Conformational substates and motions in myoglobin

External influences on structure and dynamics

Mi Kyung Hong,* David Braunstein,* Benjamin R. Cowen,* Hans Frauenfelder,* Icko E. T. Iben,‡ Judith R. Mourant,* Pál Ormos,§ Reinhard Scholl,* Alfons Schulte,‖ Peter J. Steinbach,* Ai-Hua Xie,* and Robert D. Young*¶

*Departments of Physics and Biophysics, University of Illinois at Urbana-Champaign, 1110 West Green Street, Urbana, Illinois 61801; ‡AT&T Bell Labs, Murray Hill, New Jersey 07974; §Biophysics Inst., Hungarian Academy of Sciences, 6701 Szeged, Hungary; ‖IBM Almaden Research Center, 650 Harry Road, San Jose, California 95120; and ¶Department of Physics, Illinois State University, Normal, Illinois 61761 USA

ABSTRACT Myoglobin, a simppe dioxygen-storage protein, is a good laboratory for the investigation of the connection between protein structure, dynamics, and function. Fourier-transform infrared spectroscopy on carbon-monoxymyoglobin (MbCO) shows three major CO bands. These bands are excellent probes for the investigation of the structure-function relationship. They have different CO binding kinetics and their CO dipoles form different angles with respect to the heme normal, implying that MbCO exists in three major conformational substates, A_0, A_1, and A_3. The entropies and enthalpies of these substates depend on temperature above ~180 K and are influenced by pH, solvent, and pressure. These results suggest that even a protein as simple as Mb can assume a small number of clearly different structures that perform the same function, but with different rates. Moreover, protein structure and dynamics depend strongly on the interaction of the protein with its environment.

INTRODUCTION

Proteins are biomolecules that perform vital roles of catalysis, transport, and storage in all living cells. Specificity and control are crucial to these tasks, and many models have been put forth to explain these features. In 1894, Emil Fischer described a mechanistic analogy for specificity in which a substrate ("the key") uniquely fits into a rigid protein ("the lock") (1). However, a growing body of evidence against this simple picture led Daniel E. Koshland, Jr., to postulate in 1958 that binding of the substrate can change the conformation of the enzyme to one more favorable for the reaction, a process he called an "induced fit" (2). In the present paper we show that studies of a simple protein shed new light on this problem.

The concept of conformational substates is central to the following discussion: experiments (3, 4) and computer simulations (5, 6) show that a protein does not exist in only one particular conformation but can assume a large number of slightly different structures, called conformational substates (CS). CS perform the same function, but usually with different rates. At low temperatures, each protein is frozen into a particular CS. At high temperatures, say near 300 K, a protein fluctuates rapidly from substate to substate. CS can be described as valleys in the conformational energy hypersurface of a protein. Similar rugged energy landscapes exist in glasses (7) and spin glasses (8). Proteins and glasses indeed share many similarities (9). Conformational substates form the conceptual basis for explaining protein dynamics and induced fits: if CS encompass a wide enough range of structures and if these structures adjust to external agents such as pH and solvent, then induced fits can be connected to other dynamic features of proteins.

MYOGLOBIN AS A LABORATORY

Proteins are complex systems, and investigations even on simple proteins when extended into previously unexplored regions of time, temperature, pressure, and solvent or when performed with new techniques reveal novel features. Here we describe results obtained with myoglobin (Mb), a dioxygen (O_2) storage protein. Mb contains a heme group as active site. O_2, carbon monoxide (CO), and other ligands bind at the heme iron (10). Twenty years ago, the binding of CO and O_2 to Mb was believed to be nearly fully understood (11). Studies over wide ranges in time, temperature, and solvent conditions demonstrate, however, that ligand binding is a dynamic process that involves many steps (3, 4, 12). Because the structure of Mb is well known, this relatively simple protein can serve as a laboratory for extended studies of protein states, motions, and reactions (13–15).

