Is the CO Adduct of Myoglobin Bent, and Does It Matter?

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ABSTRACT
Early reports of a severely bent CO adduct in myoglobin inspired the idea that heme proteins discriminate against CO, relative to O_2, via steric hindrance imposed by a distal histidine residue. Recent results showing that the bound CO is only slightly distorted do not by themselves overthrow the steric hypothesis, because the steric energy could be stored in displacements of the protein. However, experimental data on site-directed mutants show that the main determinant of ligand affinity changes is the polarity of the binding pocket and that H-bonding by the distal histidine accounts for about 85% of the O_2/CO discrimination while steric hindrance accounts for the remaining 15%.

Introduction

One of the more enduring controversies in chemical biology is whether nature has solved the problem of carbon monoxide toxicity by building steric hindrance into the binding pocket of the physiological oxygen carriers myoglobin and hemoglobin.[1−8] The natural affinity of the heme prosthetic group is higher for CO than for O_2, by a factor of roughly 20 000, but this ratio is reduced to 25−200 in the proteins.[9] The natural CO affinity is so high that we would all be asphyxiated by our own CO, which is produced naturally by catabolism of heme itself, were it not for the proteins' discrimination against CO.

The steric hindrance idea was advanced by Collman et al.[10] more than 20 years ago. Noting that O_2 and CO have different geometric preferences when bound to the heme iron atom of these proteins, they hypothesized that discrimination against CO and in favor of O_2 is achieved by steric repulsion of distal residues in the protein. CO prefers to bind in an upright fashion, with linear FeCO bonding, while O_2, with two extra electrons, is naturally bent. Both myoglobin and hemoglobin have distal histidine side chains positioned over the Fe atom, which can plausibly interfere with upright binding, and thereby discriminate against CO. This attractive idea has made its way into biochemistry textbooks[11] (Figure 1), but it has come under increasing scrutiny and attack over the years.

This Account is an attempt to clarify this controversy. Our point of view stems from our work in vibrational spectroscopy and the vibrations of heme-bound ligands.[12,13] Of particular relevance is the emergence of density functional theory as a method for accurately calculating vibrational spectra via the potential energy surface. The potential energy surface naturally focuses attention on energy differences among alternative ligand geometries and protein structures. Structural data alone cannot define the determinants of ligation affinities; the energetic contributions of differing structural elements, as well as contributions from the surrounding environment, must be evaluated. This observation may seem a truism, but its neglect has, in our opinion, clouded the debate over the importance of steric factors in myoglobin and hemoglobin.

MbCO Structures

Collman et al. supported their steric proposal by noting that X-ray[14] and neutron[15] crystal structures of the myoglobin CO adduct, MbCO, revealed a pronounced bend in the Fe−C−O angle, with reported values of 120−140°, presumably as a result of contact with the distal histidine side chain. However, these structures have engendered considerable skepticism, and the very large deviations from linearity are no longer credited. The heme binding cavity exhibited rather diffuse electron density (Figure 2), which was attributed to disorder in the CO conformation. The data were modeled with partial occupancy of alternative conformations. Ray et al.[16] later analyzed the positional uncertainties of the atoms and concluded that the Fe−C−O angle must be uncertain to at least ±25°. Several other MbCO X-ray crystal structures have since been determined, which show much less apparent disorder of the CO.[17−20] In retrospect, it seems likely that the earlier data were affected by oxidation of the protein to metMb over the course of data collection. MetMb contains Fe(III)−OH_2, and sometimes a second water molecule which is hydrogen bonded to the coordinated water.[17] Thus, the electron density in the ligand pocket could reflect the partial replacement of CO by bound water. Protein stability was less an issue in the more recent determinations, which benefited from cryoscopic techniques and advances in X-ray sources and detectors, developments that have reduced collection times.

The newer crystal structures do not show the very large deviations from linearity reported earlier. Neither do crystal structures of model Fe porphyrins on which covalent superstructures have been erected in order to

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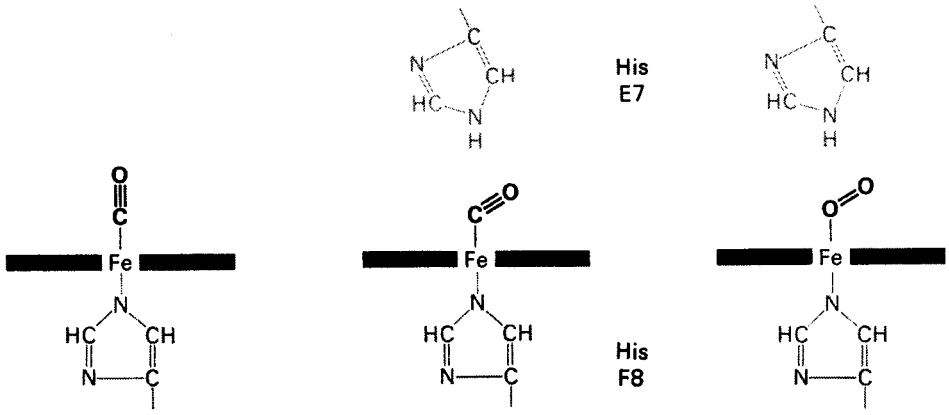

FIGURE 1. Diagram of the steric mechanism for steric inhibition of CO, but not O_2 binding (adapted from ref 11).

