The Transition from Inhomogeneous to Homogeneous Kinetics in CO Binding to Myoglobin

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ABSTRACT Heme proteins react inhomogeneously with ligands at cryogenic temperatures and homogeneously at room temperature. We have identified and characterized a transition from inhomogeneous to homogeneous behavior at intermediate temperatures in the time dependence of CO binding to horse myoglobin. The turnover is attributed to a functionally important tertiary protein relaxation process during which the barrier increases dynamically. This is verified by a combination of theory and multipulse measurements. A likely biological significance of this effect is in the autocatalysis of the ligand release process.

INTRODUCTION

Ligand binding to myoglobin (Mb) is a model biophysical reaction, extensively studied over a large range of times and temperatures (Austin et al., 1975; Doster et al., 1982, 1989, 1990; Ansari et al., 1985, 1987, 1992; Ormos et al., 1990; Steinbach et al., 1991; Young et al., 1991; Campbell et al., 1987; Petrich et al., 1991; Gibson et al., 1992; Šrajer et al., 1991; Tian et al., 1992; Hong et al., 1991; Post et al., 1993). In the experimental data of Fig. 1, horse Mb-CO is photolyzed with a nanosecond laser and the survival probability of the deligated heme is followed by transient absorption over many decades in time. Eventually, all hemes rebind the CO and the signal vanishes. The time course of this process is highly nonexponential.

At low temperatures the decay curves are smooth and close to a straight line on a log-log scale. Austin and coworkers (Austin et al., 1975) explained this near power-law behavior as arising from a static distribution of protein conformations. Double pulse experiments (Austin et al., 1975), showed that at 70K the behavior is independent of the number of photolyzing pulses. This implies that at low temperatures the protein structure is inhomogeneous, each molecule possessing a different tertiary structure and ligand binding rate coefficient. In contrast, at room temperature rebinding is single-exponential suggesting a homogeneous ensemble where all hemes have the same average rebinding rate. The question therefore arises: How does the transition from inhomogeneous to homogeneous kinetics take place? We investigate this problem by studying the reaction at intermediate temperatures, expecting to observe inhomogeneous kinetics at short times and homogeneous kinetics at long times, after conformational interconversion takes place.

Focusing on the temperature regime of 150–210K, one notes the emergence of structure in the rebinding curves in the form of "kinks" and "bends." In addition, rebinding slows down with increasing temperature. For example, at 200K the reaction is already *slower* than at 190K. This has first been interpreted (Austin et al., 1975; Doster et al., 1982) by sequential kinetics of ligand escape from the heme pocket. It was later pointed out (Agmon and Hopfield, 1983) that the minimal description incorporating both ligand and conformational state is in terms of a two-dimensional surface such as shown in Fig. 2. When it is adjusted to fit the inhomogeneous kinetics below 150K, one finds that the protein conformation of a newly photodissociated heme is out of equilibrium. While the frozen heme protein cannot readjust, at higher temperatures one expects protein relaxation to stabilize the unbound state. As a result ligand rebinding should slow down.

Such an autocatalytic, "self-trapping" effect has been suggested for hemoglobin kinetics (Scott and Friedman, 1984; Friedman, 1985), in which the Raman frequency of the iron-histidine stretch decreases with time, indicating that tertiary relaxation diminishes the ligand affinity of the heme. Faster and more accurate measurements of sperm-whale (SW) MbCO kinetics above the solvent's glass transition temperature (ca. 185K) indicate an increase of over 10 kJ/mol in the effective barrier for rebinding (Steinbach et al., 1991; Tian et al., 1992). Horse MbCO shows similar kinetics to that of SW MbCO for $T < 150$K, but exhibits more structure in the decay curves at higher temperatures (Doster et al., 1989, 1990; Post et al., 1993). If this structure is indeed related to protein relaxation, the horse-Mb system should be a good candidate for probing the relaxation process.

It is important to search for independent measures of protein relaxation. A distinctive spectral feature of deoxy hemes is their weak near-IR (760 nm) band, which blue shifts following photodissociation. Since this band is assigned to iron-porphyrin charge transfer (Eaton and Hofrichter, 1981), it is sensitive to the out of plane location of the iron. At low temperatures it is plausible to expect inhomogeneous line-shape broadening by a protein-induced distribution of iron distances. The fast binding conformations disappear first

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from the spectrum, leading to spectral shifts which are due to "kinetic hole burning" rather than to relaxation (Campbell et al., 1987; Agmon, 1988). By mapping spectral inhomogeneities into barrier heights one concludes that the barrier height increases by about 12 kJ/mol between low and high temperatures (Steinbach et al., 1991).