Myoglobin binds CO reversibly, and it has been known for many years that the bound CO shows a number of stretch bands in the mid-infrared region between 1,900 and 2,000 cm^{-1} (16). These infrared bands are sensitive to the state of the protein. The ratios of the band areas depend strongly on surroundings (17) including pH (18), hydration (19), temperature (20, 21), and pressure (21). We consequently use these bands to explore properties of substates in Mb. Because multiple bands occur in essen-

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tially all heme proteins that bind CO, we expect that the general results of the Mb study will be valid for other heme proteins, but that functionally important features may differ from protein to protein.

EXPERIMENTAL APPROACH AND DATA

We have measured the infrared spectrum of MbCO in the region of the CO stretch bands (1,900–2,000 cm^{-1}) as a function of temperature (T), pH, and solvent on a Fourier-transform infrared spectrometer (Mattson Sirius 100). Spectra were taken with 2 cm^{-1} resolution and a sensitivity of the order of 10^{-4} OD by averaging 500 scans. Samples were prepared from lyophilized sperm whale myoglobin type II powder (Sigma Chemical Co., St. Louis, MO). Buffers were 0.4 M citrate-phosphate for samples near pH 5, 0.1 M potassium phosphate for samples near pH 7, and 0.4 M carbonate-bicarbonate near pH 9. The pH was measured at room temperature after protein was added to the solvent. Quoted pH refer to these values. Solvents included 75% glycerol/buffer (by volume), 60% ethylene glycol/buffer (by volume), and deuterated water (D_2O). Metmyoglobin was gently stirred under a CO atmosphere overnight and reduced to MbCO by the addition of an excess of CO-saturated sodium dithionite. The sample holder was mounted in a closed-cycle helium refrigerator (model 21; CTI-Cryogenics Div., Waltham, MA) equipped with NaCl windows and a temperature controller (model DTC-500; Lake Shore Cryotronics, Inc., Westerville, OH).

Fig. 1 shows, as a characteristic example, the IR spectra of MbCO at three different pH values as a function of temperature. Two features of these spectra are immediately apparent: (*a*) three major CO stretch bands are clearly distinguishable. We denote them in order of decreasing wave number by A_0 ($\nu_{CO} \approx 1{,}966$ cm^{-1}), A_1 ($\nu_{CO} \approx 1{,}945$ cm^{-1}), and A_3 ($\nu_{CO} \approx 1{,}930$ cm^{-1}) and refer to them as A bands. (*b*) The relative areas and widths of the three bands depend markedly on temperature and pH, but their peak wave numbers change little. The three IR bands have also been observed with Mb embedded in a variety of solvents, dry and wet films, and crystals (17, 19, 21, 22).

It is well known that commercial myoglobin is not homogeneous (23). The appearance of the different A bands could consequently be ascribed to different protein fractions. The fact that the bands exchange proves, however, that they occur in the same component.

Additional experiments provide more characteristics of the CO stretch bands: (*c*) the angle α between the CO dipole and the heme normal has been determined with a photoselection technique (24, 25), giving $\alpha(A_0) = 15° \pm 3°$, $\alpha(A_1) = 28° \pm 2°$, $\alpha(A_3) = 33° \pm 4°$. Within each band, the angle α is distributed and depends on wave number. (*d*) We have previously measured the rebinding of CO photodissociated by a laser flash by monitoring separately in the three A bands and find that each A band binds CO with a different rate; A_0 binds fastest and A_3