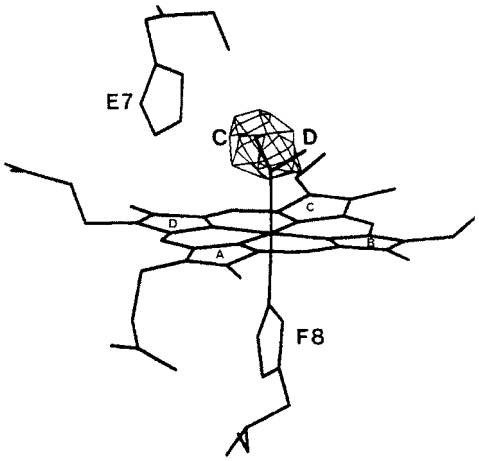

FIGURE 2. Structural diagram of the Mb binding pocket, showing the ligand electron density in the MbCO X-ray crystal structure determined by Kuriyan et al. (adapted from ref 14).

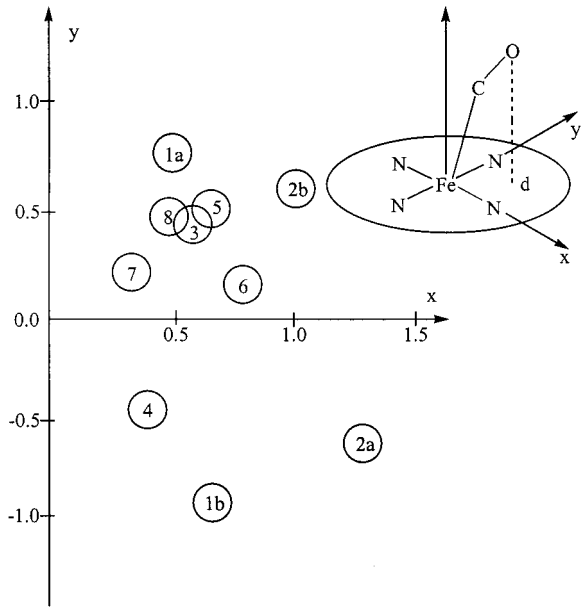

FIGURE 3. Projection of the O atom displacement from the heme *z* axis in MbCO crystal structures: points 1a and 1b, native $P2_1$ MbCO crystal, PDB file 1mbc, ref 14; points 2a and 2b, native $P2_1$ MbCO crystal, PDB file 2mb5, ref 15; point 3, "wild-type" D122N mutant of sperm whale myoglobin that crystallizes in a *P*6 space group, PDB file 2mgk, ref 17; points 4, 5, 6, native $P2_1$ MbCO crystals at pH 4, 5, and 6, PDB files 1spe, 1vxc, 1vxf, ref 18; point 7, $P2_1$ MbCO crystal (1.15 Å resolution), PDB file1bzr, ref 19; point 8, $P2_1$ MbCO crystal (1.2 Å resolution), PDB file1a6g, ref 20.

constrain the CO sterically.[21−24] However, none of these structures have entirely upright FeCO units, and they exhibit a range of distortions. The reported results are best compared in a plot of the O atom displacement from the normal to the heme plane passing through the Fe (Figure 3), first introduced by Sage.[6] This displacement is a result of two angular distortions, tilting and bending (see Figure 5), both of which vary from structure to structure. Even if the data from the initial X-ray and neutron structures (points 1a,b and 2a,b) are excluded from consideration, the O atom displacements range from 0.3 to 0.7 Å. The two most recent MbCO X-ray structures, obtained at very high resolution, 1.15[19] and 1.2[20] Å, fail to narrow this range; the O atom displacements are 0.3 and 0.6 Å.