Our results are based on a recent analysis of the nonexponential phase in two-dimensional (2d) diffusional dynamics (Agmon and Rabinovich, 1992; Rabinovich and Agmon, 1993). The conclusion from our theoretical treatment is that the logarithmic derivative of the survival probability (the "beta function," $B(t)$), should show a maximum and a minimum, displaced by about two logarithmic units. These are the "kinks" and "bends" in the decay curves above 160K (Fig. 1). Within this "relaxation footprint," the maximum signals the "onset" of protein relaxation, while the minimum represents its "termination." Using multipulse experiments, we show that these times correspond to the end of the inhomogeneous and the beginning of the homogeneous phases in MbCO kinetics, respectively. Thus the transition from inhomogeneous to homogeneous kinetics is due to protein relaxation.

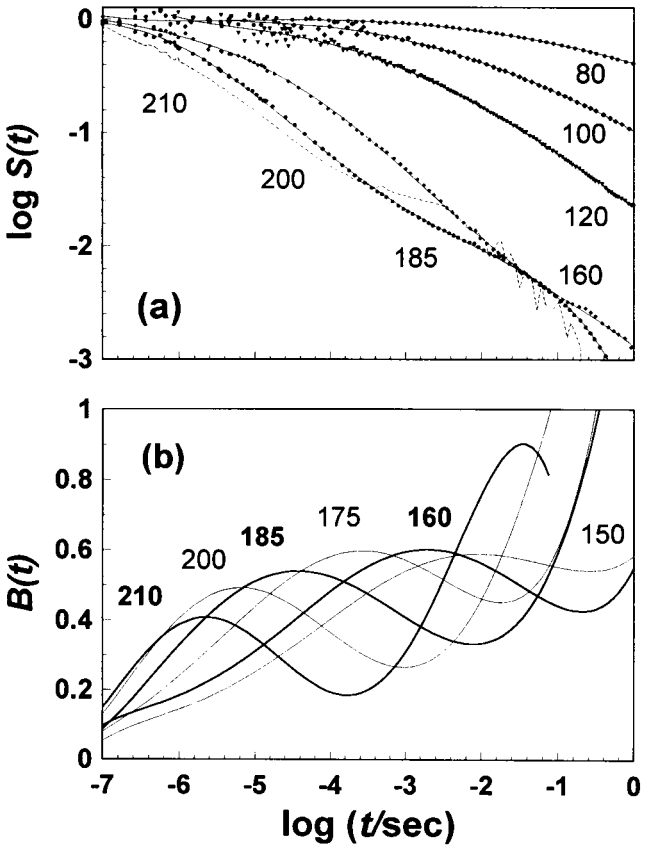

FIGURE 1 CO binding to horse myoglobin: (*a*) Deligated Mb survival probability in the low ($T < 120$K) and intermediate temperature regimes. An eighth order polynomial fit to the measured data is shown at all temperatures except 200 and 210K, for which data points were connected by dashed lines. (*b*) The slopes of the data from *a* in the intermediate temperature regime, obtained by differentiating the polynomial fits.

THEORY

Model description and justification

In a complex system such as a protein, description of a chemical reaction in terms of a single coordinate may be inadequate. The distributed low-temperature kinetics indicate that different protein conformations react with different rates (Austin et al., 1975). It is thus logical to introduce an explicit "protein coordinate" for representing the conformational variable. We consider (Agmon and Hopfield, 1983) the geminate CO-Mb binding reaction occurring on a 2d potential surface, $U(x, y)$. Comparison with experiment will indicate if and when it should be extended by adding more degrees of freedom (Šrajer et al., 1988).

In Fig. 2, x is the CO-Fe distance, where Fe is the iron atom in the center of the porphyrin ring. Small x values represent bound Fe-CO (iron spin state of $S = 0$). Large x values represent dissociated CO in the heme pocket (spin $S = 2$). The cusp at intermediate x values arises from the intersection of the two diabatic potential surfaces, presenting a barrier for CO binding. A reflecting wall is imposed at large x values, preventing ligand escape to solution. We thus focus on the geminate phase, eliminating complications of bimolecular recombination from solution. In the low temperature limit rebinding is geminate over the whole experimentally accessible time window. Even at room temperature the ligand does not seem to escape from the pocket before about 500 ns.