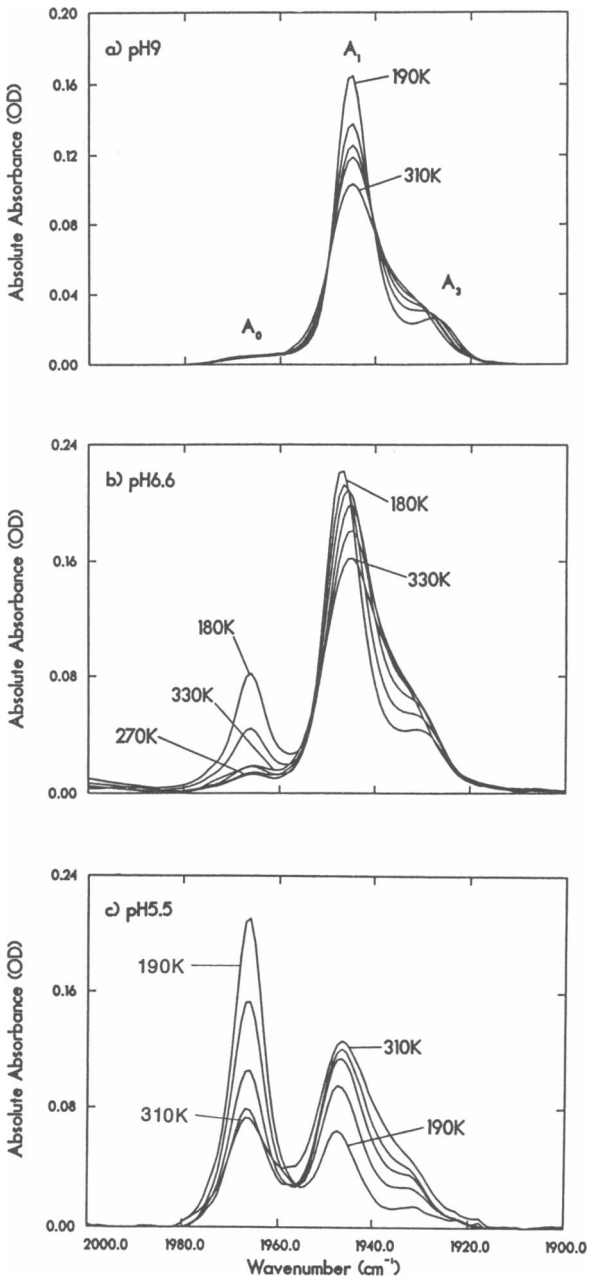

FIGURE 1 Infrared absorbance spectra of MbCO at three values of pH as a function of temperature. Spectra are shown at 30 K intervals. Solvent: 75% glycerol/buffer (by volume). The protein concentrations are (*a*) 10 mM for pH 9, (*b*) 15 mM for pH 6.6, and (*c*) 5 mM for pH 5.5.

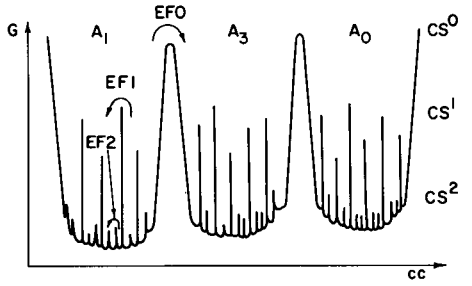

FIGURE 2 The arrangement of conformational substates in MbCO. The figure shows the Gibbs energy of MbCO as a function of a representative conformational coordinate cc. The three large potential wells represent the three substates of tier 0 characterized by the different CO stretch bands. Substates of tiers 1 and 2 are also shown. EFi represent equilibrium fluctuations among CS^i, $i = 0, 1, 2$.

slowest (21). Moreover, all three rebinding processes are nonexponential in time below ~180 K. (*e*) Spectral and kinetic hole-burning experiments on the Soret and the infrared *A* bands demonstrate that the bands are inhomogeneously broadened (26, 27, 28). This observation, together with the results described in (*c*) and (*d*), shows that the spectra and the function (CO binding) of Mb are coupled to a structural heterogeneity.

The error in determining the area of the CO stretch bands, ~15%, is mainly systematic and due to a combination of three effects: the choice of the baseline (straight line or higher order polynomial); the uncertainty of whether there is an underlying fourth peak (A_2) or whether some of the peaks are asymmetric; and the difficulty in separating A_3 from A_1. However, the general feature of the ratio of A_0/A_1 vs. $1/T$ shown in Fig. 4 is independent of the fitting procedure implemented.

CONFORMATIONAL SUBSTATES IN MYOGLOBIN

We interpret the data given in *a–e* by saying that MbCO can exist in three conformational substates of tier 0, also denoted by A_0, A_1, and A_3 and shown schematically as three large wells in Fig. 2. The three substates satisfy the proper definition: they have approximately the same overall structure; otherwise x-ray diffraction would not yield a reasonable MbCO structure (29). However, the structures of each *A* substate differ in detail as proven by the different angles α. This conclusion is consistent with the x-ray structure which shows multiple conformations for the CO and disorder in several residues near the heme pocket (29). The three *A* substates all bind CO, but with different rates (21). Substates of tier 0 are the simplest case of isozymes.