Spectroscopic data have also discounted the possibility of severe FeCO bending, but they do allow modest O atom displacements. Vibrational frequencies associated with stretching of the Fe−C and C−O bonds for a wide range of proteins and model porphyrins (having in common only the presence of a neutral imidazole sixth ligand) all fall on a single back-bonding correlation[13,16,25] (Figure 4). The variations along this correlation are explicable on the basis of the electrostatic field in the vicinity of the bound CO,[16,25] which polarizes the FeCO back-bonding system, driving the Fe−C and C−O frequencies in opposite directions. Deviations from this correlation are expected if the Fe−C−O angle is bent, because the Fe−C and C−O bonds would both weaken. Modeling of this effect via DFT[13] calculations indicate that O atom displacements of 0.5 Å or less are consistent with the scatter of the data (Figure 4). In the same vein, variations in NMR and Mossbauer parameters can all be explained on the basis of back-bonding changes as modulated by electrostatic fields.[26] Again, DFT analysis of these data indicate a slightly distorted structure with tilt and bend angles of 4° and 7°.[27]

Even smaller distortions had been indicated by IR polarization measurements on the CO stretching vibration, but reinterpretation of the results has relaxed the apparent narrowness of the constraint. The polarization can be established relative to the heme plane, either in oriented single crystals[28] or by photoselection of MbCO

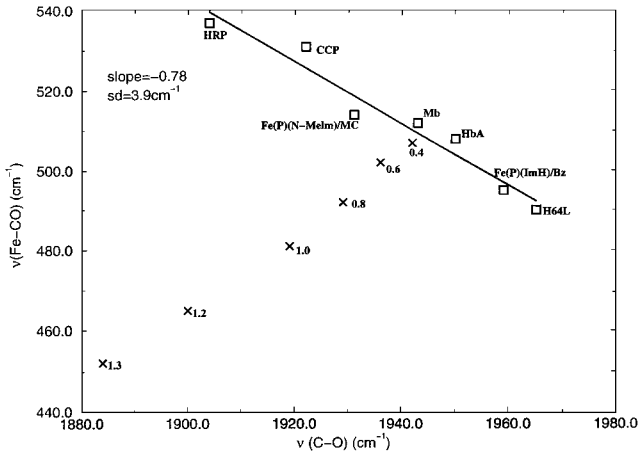

FIGURE 4. Predicted dependence of the FeCO frequencies (x's) on O atom displacement along the minimum energy path obtained by full vibrational calculations on structures constrained to have the indicated displacements (Å). The squares represent experimental data for heme proteins and models.[16]

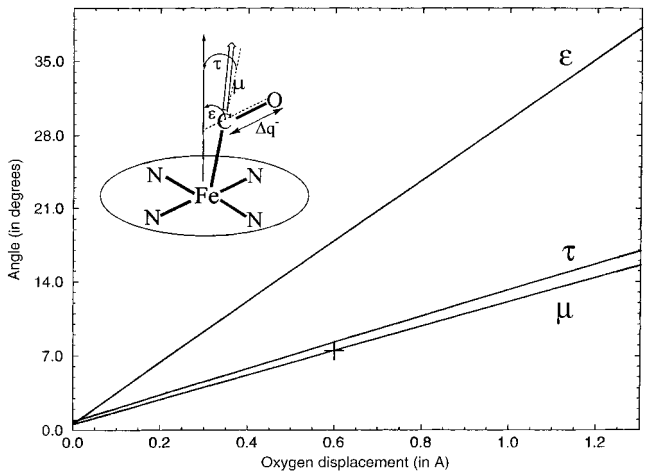

FIGURE 5. Calculated CO stretching transition dipole direction (μ) along the DFT minimum energy distortion path.[33]

molecules by partial photolysis, either in frozen solution,[29] or by using picosecond laser pulses to freeze out protein tumbling in solution.[30] Photoselection measurements had earlier suggested substantial degrees of CO inclination from the heme normal, but Lim et al.,[31] by carefully controlling the experimental variables, showed that the inclination is actually less than 7°. Meanwhile, Sage and co-workers[28] had determined the polarization angle in single crystals to be 6.7 ± 0.9°, so the two techniques are in excellent agreement.

However, they both rest on the assumption that the IR polarization measures the CO bond vector. In fact, it measures the IR transition dipole direction, which is not the same thing. Although the CO oscillator is well isolated kinematically, stretching the CO bond alters the electronic distribution of the entire molecule. Because of the importance of π back-bonding from Fe to the CO, and also to the porphyrin ring, this is not a small effect. It accounts for the IR intensity, and also the frequency shift in an applied electric field (vibrational Stark effect), being much larger for heme-bound than for unbound CO.[32] As the FeCO unit bends, the CO stretch sets up a dipole in the porphyrin ring which opposes that of the CO bond itself.[13] As a result, the transition dipole inclination is less than that of the CO bond. This effect has been calculated by DFT[13,33] and is shown in Figure 5. A 7° inclination of the transition dipole implies a 0.6 Å displacement of the O atom, consistent with the recent crystal structures.

