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A TEST OF THE ROLE OF ELECTROSTATIC INTERACTIONS IN DETERMINING THE CO STRETCH FREQUENCY IN CARBONMONOXYMYOGLOBIN

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The vibrational frequency of CO bound to myoglobin can be varied by up to 60 cm^{-1} by making site-specific mutations in the distal pocket. These changes may result from specific chemical interactions between distal amino acids and the CO or from changes in the electrostatic field of the distal pocket. In this paper, we separate the relative contributions of these two effects by comparing the IR spectra of the carbonmonoxy complexes of human myoglobin mutants V68N, V68D, and V68E. The effect of replacing valine with these polar amino acids on the electrostatic environment of the distal heme pocket has been independently determined earlier by measurements of the heme reduction potential and electronic absorption spectral band shifts. While all three mutations result in a negative dipole pointing towards the CO ligand, the CO stretch frequency shifts differently in each case. These differences are attributed to specific chemical interactions between the amino acids and the CO ligand.

The infrared (IR) spectra of carbonmonoxy complexes of heme proteins provide information on ligand bonding to the heme iron and the local environment on the distal side of the heme pocket. The stretch frequency of CO, ν_{CO}, when bound to heme proteins or model compounds is significantly shifted to lower energy from its gas phase value of ~2200 cm^{-1} to the range 1900 - 2000 cm^{-1}. There has been much discussion of the interpretation of these frequency shifts, ranging from the presence of distinct CO binding geometries (e.g. the CO tilt angle) to changes in the local electrostatic field in the distal pocket. Even in the case of wild type sperm whale myoglobin (Mb), at least three distinct IR bands are observed (1). Possible relationships between this spectral heterogeneity and the complex dynamics of ligand recombination have been extensively discussed (2). Although earlier linear dichroism studies led to the interpretation that different CO tilt angles are responsible for different spectrally distinct subpopulations (3,4), and some early crystallographic data are consistent with this heterogeneity (5), recent single crystal polarized spectroscopy (6) and careful transient dichroism measurements (7) are more consistent with little variation in the CO tilt angle for different spectrally distinct subpopulations, and further are consistent with a much smaller tilt angle than had previously been determined.

Alteration of distal pocket residues changes both the frequencies of the IR bands and the heterogeneity of the spectrum (8,9). Shifts in ν_{CO} of up to 60 cm^{-1} occur upon single amino acid substitutions in the distal heme pocket. The IR spectra of these mutants vary when the polar

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character of residues at position 64 and 68 is altered despite little variation in the crystallographically determined orientation of the CO (10). Moreover, Oldfield and coworkers have correlated ^{17}O and ^{13}C chemical shifts with ν_{CO} and developed a model in which "charge field interactions" between CO and distal pocket residues alter the iron-CO bonding and result in changes in the IR and NMR spectra (11,12). Based on these observations, Springer and co-workers proposed in a recent review that the CO stretch frequency may be a gauge of the electrostatic field near the ligand binding site (13).

The expression "electrostatic interactions" has been used rather loosely to describe a multitude of possible interactions. On the one hand it is possible that the observed CO spectral shifts in mutants arise from internal Stark shifts due to the interaction between the electric field of the protein and the change in dipole moment, $\Delta\mu$, associated with the CO stretch. Based on limited calculations (14,15) and experiments (16), $|\Delta\mu|$ for the CO stretch frequency is about 0.05 D, which translates into a Stark tuning rate, if the electric field were parallel to the direction of $\Delta\mu$ (likely parallel to the CO bond axis), of about 5×10^{-7} $cm^{-1}/(V/cm)$ (16). Thus, a shift in the CO stretch frequency of 10 cm^{-1} would require a local electric field of about 2×10^7 V/cm. Two quantitative estimates have been obtained for the electric fields in a protein by measurements of Stark shifts in electronic absorption spectra. In the case of a simple peptide which forms a helix, the electric field due the helix dipole was found to be on the order of on the order of 4×10^6 (17); for a transient dipole produced by electron transfer in photosynthetic reaction centers, the electric field is on the order of 5×10^6 V/cm (18). In both cases the charges responsible for the field are quite distant from the probe chromophore (on the order of 10 Å), and the charges are screened from their values computed in vacuum. Thus, if a charged residue were within a few Å or less of the bound CO in a relatively non-polar environment and with the optimal orientation, electric fields large enough to produce the shifts in ν_{CO} observed in Mb mutants are conceivable. Oldfield, Dykstra and coworkers have calculated the local electric field in the distal pocket of sperm whale Mb for four different conformations of His 64 and found that these subtle variations in electric field may be sufficient to explain the heterogeneity in IR spectra as well as the ^{13}C and ^{17}O NMR spectra (19).