The data described in *a–e* also suggest that substates are arranged in a hierarchy (13) shown in Fig. 2. Each of the three *A* substates (tier 0) must consist of a large number of substates of tier 1 (CS^1) because rebinding in each CS^0 is separately nonexponential in time. Moreover, the measurement of the angles α_i shows that each angle is distributed and can assume values within a range of a few degrees about the mean angle quoted above (24). Rebinding is mainly controlled by the protein through the proximal histidine (30, 31). The *protein structure* in a particular substate CS^1 then will determine the spectral characteristics, the angle α, and the CO binding rate.

Two different structural models for MbCO, sketched in Fig. 3, are suggested by the results discussed so far. In Fig. 3 *a*, the overall protein structure is the same for all CS^0, and the substates differ mainly in the orientation of the CO dipole with the heme. In Fig. 3 *b*, the entire

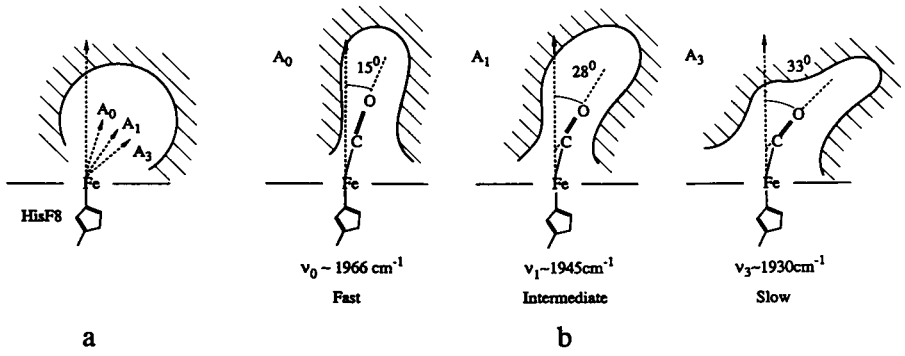

FIGURE 3 Two different models for the conformational substates of tier 0. (*a*) The three substates A_0, A_1, and A_3 have the same protein structure, but the CO molecule can assume three different orientations within the heme pocket. (*b*) Mb can assume three different overall structures. Each substate permits only a small range of CO orientations centered around a mean angle between CO dipole and heme normal. The shapes of the heme pocket do not correspond to the (unknown) real ity, but are drawn only to illustrate the concepts.

protein structure differs in the three substates and in each CS^0 only one angle α_i occurs. In both models, the structure near the heme differs in different substates of the first tier, CS^1, resulting in small variations of α about its average. In Fig. 3 we depict the simplest situation in which the heme is planar and not domed or buckled and the CO is tilted and bent. In general, doming and buckling must also be considered (32).

All evidence points to Fig. 3 *b*: Makinen et al. already stressed that the protein environment determines which of the three CO stretch bands dominates (17). Our observations strengthen this conclusion: transitions between the A substates do not occur if the solvent is a glass or a solid and does not permit the protein molecule to move (21). Even if MbCO is photolyzed, transitions among the A substates cannot be induced below the glass temperature (21). The decision between the two models could be clinched by determining both the x-ray structure and the infrared spectrum for a number of crystals with different pH. Here we adopt Fig. 3 *b* in which each substate of tier 0 possesses a different overall protein structure.

THERMAL PROPERTIES OF PROTEINS

The temperature dependence of the three CO stretch bands yields new insight into protein dynamics (9, 21). Fig. 4 displays the ratio A_0/A_1 for MbCO in two solvents. The data show two features that call for explanation: on lowering the temperature from ~320 K, the three bands display characteristically different behavior, to be discussed below. In addition, near 200 K the normalized areas of the bands become essentially temperature independent. We interpret the behavior as follows. Above a glass temperature T_{sg}, each protein molecule fluctuates rapidly from one substate CS^0 to another so that the three CS^0 are in thermal equilibrium on the time scale of our experiment. Below T_{sg}, however, the exchange is frozen on the experimental time scale, and each protein molecule is in a glasslike, metastable state. We have discussed the transition region previously (9) and concentrate now on the equilibrium behavior above 200 K. In the present paper we only discuss the substates A_0 and A_1 because they yield the best data at pH 5.5 and 6.6. The same approach can, however, also be extended to A_3 (22).