The protein coordinate, y, could modulate heme reactivity in several ways for example, by coupling to the iron out-of-plane distance via the proximal histidine link. In the deoxy state, the Fe is displaced some ¼–½ Å toward the proximal side of the porphyrin ring plane (Perutz et al., 1987). In the spirit of the Hammond postulate and Brönsted correlation (Hammond, 1955; Agmon, 1981), stabilization of the deoxy state by increase in the out-of-plane distance should increase the barrier height. Commensurate with the dif-

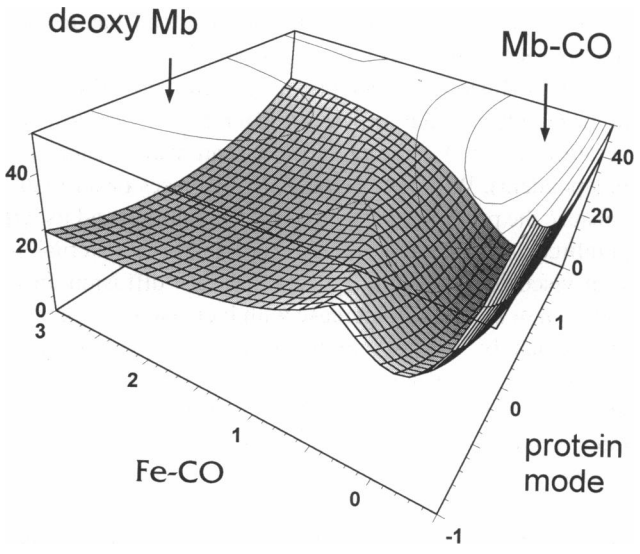

FIGURE 2 The 2d potential energy surface for CO binding to sperm-whale myoglobin.

ference in Fe position for MbCO and Mb+CO, it is reasonable to assign different equilibrium positions in y for the two binding states. This leads to a displaced double well potential as shown in Fig. 2.

Prior to photodissociation, the CO is bound to an equilibrated heme. The predissociation distribution within the $S = 0$ part of the potential is therefore proportional to $\exp[-U(x, y)/k_BT]$, where k_B is Boltzmann's constant. In analogy with the Franck-Condon principle, dissociation of the small ligand is considerably faster than a bulky protein rearrangement. Photolysis shifts the distribution to large x values while keeping y constant. This becomes the initial distribution for the rebinding process. When the protein is frozen, one has a static, inhomogeneous distribution of protein conformations giving rise to a distribution of barrier heights (Austin et al., 1975) and thus to the low temperature, nonexponential kinetics. The potential in Fig. 2 has been adjusted (Agmon and Hopfield, 1983) to reproduce SW MbCO kinetics for $T = 40$–140K.

Above 150K, the protein starts to fluctuate during experimentally accessible times. Since the initial distribution is displaced away from the most stable deoxy conformation, one expects relaxation in y during which the deoxy state is stabilized and recombination becomes less probable. The motion of both ligand and protein is stochastic and may thus be described by diffusive dynamics on a potential energy surface (Agmon and Rabinovich, 1992). The diffusive motion of the ligand arises from collisions with fluctuating protein moieties (Gibson et al., 1992), just as the diffusion of a molecule in solution is propelled by the random forces exerted on it by collisions with solvent molecules (Chandrasekhar, 1943). At low temperatures, the protein is "frozen" hence the motion of the entrapped ligand may resemble more closely "ballistic" motion (Agmon and Hopfield, 1983). This can be accounted for by letting D_x be independent of T within the frozen protein. With this provision, the Kramers and Arrhenius reaction rate expressions are governed by the same exponential factor so that, for practical purposes, the two pictures coincide.

The "diffusion coefficients" in the two degrees of freedom, D_x and D_y, may be vastly different. At very low temperatures $D_y = 0$ while D_x is finite (and nearly temperature independent). With increasing T, D_y increases faster than D_x because the protein is partly exposed to solution and its large amplitude motions couple more intimately to external solvent viscosity. One thus expects that the "diffusion anisotropy," $\eta \equiv D_y/D_x$, will increase with increasing temperature. Unfortunately, one does not determine $\eta(T)$ experimentally. Given the potential surface fitted to the low temperature (say, 40–140K) kinetics, we propose to identify properties of the intermediate temperature kinetics (say, 150–210K) which are *independent* of η. Such properties would depend only on the potential and, of course, on T. Their investigation could allow a meaningful test of theoretical predictions which is free of adjustable parameters.