Another possible source of spectral dispersion in the IR spectrum of CO bound at the heme involves variations in bonding, either the Fe-CO bond, which would include differences due to variations in the *trans* ligand effects (20) as well as tilt angle, or specific interactions between the bound CO and distal amino acids (e.g. hydrogen bonds) or water in the distal pocket. We will call these "chemical interactions" and distinguish them from "electrostatic interactions" as defined operationally in the previous paragraph. It is likely that both electrostatic (Stark shift) and chemical interactions will vary from mutant to mutant or within subpopulations of a single mutant detected by dispersion in ν_{CO}.

It may be possible to distinguish these two mechanisms using mutants for which independent information is available on electrostatic interactions. Other local probes of electrostatic interactions include shifts in pKa's of ionizable residues (21) and in the redox potentials of bound prosthetic groups. A number of years ago we prepared a series of charged and polar mutants of valine 68 in human myoglobin and characterized both changes in the heme iron redox potential

(22) and spectral shifts of bound chromophores (23) as a probe of local electrostatic environment. Given this independent quantitative information, we thought it would be useful to obtain parallel information on ν_{CO} in this series of mutants to attempt to separate the chemical and electrostatic contributions.

MATERIALS AND METHODS

Protein purification and sample preparation. Human Mbs WT, V68E, V68D and V68N were expressed and purified from *E. coli* as described elsewhere (23,24). All four proteins also contain the surface mutaion C110A, which has been introduced to improve the yields upon purification; this mutation does not siginificantly perturb the structural and ligand binding properties. Samples contained 2 - 3 mM Mb in 75% glycerol/water, pH = 7.0. Protein solutions were equilibrated under CO for 45 minutes; a minimum amount of sodium dithionite was then added to reduce the iron. The solutions were placed under CO for an additional 30 minutes. The samples were loaded into an IR cell with CaF_2 windows and a 100 micron spacer, attached to the ccold finger of a closed cycle helium cryostat.

FTIR spectra. FTIR spectra were measured on a Bruker IFS113 FTIR spectrophotometer equipped with a Hg/Cd/Te detector. Data was collected at 2 cm^{-1} resolution. Samples were cooled to 10 K using a closed cycle helium refrigerator. At 10 K, spectra were taken before and after irradiation with visible light. The difference spectrum (pre-illumination minus post-illumination) yields the spectrum of the CO ligand corrected for the solvent and protein background because the CO does not recombine to the iron at this temperature (3).

RESULTS AND DISCUSSION

The IR spectra for V68E, V68D, V68N and WT at 10 K are presented in Figure 1, and the parameters are listed in Table I. At 10 K and pH = 7, the spectrum of V68D has two major bands at 1972 cm^{-1} and 1951 cm^{-1}, both shifted to higher energy from the wild type values of 1945 cm^{-1} and 1967 cm^{-1}. Arginine is similar in size and shape to aspartic acid; yet the IR spectrum of V68N at 10 K has two bands at 1927 cm^{-1} and 1912 cm^{-1}, significantly shifted to lower energy compared to V68D. The V68E spectrum has one major band at 1937 cm^{-1} at 10 K and pH 7.

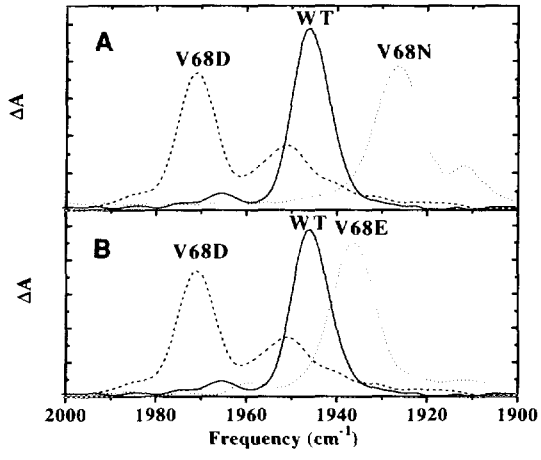

Figure 1. IR difference spectra (pre-illumination minus post-illumination) of MbCO recorded at 10 K, 75% glycerol/buffer, pH = 7.0. **A.** IR spectra of WT, V68N, and V68D MbCO. **B.** IR spectra of WT, V68E, and V68D.

