuclear relaxation for $S = 5/2$ has been applied to the manganese(II) case.[28] The T_{1M}^{-1} values can be numerically computed following the Solomon approach after the ZFS has been included in the static spin Hamiltonian.

The value of τ_c is given by eq 4,

$$\tau_c^{-1} = \tau_s^{-1} + \tau_r^{-1} + \tau_M^{-1} \quad (4)$$

where τ_s is the electronic relaxation time and τ_r is the rotational correlation time. The latter is on the order of 10^{-9} s for a protein of 17 kDa,[29,30] whereas the electronic relaxation time is expected to be 1 order of magnitude smaller (*vide infra*). τ_M is much longer and of no importance in this case.

In the absence of (or in addition to) a binding site for water, water proton relaxation can occur when a water molecule approaches the paramagnetic metal ion without a fixed time in a given site. This is called outer-sphere relaxation,[20,21] the mechanism being dipolar in origin, eq 5:

$$\frac{1}{T_{1OS}} = \left(\frac{\mu_0}{4\pi}\right)^2\left(\frac{32\pi}{405}\right)\gamma_I^2\gamma_S^2\left(\frac{\hbar}{2\pi}\right)^2 S(S+1)(1000N_A)\left(\frac{[M]}{dD}\right) \{3J_{OS}(\omega_I) + 7J_{OS}(\omega_S)\} \quad (5)$$

where N_A is Avogadro's constant, d is the distance of closest approach, and D is the sum of the diffusion coefficients of the solute and solvent.[31,32] J_{OS}s are the spectral densities, eq 6,

$$J_{OS}(\omega) = \frac{1 + 5z/8 + z^2/8}{1 + z + z^2/2 + z^3/6 + 4z^4/81 + z^5/81 + z^6/648} \quad (6)$$

with $z = (2\omega\tau_d)^{1/2}$; τ_d is the translational diffusion correlation time, eq 7:[29]

$$\tau_d = d^2/D \quad (7)$$

The spin Hamiltonian describing the EPR spectra of iron sites of less than cubic symmetry includes two ZFS parameters, **D** and **E**. An axial distortion from cubic symmetry is represented by the **D** term, while a planar or rhombic distortion is expressed in the **E** term. The spin Hamiltonian can then be represented as a sum of these two terms and the Zeeman energy, eq 8:[33]

$$H = \beta B \cdot g \cdot S + \mathbf{D}[S_z^2 - 1/3S(S+1)] + \mathbf{E}(S_x^2 - S_y^2) \quad (8)$$

The relative magnitudes of these terms will determine the fields at which EPR transitions are observed, assuming quartic terms are negligible. The Zeeman term is small for $S = 5/2$ Fe(III), especially compared to **D**, so that the position of spectral transitions (g values) will depend mainly upon the **D** and **E** values. For a high-spin ferric ion with appreciable ZFS (2**D**), the in-plane g values for the ground-state doublet are, to second order in **E**/**D** and η,

$$g_{x,y} = 6.01 \pm 24\mathbf{E}/\mathbf{D} - 18.7(\mathbf{E}/\mathbf{D})^2 - 12\eta^2 \quad (9)$$

where **E**/**D** is the ratio of rhombic to axial ligand field strength and $(6\eta)^{1/2}$ indicates the admixture of the $S = 5/2$ and $S = 3/2$ (quartet) spin states.[34]

Results and Discussion

The paramagnetic contribution to the longitudinal relaxation of native human myoglobin was obtained by subtracting the normalized CN-met (cyanometmyoglobin) NMRD values from the native metmyoglobin dispersion curve (Figure 2). Analogous results for all four proteins are shown in Figure 3. The profiles of metmyoglobin and its behavior with temperature are very similar to those of methemoglobin. The values of T_{1p}^{-1} are small, in accord with results already reported[20] for hemoglobin. The values of T_{1p}^{-1} for the mutants are higher, in agreement with expectation, since a hydrophobic group (Val) has been replaced by more hydrophilic residues.