The relaxation footprint

The effect of diffusion anisotropy on multidimensional barrier crossing has been the subject of intensive theoretical research (Klosek-Dygas et al., 1989; Berezhkovskii and Zitserman, 1990, 1991; Agmon and Kosloff, 1987; Agmon and Rabinovich, 1991; Rabinovich and Agmon, 1991), mostly focused on the long-time exponential decay. Only recently has the intermediate-time, nonexponential behavior been analyzed (Agmon and Rabinovich, 1992; Rabinovich and Agmon, 1993). The full model requires the numerical propagation of the probability density on the given potential surface namely, solving a multidimensional Smoluchowski equation. In certain anisotropy regimes, it is possible to apply useful approximations. The approximate theory summarized below is valid for small values of η. It has been verified by comparison with 2d numerical propagations (Agmon and Rabinovich, 1992). It is expected to be valid in the transition region say, up to 220K, but not necessarily at room temperature, where η could approach (perhaps exceed) unity.

When $\eta < 1$ the ligand diffuses faster than the protein fluctuates. This separation of time-scales may be exploited, in the spirit of a Born-Oppenheimer approximation, to reduce the 2d Smoluchowski equation (SE) to an effective one-dimensional SE in the slow coordinate, y, with a sink term, $k(y)$, representing the dependence of the binding rate on the protein conformation (Agmon and Hopfield, 1983; Berezhkovskii and Zitserman, 1990; Rabinovich and Agmon, 1991)

$$\frac{\partial p(y,t)}{\partial t} = \left[D_y \frac{\partial}{\partial y}\exp\left(-\frac{U(y)}{k_BT}\right)\frac{\partial}{\partial y}\exp\left(\frac{U(y)}{k_BT}\right) - k(y)\right]p(y,t). \quad (1)$$

From the transient distribution of protein conformations, $p(y, t)$, one may calculate the survival probability of the deligated heme, $S(t) \equiv \int p(y, t)\, dy$. In the limit of a frozen protein, $D_y = 0$, Eq. 1 reduces to first order rate equations for each conformation, $dp(y, t)/dt = -k(y)p(y, t)$. This describes the evolution of a noninterconverting conformational distribution.

When $D_y > 0$ a first-order rate equation holds for the survival probability rather than for the probability density itself (Agmon and Rabinovich, 1992; Rabinovich and Agmon, 1993). Therefore

$$S(t) = \exp\left(-\int_0^t \langle k\rangle_{t'}\, dt'\right), \quad (2)$$

only that the rate coefficient is time-dependent,

$$\langle k\rangle_t \equiv \int k(y)\, p(y, t)\, dy/S(t). \quad (3)$$

Equation 2 follows by integrating Eq. 1 over y. No additional approximations are invoked thus far.

The time dependence of $\langle k \rangle_t$ is due to evolution of $p(y, t)$. It may be classified into two distinct physical mechanisms:

- "Kinetic hole burning" in a static, nonrelaxing distribution from which the fast-reacting conformations disappear first (Campbell et al., 1987; Agmon, 1988; Steinbach et al., 1991). This occurs in the $\eta \to 0$ limit in which Eq. 2 reduces to the familiar expression $S(t) = \langle \exp(-k(y)t) \rangle_0$.
- Relaxation of the protein characterized by a transient shift in the peak of its distribution function. As long as $\langle y \rangle_t$ is changing, Eq. 2 produces non-exponential kinetics even when the initial width of $p(y, t)$ is negligibly small.

The *same* coordinate, y, is responsible for both low temperature distributed kinetics and intermediate temperature relaxation. This differs from the approach (Steinbach et al., 1991) which postulates two independent coordinates, one which determines the shape of the distribution and other determines its shift. One expects that the variation in $\langle y \rangle_t$ due to high temperature relaxation is considerably larger than that due to kinetic hole burning. To a first approximation, one may neglect the width of the initial conformational distribution in discussing relaxation effects.