What then is the "real" structure of MbCO? It is not severely bent, as earlier indicated, but neither is it entirely upright. The O atom displacement is somewhere between 0.3 and 0.6 Å, with tilt and bend angles of 5−10°.[19,20] Small tilt (4°) and bend (6°) angles were also calculated by Lopez and Kollman,[34] using molecular dynamics on the MbCO structure. (In their abstract, Lopez and Kollman reported a more distorted structure, with 20° of bend and 6° of tilt, but this was based on a calculation with the NδH tautomer of the distal histidine, the tautomer reported from neutron crystallography.[15] Calculations with the NϵH tautomer, which is almost certainly the correct tautomer for the CO adduct—see below—gave the less distorted structure.)

Bending Energy

Does the downward revision of the extent of FeCO distortion in MbCO disqualify the steric hindrance proposal of Collman et al.? It does not. The steric hindrance could equally well be accommodated by displacements of the protein itself. The study of superstructured porphyrins has made it clear that steric forces can strongly discriminate between CO and O_2,[2,8,23] yet crystal structures of the CO adduct show only small FeCO distortions.[8,21−24] There are, however, large displacements of the superstructure, together with distortion of the porphyrin ring. Ironically, the original reports of large FeCO distortions in MbCO ultimately obscured the steric hindrance hypothesis by focusing too much attention on this structural anomaly.

How much steric energy do the newer, less-distorted MbCO structures imply? Until recently, the FeCO bonds had been thought to be quite stiff, because the vibrational frequency of the Fe−C−O bending mode, ~570 cm^{-1}, is high, higher than the frequency of the Fe−C stretching mode (~500 cm^{-1}).[35] However, Ghosh and Bocian[4,36] discovered via a DFT analysis of the vibrations that the elevated bending frequency results from a large positive interaction force constant between the bending and tilting coordinates, and that the energy required to distort the FeCO unit is actually quite modest. We confirmed this result at a higher level of DFT[2] and calculated the energy as a function of the O atom displacement by constraining only this parameter, for an imidazole−(Fe^{II} porphine)−CO model, and allowing all other structural parameters to relax to the minimum energy geometry.[13] The resulting minimum energy distortion curve (Figure 6) is much lower than the curves calculated if the distortion were limited to only bending or only tilting. The range of distortions derived from the more recent structures requires small amounts of energy, 0.3−1.1 kcal/mol for 0.3−0.6 Å displacements of the O atom.[13]

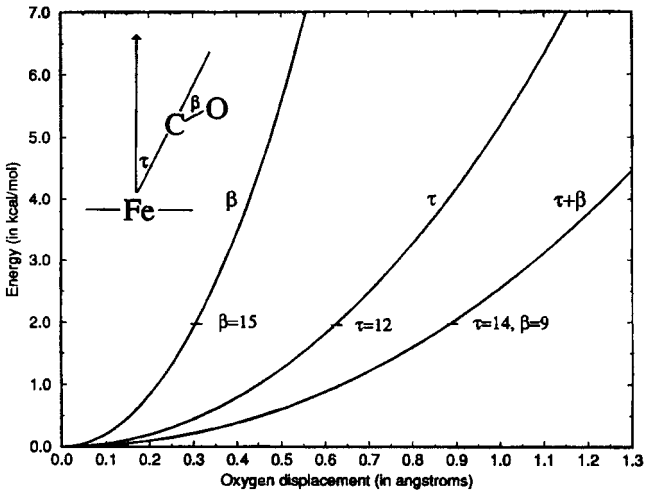

FIGURE 6. Energy for displacement of the O atom from the heme normal calculated via DFT for bending (β), tilting alone (τ), and a minimum energy combination of bending and tilting.[13]

The steric energy stored in protein displacements is harder to evaluate. Long ago, Case and Karplus[37] estimated 7 kcal/mol as the upper bound on the energy that the protein could exert on the CO ligand, via a molecular mechanics calculation of side-chain reorientations on a rigid backbone. More recently, Lopez and Kollman[34] used free energy perturbaton methods to calculate the van der Waals energy contribution to the discrimination between CO and O_2, and they concluded that it is only on the order of 0.5 kcal/mol. However, evaluating the energies of the many small displacements which are evident in the crystal structures puts severe demands on the available force fields.

Elsewhere we have assumed that the protein stores as much energy as the FeCO distortion energy, and that doubling the distortion energy gives a reasonable estimate of the total steric energy, i.e., 0.6−2.2 kcal/mol for 0.3−0.6 Å O atom displacements. However, the energy need not be equally partitioned (the forces on interacting atoms must be equal and opposite, but the associated energies need not be equal), and it is possible that the protein stores "excess" steric energy. This possibility is illustrated by the superstructured porphyrins. For example, the CO affinity of the 1,2-dimethylimidazole adduct of Fe^{II}-PocPiv,[21] a derivative of "picket-fence" porphyrin in which a capping benzene ring is attached to three of the pivaloylamido pickets by methylene bridges, is 2.5 kcal/mol smaller than it is for $Fe^{II}Piv_3$5Cim, another picket-fence porphyrin derivative which lacks the benzene cap, but in which one of the pickets is attached to an internally coordinated imidazole.[38] The electronic environments of these two porphyrins are very similar, so that the affinity lowering is attributable to the steric hindrance of the cap. The crystal structure of the CO adduct shows a small FeCO distortion, with a 0.4 Å O atom displacement, corresponding to an FeCO distortion energy of 0.5 kcal/mol, on the basis of the DFT curve. This is only 20% of the 2.5 kcal/mol total steric energy. The remainder must be provided by the substantial distortions in the porphyrin and its superstrucure.[21]