Table I. Parameters for IR spectra, visible spectra and reduction potentials for human Mb mutants

Mutant	ν (cm^{-1})[a]	λ_{max} (nm) LMCT band metaqo Mb	λ_{max} (nm) Q_1 band MbCO	$E^{o'}$ (mV)[b]
WT	1967 (minor)[c]	632	578	57.0
	1945 (major)[c]			
V68N	1927[c]	628	572	-23.8
	1912[c]			
V68D	1972	622	574	-132.1
	1951			
V68E	1937	620	576	-136.8

[a] Spectra recorded at 10 K; [b] $E^{o'}$ relative to NHE, measured at room temperature; see Varadarajan et al, 1989a; [c] Balasubramanian et al., 1993.

While the CO stretch bands of V68N and V68D shift in opposite directions, other properties sensitive to local electrostatic interactions change in the same direction. For example, the reduction potentials for V68N and V68D are both significantly decreased compared to those of WT (Table I). In addition, the visible absorption band at 632 nm (15822 cm^{-1}) in WT metMb shifts to higher energy in V68D (622 nm, 16077 cm^{-1}) and V68N (628 nm, 15924 cm-1), while in MbCO the band at 578 nm (17301 cm^{-1}) in WT shifts to 572 nm (17482 cm^{-1}) in V68D and 574 nm (17421 cm^{-1}) in V68N. A decrease in reduction potential and shift to higher energy in the electronic absorption spectrum of the ligand-to-metal charge-transfer (LMCT) bands are consistent with the introduction of a dipole which points away from the heme pocket, i.e., the negative end points towards the heme iron. The magnitude of the dipole is different, with the carboxylic acid having more partial negative charge than the asparagine, but the direction is similar.

The CO stretch in the V68E mutant is shifted modestly to lower energy relative to wild type and is similar to the spectrum of V68Q and V68L in sperm whale MbCO (1). The glutamic acid side chain is longer and more flexible than the aspartic acid group and can orient such that the carboxylate group does not point at the CO ligand; this is the orientation of the leucine side chain in the crystal structure of the sperm whale V68L mutant (10). However, there are substantial changes in the electronic absorption spectrum of the metaquo and CO forms of V68E (Table 1).

^1H nmr data of the CO forms of V68N and V68D reveal that there are no major perturbations in the distal pocket structure caused by these site-specific substitutions (23). Furthermore, aspartic acid and asparagine are very similar in size and shape. Based on these structural observations, the steric interaction with the CO should be similar in the two mutants and cannot explain the large differences observed in the IR spectra. These two mutations perturb the electrostatic environment of the distal pocket as evidenced by the changes in reduction potential and

visible absorption spectra (Table I; 22-23). These data are consistent with a model in which a dipole is directed towards the heme and the CO binding site, with the negative end of the dipole facing the CO ligand. In theoretical treatments of vibrational Stark effects on CO, the spectrum of the CO stretch mode shifts to higher energy when interacting with the electric field of a small negative charge (14); this is consistent with the observed spectrum in V68D, yet inconsistent with the large shift to lower energy in the IR spectrum of V68N. Thus, while the effect of a local electrostatic field may explain the V68D IR data, such a model can not explain the IR spectrum of V68N.

When an asparagine group is at position 68, it is capable of either hydrogen bonding or donating the free electron pair of the amide group to the antibonding orbitals of the CO. Either interaction would lead to a shift to higher energy for the band, as observed. According to the ^1H nmr data, the aspartate in V68D is unprotonated, and engages in a salt bridge interaction with His 64 (23); Asp 68 is unable to hydrogen bond with the CO, while Asn 68 can. Hydrogen bonds to carbonyl groups in small organic molecules result in shifts of ν_{CO} ~20 - 30 cm^{-1} to higher energy (25, 26), which is consistent with the shifts observed here. Thus, the differences in the IR spectra of V68D and V68N can be explained by the presence of hydrogen bonding, a specific chemical interaction, between Asn 68 and the CO in V68N, not by electrostatic interactions.

The presence of a sensitive probe of local dielectric within a functional protein is a valuable tool. Local probes into the electrostatic properties of protein interiors have been important in determining, in a general sense, how dielectric and solvation properties determine protein function. The observed CO stretch frequencies in the mutants V68N, V68D and V68E, however, are consistent with a model in which both chemical interactions and electrostatic fields determine the CO stretch frequency. Because these interactions can have opposing effects on the stretch frequency, the IR spectrum itself is not a reliable independent probe of the electrostatic fields within the heme pocket.

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