Equation 2 can be used to characterize intermediate-time power-law regimes: When $S(t) = A/t^{\alpha}$, the power α is given by $\alpha = -d \ln S/d \ln t$. Therefore we define a "power-law regime" as a region where the logarithmic derivative of the survival probability,

$$B(t) \equiv -d \ln S(t)/d \ln t = t\langle k(y) \rangle_t, \qquad (4)$$

is constant. Such regions are centered at extrema of this "beta-function," $B(t)$. These extrema may result from the "onset" or "termination" of the relaxation process. For a qualitative understanding of the effect, it suffices to consider a *single* initial conformation y_0 for example, that at the peak of the low temperature inhomogeneous conformational distribution. Prior to the "onset" of y relaxation $\langle k \rangle_t = k(y_0)$. The rebinding rate coefficient for a fixed conformation, y_0, is time-independent. Hence at short times $B(t)$ is linear in t and $S(t)$ decays exponentially. After the "termination" of relaxation $\langle k \rangle_t = \langle k \rangle_\infty$, so that $B(t)$ once again grows linearly with time, but with the smaller slope $\langle k \rangle_\infty$. This follows because from the bottom of the deoxy Mb well there is a higher climb to the ridgeline separating reactants from products (Fig. 2).

Two conceivable interpolations between the linear asymptotic behaviors of $B(t)$ are: (i) Monotonic increase or (ii) through a maximum and a minimum. The second case occurs when energetic stabilization by protein relaxation is sufficiently large, as we shall prove below. Thus either zero or *two* power-law phases are predicted, corresponding to the maximum and minimum in $B(t)$. This characteristic shape of the beta-function is the "footprint" that the relaxation process leaves on the time dependence of the binding process.

Imagine that, rather than protein relaxation "perpendicular to the reaction coordinate" (Agmon and Hopfield, 1983), two consecutive kinetic steps take place *along* the reaction coordinate. The first may be geminate rebinding, which is distributed at low temperatures, and the second ligand escape from the heme pocket (Austin et al., 1975). Depending on the values of the rate parameters, such a kinetic scheme may also give rise to either type of behavior. Since at low temperatures only the geminate step is observed, it is impossible in the consecutive mechanism to predict the shape of the beta-function based on low temperature data alone. In contrast, in a 2d geminate model the shape of the potential surface, which is obtained from the low T data, determines the behavior of $B(t)$ as demonstrated below.

Simple analytical expressions

We now apply simple approximations to obtain a criterion for the existence and location of the extrema in $B(t)$, characterizing the onset and termination of the relaxation process. The approximations make use of the relatively simple form of the potential surface in Fig. 2 (Agmon and Hopfield, 1983). For this potential, the protein mode is harmonic: In the bound state $U(0, y) = f(y - y_{eq})^2/2$, while in the unbound state $U(\infty, y) = fy^2/2$. Subsequently, it can be shown that the rebinding rate coefficient is approximately exponential

$$k(y) = k_0 \exp(ay), \qquad a = fy_{eq}/(3k_B T), \qquad (5)$$

where k_0 is a y-independent (but T-dependent) pre-exponential factor. If, rather than the Arrhenius expression (Agmon and Hopfield, 1983), the 1d Kramers formula is used for k_0, it becomes proportional to D_x and acquires some y dependence. This, however, may be neglected in relation to the y dependence in the exponent. The results below do not depend on the value of k_0.

Next, consider an approximation for the time course of the average protein conformation. Since the protein mode is harmonic, in the absence of reaction it relaxes exponentially (Chandrasekhar, 1943)

$$\langle y \rangle_\tau = y_0 \exp(-\tau), \qquad \tau \equiv k_{rel} t, \quad k_{rel} = D_y f/k_B T. \qquad (6)$$

The initial protein conformation, y_0, is typically the most probable conformation of the equilibrated bound state (namely, $y_0 = y_{eq}$). The neglect of the reaction along x is justified once η is sufficiently large. Since, to begin with Eq. 1 is valid for small η values the treatment presented here is most appropriate for intermediate values of η. This presumably corresponds to the intermediate temperature regime discussed in the present work.