Could the residue displacements observed in MbCO relative to those in deoxyMb[14−20] carry significant steric energy? We return to this question after first considering electrostatic effects.

Electrostatics and H-Bonding

The alternative to the steric hypothesis is that discrimination in favor of O_2 results from a stabilizing H-bond interaction with the distal histidine. This idea can be traced back to a suggestion of Pauling in 1964[39a] and has been supported by model studies from the 1980s onward which demonstrated an important influence of polarity on the binding of O_2, but not CO, to the heme group. (For reviews, see refs 8 and 23). An H-bond between the distal histidine and bound O_2 has been clearly identified by neutron diffraction[39b] and NMR,[39c] and a weak H-bond to bound CO is suggested by D_2O sensitivity of the FeCO vibrations.[39d] Both ligands rely on back-donation of electrons from the heme Fe(II), but the transfer of charge is much greater for O_2 than for CO. Consequently, polar interactions with distal residues carry more energy for O_2 than for CO.

This differential effect has been extensively investigated in myoglobin by Olson, Phillips, Sligar, Springer, Wilkinson, Ikeda-Saito, and co-workers, using site-directed mutagenesis.[9] Altering the polarity of residues in the binding pocket produces major changes in the affinity and dissociation rate for O_2 but minor ones for CO. Paradoxically, it is the CO adduct that provides a convenient measure of the pocket polarity,[9,32] because of the accessibility of the CO and FeC stretching frequencies in the IR and Raman spectra and the ^{13}C chemical shift in the NMR spectrum. All of these respond sensitively to polarity changes because of the resulting shift in the extent of back-bonding. However, the energy differences are small, and the CO dissociation rates correlate only weakly with these spectral parameters.[9]

The O_2 dissociation rates, on the other hand, correlate strongly with the CO spectral parameters. Phillips et al.[40] have recently shown that this correlation can be attributed quantitatively to the pocket polarity. For both ligands, the log of the dissociation rate correlates linearly with the electrostatic potential at the ligand atoms, calculated via a linearized Poisson−Boltzmann method for a series of mutant myoglobin crystal structures. However, the slope of the correlation is 4 times larger for O_2 than for CO (Figure 7). Since the log of the dissociation rate is expected to be directly related to the bond strength, the electrostatic correlations imply that a given polar influence, e.g., an H-bond, carries 4 times more energy for O_2 than for CO. Encouragingly, the H-bond energy calculated via DFT by Sigfridsson and Ryde[41] for imidazole interacting with a ligated heme was also 4 times greater for O_2 than for CO. The absolute energies, 7.6 and 1.9 kcal/mol, respectively, were recognized by the authors to be too high, but the ratio is in agreement with the experimental results of Phillips et al. on myoglobin. Using free energy perturba-

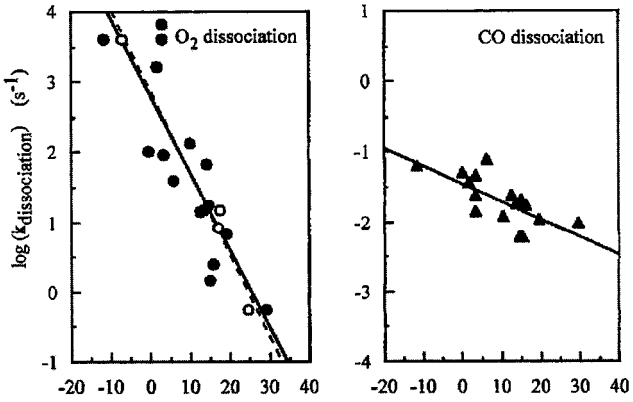

FIGURE 7. Correlations of O_2 and CO dissociation rate constants with the electrostatic potential at the O atom (units in kcal/mol) calculated using MbO_2 (open circles) or MbCO (filled symbols) crystal structures (adapted from ref 40).

tion methods and the AMBER empirical force field,[42] Lopez and Kollman[34] calculated 1.9 kcal/mol as the difference in the histidine−ligand electrostatic energy between O_2 and CO for the N−H tautomer; curiously, however, almost the same difference, 1.7 kcal/mol, was calculated when MbCO had the N−H tautomer.