The first striking result is that the four (if the native protein is included) myoglobins behave so differently. The low magnetic field relaxivities span a factor of 3, and the inflection points of

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Table 1. NMRD Parameters for Native and Val68X Human Myoglobins[a]

	native		Asp		Asn		Glu	
τ_M (s)	107	115	15	14	32	20		
τ_d (ps)	214	84	64	22	139	86	690[b]	510[b]
d (Å)	15	10	5.7	3.3	8.2	6.8	4.6[c]	4.4[c]
$D \times 10^5$ (cm^2 s^{-1})	10	13	5.2	5.0	4.8	5.3		

[a] Temperatures are 278 (left column) and 298 K (right column) in each case. [b] τ_s (ps) value. [c] r (Å) value.

the dispersions occur at a few megahertz in the native, Glu, and Asn proteins, whereas this inflection is ill-shaped and around 20 MHz for the Asp derivative. By decreasing the temperature, the relaxivity dramatically decreases for the Asp, Asn, and native systems, whereas it increases for the Glu derivative. For the Asp and Asn proteins, the values of the field of inflection decrease with increasing temperature, but the reverse is observed for the Glu derivative. The interpretation of such complex behavior can be attempted within a general model in which a coordinated water molecule and a second water molecule are coupled to the paramagnetic center. The former molecule would be in slow exchange, as suggested for metmyoglobin, and the main contribution to relaxivity would be given by eq 2, where τ_M is much larger than T_{1M}. This contribution is magnetic field independent and increases with increasing temperature, because τ_M changes in the same way.[21] The Asp and Asn metmyoglobin mutants satisfy the temperature dependence if (as is the case in the native protein)[35] there is a slowly exchanging water molecule in the sixth coordination position of the heme. The Glu mutant does not fit this scheme.

The second water molecule coupled to the paramagnetic center is responsible for the overall shape of the profile. This molecule would be in rapid exchange (τ_M would not contribute to T_{1p}), and the profile could be understood in the Solomon framework (eq 3) if there is a binding site with a residence time that is long with respect to the diffusion time, or through an outer-sphere model (eq 5). Whatever the model, the qualitative interpretation of the data is that the Asp derivative has a second water molecule close to the iron that is diffusing rapidly. In the Asn and Glu proteins, this type of water proton makes a smaller contribution to the relaxivity. It is likely that there are more protons interacting with the paramagnetic center in the Asp derivative than in the case of fluoromethemoglobin, since the low magnetic field relaxivities are comparable but τ_c is larger for the latter protein.[20] In fluoromethemoglobin the fluoride is covalently bound to iron and H-bonded to a water proton.

A best fit of the data employs a sum of eqs 2 and 5 (for the slow exchanging and the fast diffusing waters, respectively). The best-fit parameters are set out in Table 1. The parameters for the native protein are similar to those of methemoglobin.[20] In point of fact, there is a large covariance among the parameters, and their values should be viewed cautiously. There is a water molecule that exchanges slowly and an outer-sphere water with a relatively large distance of closest approach. Accordingly, d is long; the value of D is slightly larger than that for methemoglobin. The parameters for the Asp and Asn derivatives are completely in line with the above qualitative discussion; there are contributions from the coordinated water and from outer-sphere water. The temperature variation of τ_M is consistent with the presence of a slowly exchanging water; the diffusion constant D is close to that of methemoglobin;[20] and the distances of closest approach are shorter than in the native protein. In all cases the fits are much poorer if eqs 2 and 3 are used (even if the ZFS is included in eq 3).

The presence of slowly exchanging water in the Glu protein can be ruled out since the relaxivity decreases with increasing temperature. (However, the 278 K profile gives a relaxivity ratio between high and low fields that is smaller than the 10:3 predicted by eq 5.) The fit to Solomon's equation (eq 3) including ZFS[28] is satisfactory; the correlation time is 5×10^{-10} s at 298 K (see Table 1), which is similar to the electronic relaxation time of hemoglobin.[20] Therefore, it is likely that the exchangeable proton coupled to the paramagnetic center in the Glu mutant has a longer residence time than in the case of the rapidly diffusing water in the Asp and Asn derivatives, owing to hydrogen bonding similar to that in fluoromethemoglobin.[20] (Of course, this proton is still in the fast-exchange region.) The calculated distance is 4.5 ± 0.3 Å, which is quite reasonable for a water in the heme pocket that is hydrogen bonded to the nonligated oxygen of a coordinated carboxylate group.