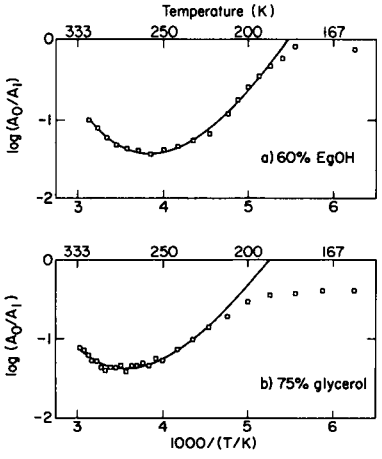

FIGURE 4 Fits to the ratio A_0/A_1 using a temperature-dependent enthalpy and entropy. (*a*) MbCO in 60% ethylene glycol/buffer, pH 7. (*b*) MbCO in 75% glycerol/buffer, pH 6.6. Above ~330 K, the protein begins to unfold and the data deviate from the fit.

The nonlinearity of the temperature dependence of the ratio A_0/A_1 in Fig. 4 is dramatic. One explanation of this behavior invokes a new substate A_0' and assumes that the protein gradually shifts from A_0 to A_0'. A similar model was postulated earlier to explain denaturation of hemoglobin by acids and temperature (33). Although this explanation permits a good fit to the data of Fig. 4 (34), it is unsatisfactory: the substate A_0' must have the same CO stretch spectrum as A_0 but must be different thermodynamically (35). We now present an alternative model, based on the known thermal properties of proteins, which yields a satisfactory explanation.

In equilibrium, the ratio of the populations in the two substates A_0 and A_1 is given by the Boltzmann factor,

$$A_0/A_1 = \exp[-\Delta G/RT] = \exp[-(\Delta H - T\Delta S)/RT], \quad (1)$$

where

$$\Delta G = G_0 - G_1, \ \Delta H \equiv H_0 - H_1, \ \Delta S = S_0 - S_1 \quad (2)$$

are the differences in Gibbs free energies, enthalpies, and entropies of the protein-solvent system for the substates A_0 and A_1. The population ratios can be found from the band areas if the relative oscillator strengths of the bands are known. We have determined the relative oscillator strengths of the bands A_0 and A_1 by examining the changes in band area under pressure. Pressure shifts the population from A_1 to A_0. The area lost from one band must equal the area gained by the other if the oscillator strengths are the same. Using data from our pressure experiments (36) we find that the areas are the same to within <10%. With equal oscillator strengths, the ratio of populations A_0/A_1 in Eq. 1 is given by the experimentally determined ratio of band areas.

Usually, enthalpy and entropy are taken to be temperature independent, and Eq. 1 then predicts a standard van't Hoff behavior for the ratio A_0/A_1. For proteins, however, enthalpy and entropy are temperature dependent. Experiments show that the specific heat of globular proteins is

TABLE 1 Thermodynamic parameters

System	Solvent	$\Delta H(0)$	$\Delta S(0)$	Δs
		kJ/mol	*J/mol K*	*J/mol K²*
MbCO	75% G pH 5.5	−22	−150	0.44
MbCO	75% G pH 6.6	−50	−380	1.26
MbCO	60% EGOH pH 7	−55	−450	1.6
MbCO	D_2O pD 5	−27	−210	0.68
metMb	pH 10	−1,600	−11,000	35
metMb	pH 4	−1,300	−8,900	29

The first four rows refer to the difference between the substates A_0 and A_1 in MbCO; rows five and six give the differences between the folded and unfolded states of metMb. The parameters are defined in Eqs. 3 and 4. *G* refers to glycerol/buffer and EGOH to ethylene glycol/buffer. The errors of $\Delta H(0)$, $\Delta S(0)$, and Δs are ~20% and include both the errors from determining the area of the CO stretch bands and from the fit of the ratio A_0/A_1 to the thermodynamic model.