The above relations suggest the use of the approximation

$$\langle k(y) \rangle_t \approx k(\langle y \rangle_t), \qquad (7)$$

in which the order of averaging has been interchanged (Agmon and Rabinovich, 1992). With its aid, we impose the necessary condition for an extremum

$$B'(t) = k_{rel} B'(\tau) \approx k(\langle y \rangle_\tau) + \tau k'(\langle y \rangle_\tau) \langle y \rangle'_\tau = 0, \qquad (8)$$

where a prime denotes differentiation with respect to the

designated variable. Substituting Eqs. 5 and 6 gives

$$\tau \exp(-\tau) = 1/(ay_0). \tag{9}$$

The solutions to this transcendental equation determine the extrema of $B(t)$. If $ay_0 < e$, Eq. 9 has no solutions and $B(t)$ is a monotonically varying function of t. If $ay_0 > e$, two solutions are obtained which we denote by τ_{max} and τ_{min}. Their subscripts correspond to the maximum and minimum of $B(t)$. It can be shown that $\tau_{max} < \tau_{min}$. For $y_0 = y_{eq}$, $ay_0 = ay_{eq} = 2\Delta U/(3k_BT)$, where $\Delta U \equiv fy_{eq}^2/2$ is the energy shift between relaxed and unrelaxed deoxy Mb. Thus the magnitude of the energy shift determines whether $B(t)$ has zero or two extrema. For MbCO, previously determined parameters (Agmon and Hopfield, 1983) lead to the inequality $fy_{eq}/(3k_BT) > e$ even at room temperature. Hence two extrema are expected over the whole temperature regime. The times t_{max} and t_{min}, by virtue of Eq. 6, scale inversely with $D_y(T)$. This protein "diffusion coefficient" is expected to increase strongly with T. Hence at low T the extrema occur at times which are too long to be observed. Conversely, at high T they become too fast and only in the intermediate temperature regime do they occur in the right time window for observation.

It is interesting to note that for large values of ay_0 there exists a useful Padé approximation for the onset of protein relaxation (Fritsch et al., 1973),

$$\tau_{max} = \frac{ay_0 - 4/3}{(ay_0)^2 - 7/3\, ay_0 + 5/6}. \tag{10}$$

For systems like MbCO, where $y_0 = y_{eq}$ and ay_{eq} is really large (ca. 15 at 185K), even the leading term, $\tau_{max} \approx 1/(ay_{eq})$, already gives a reasonable estimate.

The prediction from Eq. 9 is compared in Fig. 3 against exact numerical solutions of Eq. 1 for two hypothetical MbCO potentials, one with $ay_{eq} > e$ and the other with $ay_{eq} < e$. In the first case $B(t)$ indeed has two extrema (bold curve) while in the second case (thin curve) it has none. The dotted vertical lines show how well the solutions to Eq. 9 determine the locations of the calculated extrema.

Equation 9 enables us to identify quantities which depend on ΔU (through τ_{max} and τ_{min}) but not on k_{rel}. Since $\tau \equiv k_{rel}t$, the ratio of the two characteristic times is

$$t_{min}/t_{max} = \tau_{min}/\tau_{max}. \tag{11}$$

Denoting the characteristic powers by $\alpha_{max} \equiv B(t_{max})$ and $\alpha_{min} \equiv B(t_{min})$, their ratio

$$\alpha_{min}/\alpha_{max} = (\tau_{min}/\tau_{max}) \exp(1/\tau_{min} - 1/\tau_{max}), \tag{12}$$

is again independent of the protein relaxation rate for $\eta < 1$. The dimensionless times, τ_{min} and τ_{max}, are determined for a given protein once and for all by fitting the low temperature kinetics. At higher temperatures, we expect to see a "relaxation footprint" involving a maximum and a minimum in $B(t)$. Equations 11 and 12 imply that, whenever the ligand motion is faster than that of the protein, the ratios of both peak positions and heights are universal constants. Below, we compare t_{min}/t_{max} with quantitative measurements. For $\alpha_{min}/\alpha_{max}$ one cannot hope to achieve the same level of agreement, because of the sensitive dependence on the exponential factor.