EPR measurements give data complementary to the results of the NMRD experiments. Since the heme Fe(III) is essentially axially symmetric (D is large),[36] electronic spin transitions occur at different energies when the magnetic field is applied perpendicular ($g = 6$) and parallel ($g = 2$) to the porphyrin plane (eq 7). Both the native myoglobin spectrum (Figure 4a) and the asparagine mutant spectrum (Figure 4b) have such transitions, and they show no evidence of broadening or splitting from rhombic

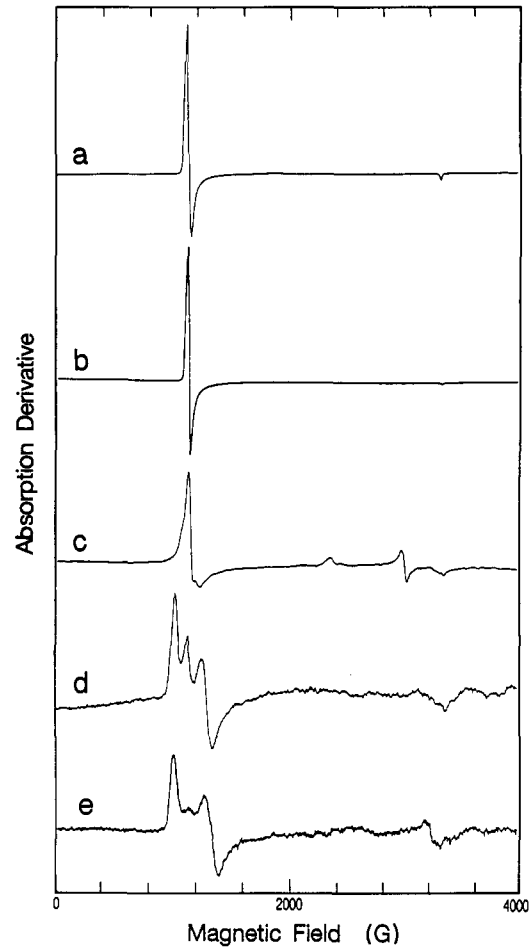

Figure 4. EPR spectra of myoglobins: (a) native human myoglobin 2 mM, 0–4000 G, 0.128-s time constant, 4-min scan, 2.5-G modulation amplitude, 100-kHz modulation frequency, 3.2×10^2 receiver gain, 3.8 K, 5-mW microwave power; (b) Val68Asn myoglobin 1 mM, 8-min scan, 0.250-s time constant, 4.1 K, other parameters as in a; (c) Val68Asp myoglobin 1 mM, 0.128-s time constant, 4-min scan, 10-G modulation amplitude, 100-kHz modulation frequency, 5×10^3 receiver gain, 3.8 K, 10-mW microwave power; (d) Val68Glu myoglobin, 3.8 K, parameters as in a except 1.6×10^4 receiver gain; (e) Val68Glu myoglobin, 50 K, parameters as in d except 2.5×10^4 receiver gain.

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distortions. The spectra of the aspartate and glutamate mutants deviate more from the native myoglobin spectrum (Figure 4, parts c and d, respectively). Both of these spectra show a splitting in the $g = 6$ transition, indicating a rhombic distortion that splits the d_{xz} and d_{yz} orbitals (eq 9). The effect is much more pronounced in the spectrum of the glutamate mutant (transitions at $g = 6.5$ and $g = 5.0$, giving $\mathbf{E/D} = 0.031$) than in that of the aspartate mutant ($\mathbf{E/D} \approx 0.002$) or fluorometmyoglobin ($\mathbf{E/D} = 0.004$).[37]