essentially linear from 100 to 320 K (37). The entropy therefore has the form $S(T) = a + b \ln (T/T_0) + cT/T_0$, where T_0 is an arbitrary reference temperature. The experimental data (37) show that the term $a + b \ln (T/T_0)$ deviates from a constant by <5% between 200 and 300 K so that the entropy can be approximated by

$$S(T) = S(0) + sT. \quad (3)$$

The temperature dependence of H follows from the relation $(\partial H/\partial T)_P = T(\partial S/\partial T)_P$ as

$$H(T) = H(0) + \tfrac{1}{2} s T^2. \quad (4)$$

With Eqs. 3 and 4, the difference in Gibbs energy between the substates A_0 and A_1 becomes

$$\Delta G(T) = \Delta H(0) - T\Delta S(0) - \tfrac{1}{2}\Delta s\, T^2. \quad (5)$$

This form of $\Delta G(T)$ leads naturally to the nonlinear temperature dependence in Fig. 4. Fitting the experimental data to Eqs. 1 and 5 yields the solid lines shown in Fig. 4 for two characteristic data sets.[1] The resulting parameters, together with parameters derived from other data sets, are summarized in Table 1. The differences $\Delta H(T)$, $T\Delta S(T)$, and $\Delta G(T)$ for MbCO in 75% glycerol-water are given in Fig. 5.

[1]Retaining the term $b \ln (T/T_0)$ in the entropy results in a term bT in $H(T)$ and $bT[1 - \ln (T/T_0)]$ in the Gibbs energy. Eq. 5 for the Gibbs energy difference then would include a term $\Delta bT[1 - \ln (T/T_0)]$ which deviates from a constant by ~10% from 200 K (reference) to 300 K whereas T and T^2 change by 50% and 125%, respectively. This term also introduces another parameter. Because Eq. 5 is already sufficient to fit the data we omit this term. The resulting uncertainties in the parameters of Eq. 5 are included in the errors quoted in Table 1.

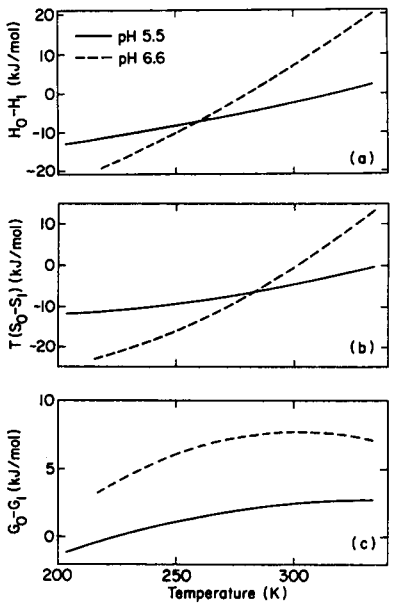

FIGURE 5 (*a*) Plot of $\Delta H(T) = H_0(T) - H_1(T)$ vs. T; (*b*) $T\Delta S(T) = T[S_0(T) - S_1(T)]$ vs. T; (*c*) $\Delta G(T) = G_0(T) - G_1(T)$ vs. T. Curves derived from fits to data in Fig. 1, *b* and *c*. Sample: MbCO in 75% glycerol/buffer (by volume). pH is 5.5 and 6.6.

CONCLUSIONS AND SPECULATIONS

The data presented above lead to a number of conclusions and speculations. We begin with facts that are established by the present experiments and proceed to speculations that must be checked by additional investigations.

(*a*) The data in Figs. 1 and 4 demonstrate that studies of protein states and protein dynamics performed over small ranges in temperature or by using only visible spectroscopy can be misleading. The spectral bands in the visible region usually are not sufficiently sensitive to protein conformation and, hence, give little detailed information about the conformational substates, such as A_0, A_1, and A_3, with rather different functional properties. Infrared and Raman techniques, applied over a wide range in temperature, are needed to extract the functionally important details.

(*b*) The data in Fig. 5 for MbCO in 75% glycerol/buffer at pH 6.6 show that the substate A_0 is lower in enthalpy and entropy than A_1 below ~280 K. The enthalpy and entropy of A_0 increase more rapidly with T than those of A_1 and above ~280 K, the A_0 substate is much less tightly bound than A_1. The rapid increase of the entropy of A_0, however, causes the population to shift increasingly from A_1 to A_0. These characteristics suggest that the substate A_0 is stabilized by additional interactions, possibly hydrogen bonds, as compared with A_1.