The correlation of the CO stretching frequency with the calculated electrostatic potential only works if the NϵH tautomer is assumed, i.e., with the NH group pointing at the CO; the correlation is poor if the NδH tautomer is assumed.[40] Although neutron crystallography gives a clear-cut location for the imidazole proton,[15] Phillips et al.[40] point out that the NδH tautomer could well have been produced by the same autoxidation to metMb that likely produced the apparent FeCO distortion, since replacement of $Fe^{II}CO$ by $Fe^{III}H_2O$ could reverse the H-bonding with the distal histidine.[43] Interestingly, binding of NO to Fe^{III}Mb also appears to favor the NδH tautomer; the NO stretching frequency is 20 cm^{-1} *lower* than it is in the H64L mutant, suggesting a negative polar interaction with the N^- lone pair.[44] Although $Fe^{III}NO^+$ is isoelectronic with Fe^{II}-CO, the positive charge apparently induces a tautomeric shift in the distal histidine.

Quantitative Evaluation of Energy Contributions from Mutants

Olson and Phillips[7] have argued that the electrostatic mechanism is mainly responsible for the ~1000-fold reduction in the ratio of the CO and O_2 affinity constants in Mb, and that steric hindrance is less important. The main evidence is the ~500-fold increase in the O_2 dissociation rate constant when the distal histidine in myoglobin is replaced with a hydrophobic residue. To be sure, the CO affinity increases, but only by a factor of ~10, and this factor can be attributed to the displacement of a water molecule which is H-bonded to the distal histidine in the deoxy form of the protein, and which is not present in the hydrophobic mutants. Thus, there appears to be little room for a steric influence by the distal histidine.

The steric vs electronic issue can be approached quantitatively with the aid of the full set of kinetic and equilibrium constants determined by Olson and coworkers. We can estimate the various binding energy contributions in the following way.

The change in the ligand binding free energy for a residue substitution in myoglobin is obtained via

$$kT\Delta \ln K_L = \Delta\Delta G_L \quad (1)$$

where K_L is the equilibrium constant for the reaction Mb + L = MbL. We decompose this change into three terms

$$\Delta\Delta G_L = \Delta E_L - \Delta St_L - \Delta E_{dx} \quad (2)$$

ΔE_L is the change in the electrostatic stabilization energy from the field in the binding pocket, while ΔSt_L is the change in the steric interaction energy that opposes ligand binding. ΔE_{dx} is an energy term for deoxyMb, whose main contribution is from the water molecule which is H-bonded to the distal histidine in the wild-type protein. This term is the same whether CO or O_2 is the ligand.

We assume that for O_2 steric hindrance is absent and $\Delta St_{O_2} = 0$. Then, ΔE_{dx} and ΔSt_{CO} can be obtained from the experimental values of $\Delta\Delta G_L$ for CO and O_2, if the electrostatic energies, ΔE_L, can be estimated independently.

To do this, we turn to the dissociation rate constants, k_L, and assume that the activation energy for ligand dissociation is just the bond dissociation energy, which is the sum of the intrinsic bond energy, D_L, and the electrostatic stabilization energy imposed by the protein,

$$E_a = D_L + E_L \quad (3)$$

where $k_L = Ae^{E_a/kT}$. This assumption ignores protein effects on the nascent products, namely the deoxy-heme and the dissociated but protein-localized ligand molecule. These effects on the activation energy may not be negligible (see below, in connection with the leucine mutant) but are likely to be small compared to the electrostatic interaction with the bound ligand. Since D_L is independent of the protein, the effect of a residue substitution on the electrostatic stabilization is obtained from

$$\Delta E_L = kT\Delta \ln k_L \quad (4)$$

(We note that ΔE_L includes changes in bond strength which are induced by the electric field of the protein, as reflected in the changes observed for the Fe−L stretching frequency.[16]) In Table 1, we list measured values for k_L and K_L for wild-type myoglobin, both sperm whale and human, and for three mutants, in which the distal histidine (residue E7) is replaced by valine, isoleucine, and leucine, hydrophobic amino acids having isopropyl, *sec*-butyl and isobutyl side chains. These were selected from among the many mutants studied by Olson and coworkers because the side chains are hydrocarbons of about the right size to replace the histidine side chain. Larger ones (e.g., Phe and Trp) may exert extra steric force, while smaller ones (e.g., Gly and Ala) may leave room for water in the ligated protein, something that has been