There is also a third peak for the perpendicular heme/magnetic field transition centered at $g = 5.8$ in the glutamate mutant spectrum, indicating that there are two high-spin Fe(III) states, one described above with $\mathbf{E/D} = 0.031$ and one with $\mathbf{E/D} \approx 0.000$ in the frozen solution. The spectrum arising from the state with $\mathbf{E/D} = 0.031$ is dominant at 120 K (Figure 4e). A thermal equilibrium of two iron(III) spin states is evident in the EPR spectrum of the aspartate mutant as well. However, in this case the two states are not both high-spin; one is low-spin ($S = 1/2$), because signals at $g = 2.8$ and 2.3 are observed (Figure 4c).[38-40] It is probable that the low-spin state is one in which the carboxylate group of the mutant aspartate residue is hydrogen bonded to a proton of a water molecule coordinated to the heme. Molecular modeling indicates that either oxygen atom in the aspartate side chain is within range to hydrogen bond to a heme-coordinated water, but neither can come close enough to bind directly to the heme.[8]

The signals in the EPR spectrum of the $^{17}OH_2$-treated glutamate mutant are the same as those in ordinary water, but the $g = 5.9$ feature in the aspartate mutant is significantly broadened (Figure 5). The observation that the other bands in the spectrum of the aspartate mutant are not broadened is not unexpected, because ligand hyperfine interactions are anisotropic.[41] However, the EPR spectrum of $^{17}OH_2$-treated native myoglobin shows preferential broadening of its high-field EPR signal ($g = 2$),[42] in contrast to that of the aspartate mutant, which shows broadening of its low-field ($g = 5.9$) feature. It could be that the rhombic distortion, which affects only the $g \approx 6$ signal in hemes, causes the low-field transition to be more susceptible to hyperfine broadening in the $^{17}OH_2$-treated aspartate mutant. It is worth noting that the slightly rhombic ferric EPR spectrum of $^{17}OH_2$-treated lipoxygenases ($\mathbf{E/D} = 0.01$ and $\mathbf{E/D} = 0.06$) also displays significant broadening in the low-field signal.[43]

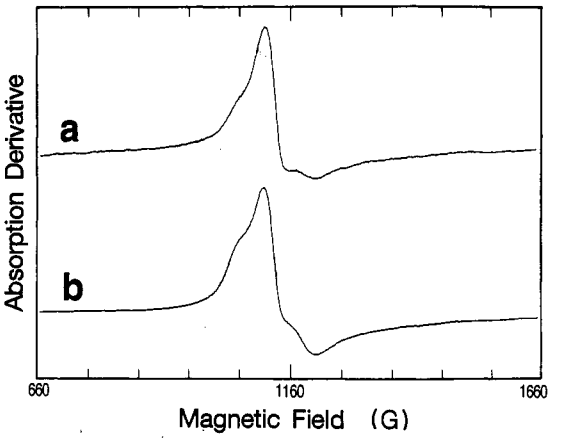

Figure 5. EPR spectra (660–1600 G) of $^{16}OH_2$-treated (top) and $^{17}OH_2$-treated (bottom) Val68Asp myoglogin. The $g = 5.9$ signal is broader in the bottom spectrum. Parameters are as in Figure 4c except that the protein concentration was ≈2 mM in both cases.

In summary, NMRD and EPR data indicate that a water is bound to the ferric heme in the asparate and asparagine human myoglobin mutants and that a carboxylate is coordinated in the glutamate derivative. Because the NMRD suggests that there is at least one rapidly exchanging hydrogen 4.5 ± 0.3 Å from the Fe(III) in the glutamate mutant, it is likely that a water molecule is hydrogen bonded to the nonligated carboxylate oxygen. Consistent with this interpretation, the magnitude of T_{1p}^{-1} is inversely related to temperature for both the glutamate mutant and fluoromethemoglobin.[44] In contrast, the aspartate and asparagine mutants have dispersion parameters and a thermal activation of T_{1p}^{-1} similar to native myoglobin, which has a slowly exchanging, Fe(III)-coordinated water molecule. The EPR spectra of the asparagine and aspartate mutants and native myoglobin are all very similar and show much less rhombic distortion than the glutamate mutant spectrum. Moreover, the high-spin heme of the aspartate mutant is in thermal equilibrium with a low-spin form, suggesting that the aspartate carboxylate is hydrogen bonded to a heme-bound water molecule.

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