With increasing T some bonds break, a situation consistent with the more rapid increase in enthalpy and entropy of A_0 relative to A_1. The fact that the difference $H_0(0)$–$H_1(0)$ is smaller at pH 5.5 than at pH 6.6 supports this interpretation. At the lower pH, more residues are protonated and the number of hydrogen bonds is smaller in A_0 as compared with A_1, leading to a smaller $\Delta H(0)$.

(*c*) The substates of tier 0, characterized by their different CO stretch bands, tilt angles α, and CO binding rates, possess different overall protein structures as in Fig. 3 *b*. The temperature-dependent entropy and enthalpy in Eqs. 3 and 4 suggest that the A substates of MbCO are stabilized by collective phenomena involving the entire protein (38–40).

Kinetic and spectral hole-burning studies in MbCO show a correlation between the barrier distribution responsible for the nonexponential time dependence of rebinding at low temperature and the inhomogeneous broadening of the Soret and the A_1 bands (28). Thus, structural features present in the bound state are involved in the barrier distribution for bond formation at the heme iron. Furthermore, kinetic hole-burning experiments on band III, a charge-transfer band of deoxy and photodissociated Mb, show a strong correlation between the barrier distribution and band III (27, 41). All the data taken together imply that the structural features responsible for the substates of tiers 0 and 1 in MbCO also exist in the unbound Mb state.

(*d*) The populations of the substates are affected by temperature, pressure, pH, and the structure of the environment. This observation shows that a protein reaction can be controlled by shifting the protein from one substate to another which has a different binding rate. The entire protein is then involved in the control, not merely a few residues.

(*e*) The existence of substates of various tiers affords a molecular model for induced fits. In this model, the protein is not simply a floppy system that can adjust its structure nearly arbitrarily, but possesses a small number of conformational substates of tier 0 with well-defined structures and properties. Smaller changes around these CS^0 are achieved by changes in the population of the CS^1. An induced fit may thus correspond to a switch between CS^0 with a concomitant change in the CS^1 populations. This speculation is supported by studies of the CO stretch bands in genetically modified Mb molecules suggesting that the mutual interaction between the bound CO and residues in the heme pocket shifts the MbCO molecule into a particular substate of tier 0 (42). Furthermore, comparison of the x-ray structure of MbCO and deoxy Mb shows that the heme pocket is significantly larger in MbCO than in deoxy Mb because the distal residues have relaxed around the CO. Also, the CO and several residues near the heme pocket are disordered and possess multiple conformations (29).

(*f*) The curves in Figs. 4 and 5 imply a connection between the substate A_0 and unfolding. Plots of the difference in enthalpy, entropy, and Gibbs energy between the folded and unfolded state of a protein show a behavior that is similar to the one displayed in Fig. 5 (38, 40). Evaluating the unfolding data for metMb given in references 39 and 40 with the Eqs. 3 and 4 yields the thermodynamic parameters given in Table 1. The coefficients have the same sign as the ones listed in Table 1 for the difference between A_0 and A_1, but are more than one order of magnitude larger. The values in Table 1 are consequently not unreasonable because the difference is taken between two folded substates of Mb and should be smaller than the difference between a folded and the unfolded state of Mb. It is possible that the A_0 substate is a precursor in the unfolding of myoglobin: above 300 K, A_0 is much less tightly bound and has a higher entropy than A_1 and A_3, and it interchanges rapidly with them. Between 340 and 350 K, the Gibbs energy of A_0 is ~7 kJ/mol greater than A_1 and 4 kJ/mol $>A_3$. In this temperature range the Gibbs energy of the unfolded state is rapidly decreasing with increasing temperature. Thus, unfolding may occur through the sequence $A_1 \rightarrow A_0 \rightarrow$ unfolded.

While we have investigated only Mb in detail, essentially all heme proteins that have been studied show multiple CO stretch bands (16, 20, 43, 44). The existence of substates of tier 0, with different overall protein structures and functional properties, is consequently implied for a large class of proteins.

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