Table 1. O_2 and CO Dissociation Rate Constants (k, s^{-1}) and Binding Constants (K, μM^{-1}) (from Ref 40) for Myoglobin Variants, and Calculated Interaction Energies (kcal/mol)[a]

variant	k_{O_2}	ΔE_{O_2}	k_{CO}	ΔE_{CO}	K_{O_2}	$\Delta\Delta G_{O_2}$	ΔE_{dx}	K_{CO}	$\Delta\Delta G_{CO}$	ΔSt_{CO}	$\Delta\Delta\Delta G_{CO-O_2}$
sperm											
E7His	15		0.019		1.1			27			
E7Val	10 000	3.84	0.048	0.55	0.011	−2.72	1.12	150	1.01	0.44	3.73
E7Ile	6 400	3.58	0.047	0.54	0.014	−2.46	1.12	170	1.09	0.50	3.55
E7Leu	4 100	3.31	0.024	0.14	0.023	−1.68	1.08	1100	2.19	1.25	3.87
human											
E7His	22		0.022		0.86			35			
E7Val	7 700	3.46	0.051	0.50	0.011	−2.58	0.88	100	0.62	0.24	3.20
E7Ile	11 000	3.67	0.044	0.41	0.0072	−2.82	0.85	130	0.78	0.33	3.60
E7Leu	5 400	3.35	0.029	0.16	0.022	−2.16	1.08	880	1.90	0.98	4.06
average		3.5		0.4			1.0			0.6	3.7
(less E7Leu)		3.6		0.5			1.0			0.4	3.5

[a] Symbols: $\Delta E_L = kT\Delta \ln k_L$; $\Delta\Delta G_L = kT\Delta \ln K_L$, where Δ is the difference between wild-type Mb (E7His) and the indicated mutant. $\Delta E_{dx} = \Delta E_{O_2} - \Delta\Delta G_{O_2}$; $\Delta S_{CO} = \Delta E_{CO} - \Delta E_{dx} - \Delta\Delta G_{CO}$; $\Delta\Delta\Delta G_{O_2-CO} = \Delta\Delta G_{O_2} - \Delta\Delta G_{CO}$.

observed in the E7Gly crystal structure.[9] Also listed are values of ΔE_L, ΔE_{dx}, and ΔSt_{CO}, calculated via eqs 1, 2, and 4.

For O_2, the three hydrophobic E7 residue replacements increase k_L by a factor of 385, on average, giving 3.5 kcal/mol for ΔE_{O_2}. For CO, k_L also increases but by a much smaller factor, 1.9 on average, giving 0.4 kcal/mol for ΔE_{CO}. The results are consistent for myoglobin from sperm whale and from humans. We identify the ΔE_L's with the strength of the H-bond interaction between the distal histidine and the bound O_2 and CO ligands. Encouragingly, the value for CO agrees well with the 0.5 kcal/mol estimate made earlier[16] from the correlation of the CO infrared frequency with k_L values. The ratio of H-bond strengths for O_2 and CO is higher than the value of 4 which was estimated above from the dependence of k_L on the calculated electric potential,[40] or from the DFT model results,[41] but this could reflect a less-than-optimal alignment of the histidine side chain with respect to the bound CO. Precisely because the bound O_2 and CO have different geometries, their interaction with specific residues will differ somewhat. Indeed, Phillips et al.[40] found that the correlation of k_L vs electrostatic potential for O_2 improved if structural data for the O_2 adducts were substituted for those of the CO adducts (see Figure 7).

The six entries in Table 1 for ΔE_{dx} give an average value of 1.0 kcal/mol for the H-bond between the distal histidine and the water molecule in the deoxyMb structure. This is a low H-bond value but is consistent with several lines of evidence cited by Olson and Phillips[7] that the occupancy factor for this water molecule is only about 90%. The six values for ΔSt_{CO} average to 0.6 kcal/mol. Thus the steric inhibition to CO binding by the distal histidine does indeed appear to be very modest, and accounts for a minor part of the discrimination energy.

The discrimination energy is the difference between $\Delta\Delta G_L$ values for CO and O_2 (last column of Table 1). We obtain an average value of 3.7 kcal/mol as a measure of the contribution of the histidine residue to the ligand discrimination. (This is twice the value calculated by Lopez and Kollman[34] using free energy perturbation and the AMBER force field[42]). This value is sufficient to account for the entire ligand discrimination effect of Mb relative to protein-free heme. Thus, the distal histidine is, indeed, the key residue in adjusting the relative ligand affinities. The 3.7 kcal/mol of discrimination energy is made up of a 0.6 kcal/mol steric contribution and a 3.1 kcal/mol electrostatic contribution, the difference between the H-bond energies for bound O_2 and CO. On the basis of these estimates, the steric contribution accounts for about one-sixth of the discrimination, the remainder being due to the stronger H-bond to O_2 than to CO.

The individual entries in Table 1 show some dispersion, as expected from the simplified nature of the model we employ. Each mutation creates numerous small changes in the inter-residue interactions. The leucine mutant is an outlier, whose anomalous character has been noted by Phillips and Olson.[7] The main anomaly is the very high value of K_{CO}, ~1000 μM^{-1}, vs ~150 μM^{-1} for the valine and isoleucine mutants. Likewise, K_{O_2} is higher for leucine, although the elevation is not as dramatic. In addition, k_L is barely altered for CO, relative to that for the wild-type protein, resulting in an anomalously low ΔE_{CO}. The value of ΔE_{O_2} is also depressed, relative to the values for the other mutants. This pattern of anomalies is the same for the sperm whale and human mutants. We speculate that the leucine side chain provides a particularly favorable environment for the nascently dissociated ligand, which is known to be located in a pocket next to the heme.[5,45,46] This stabilization can explain the increase in fraction of geminate CO recombination which is observed immediately after photolysis of the CO adduct of the Leu64 mutant.[47,48] An increase in internal rebinding increases the overall association rate constant and lowers the dissociation rate constant, producing a lower apparent ΔE_L value. This extra stabilization could also account for a higher ligand affinity because the free energies for forming the heme-bound and protein-bound ligand states are additive.

If the leucine mutant is excluded from the averages, then the energy estimates change only slightly. ΔE_L increases by 0.1 kcal/mol, for both ligands, leaving the electrostatic contribution to the discrimination at 3.1 kcal/mol. ΔE_{dx} is unaltered, while ΔSt_{CO} diminishes, from 0.6 to 0.4 kcal/mol, thus accounting for one-ninth of the total discrimination energy.

These estimates of ΔSt_{CO}, 0.6 or 0.4 kcal/mol, are at the low end of the range of estimates based on the DFT-computed distortion energies, implying that, unlike the

superstructured porphyrin model compounds, the distal histidine stores very little excess steric energy. This is not entirely surprising since the crystal structures show the E7 imidazole side chain to be rather flexible, as indicated by high-temperature factors[19] or significant population of alternative orentations.[20]

Recently, Kachalova et al.[19] have proposed a different steric mechanism that could potentially carry excess energy, like the model compounds. Analysis of their high-resolution structures revealed a ligation-induced concerted motion of the distal (F) and proximal (E) helices, which hold the heme like a clamshell. (Elsewhere we have proposed that a similar concerted motion is the first step in the allosteric transition of hemoglobin[49]). They noted, that unlike histidine E7, another contacting residue, valine E11, is well-packed in the pocket but is displaced 0.5 Å betweeen MbCO and deoxyMb, and they proposed that this displacement is linked to the concerted helix movements.

Replacement of valine E11 with the smaller alanine residue (which has no effect on the CO dissociation rate, as expected) does selectively increase the CO binding constant, but only by a factor of 2,[9] showing that the contribution to ligand discrimination by the steric hindrance of valine E11 is worth only 0.4 kcal/mol. Substitution of E11 by the larger isoleucine residue[9] inhibits CO binding by a much larger factor, ~13, showing that steric hindrance at this position is certainly possible. However, the affinity of myoglobin for O_2 is also reduced significantly, ~6-fold, by the Ile68 substitution. Thus, although there is selective steric discrimination against CO binding, the effect is again only a factor of ~2.

Summary

Early reports that the FeCO unit is severely bent in MbCO are generally agreed to have been incorrect. All recent experimental data point to a small and somewhat variable distortion, with the O atom displaced from the heme normal by 0.3−0.6 Å. The energy required for this extent of distortion is quite small, because of interaction between bending and tilting coordinates. DFT computation yields 0.3−1.1 kcal/mol. However, these facts are not in themselves sufficient to overthrow the steric mechanism for ligand discrimination, because they do not address the amount of steric energy stored by the protein. The finding that FeCO is only slightly distorted in sterically encumbered model compounds with significantly diminished CO affinity underscores the possibility that the protein could, in principle, store significant steric energy.

Nevertheless, the results of site-directed mutagenesis establish fairly clearly that this does not happen. Analysis of the kinetic and equilibrium data indicates that the steric inhibition of CO binding is worth about 0.5 kcal/mol. The rest of the ca. 3.5 kcal/mol of discrimination in favor of O_2 is attributable to H-bonding by the distal histidine side chain, which is much stronger to bound O_2 than to bound CO.

There are two important differences in the properties of heme-bound CO vs O_2. The first is the geometry difference: FeO_2 is naturally bent, while FeCO is linear. The second is the polarity difference: more negative charge is transferred to the ligand in FeO_2 than in FeCO. Both offer a potential mechanism for ligand discrimination. Nature has mainly exploited the polarity difference in achieving discrimination. One can speculate that it is easier to place H-bond donors at strategic positions in the protein than it is to engineer a protein scaffold near the iron atom that is rigid enough to exploit small geometry differences between the bound ligands.

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