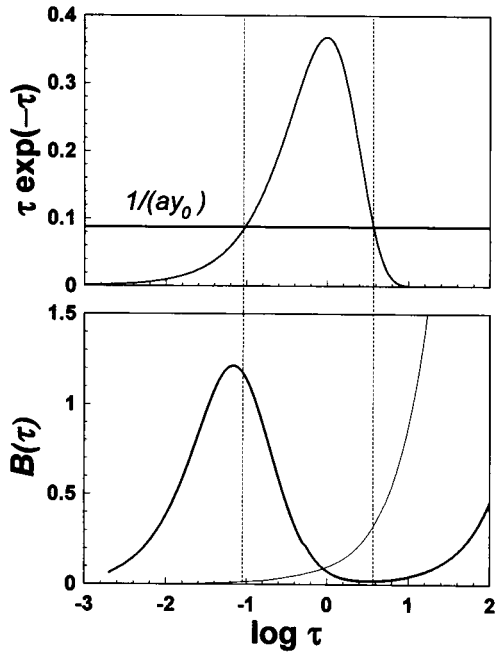

FIGURE 3 Exact beta-functions calculated from propagating the Smoluchowski equation 1, starting from a delta-function at $y_0 = y_{eq}$. The calculations use SW-MbCO parameters (Agmon and Hopfield (1983), Table I), except that the protein force-constant is either $f = 10$ or $f = 1$ kcal/(mol · au^2). These values correspond to $1/ay_0 = 0.9$ (*bold curve*) and 9 (*thin curve*), respectively. For $1/ay_0 = 0.9$, two extrema are predicted from the solution of Eq. 9 as demonstrated in the upper panel.

A parameterized data fit

The analysis presented above may look somewhat abstract to people used to a parameterized fit of experimental data. In order to obtain a quantitative fit using a 2d potential surface one requires the temperature dependence of the protein relaxation rate, $k_{rel}(T)$. In addition, the surface may have to be fine-tuned to improve the agreement at higher temperatures. Such a quantitative comparison has not yet been performed. However, simpler parameterized fits of the data in the intermediate temperature range have been obtained using either an inhomogeneous (Steinbach et al., 1991) or a homogeneous (Post et al., 1993) model for protein relaxation.

In the homogeneous model, as in our 2d picture, relaxation and conformational mixing occur simultaneously. The rebinding kinetics is assumed to be governed by a time-dependent rate coefficient, $k(t)$, in which the activation enthalpy, $H^\dagger(t)$, increases due to protein relaxation (Post et al., 1993),

$$k(t) = A \exp\left[-\frac{H^\dagger(t)}{k_BT}\right],$$
$$H^\dagger(t) = H_f^\dagger - \Delta H^\dagger \exp[-(k_{rel}t)^\beta]. \tag{13}$$

In this process, the activation enthalpy relaxes from an initial value $H_0^\dagger$ to a final value $H_f^\dagger$ in a stretched-exponential fashion. $\Delta H^\dagger \equiv H_f^\dagger - H_0^\dagger$ is the relaxational activation enthalpy shift. In the parameterization approach, only the Arrhenius pre-exponent, A, and the activation enthalpy ($H_0^\dagger$) distribution, are known from fitting the low temperature kinetics. Thus not only $k_{rel}(T)$ need be fitted to the experimental data at each temperature, but also $\beta(T)$ and $H_f^\dagger(T)$.

The survival probability is given by an expression similar to Eq. 2, with $k(t)$ replacing $\langle k \rangle_t$ and is subsequently averaged over the low temperature distribution of activation enthalpies. Commensurate with our earlier observation that the width of the initial inhomogeneous distribution has only a small effect on the relaxational enthalpy shift, we replace it by a delta-function at the peak of the distribution ($H_0^\dagger \approx 12$ kJ/mol). With this assumption, a similar derivation leads to

$$\tau^\beta \exp(-\tau^\beta) = k_B T/(\beta \Delta H^\dagger), \qquad (14)$$

which extends Eq. 9 to stretched exponential relaxation. As before, $\tau \equiv k_{rel} t$, although the numerical values of k_{rel} may differ. The maximum of the function on the l.h.s. is again $1/e$. Thus Eq. 14 has (two) solutions only if $\Delta H^\dagger > e k_B T/\beta$. The observation of a relaxation footprint implies that the protein exhibits a nonvanishing activation enthalpy shift.

HORSE MYOGLOBIN EXPERIMENTS

Materials and methods

Samples were prepared from salt-free, lyophilized powder of horse Mb (Sigma Chemical Co., St. Louis, MO). The material was dissolved (3 mM) in 75% glycerol-water (v) containing 0.1 M phosphate buffer at pH 7. The protein solution, equilibrated with 1 atm of CO, was then reduced by adding 10-fold excess of $Na_2S_2O_4$ under unaerobic conditions. The sample cell consisted of two glass plates separated by a 100 μm teflon spacer. The Mb concentration was adjusted to give 1–1.5 OD at 423 nm. The cell was mounted in a He-flow cryostat (Oxford) allowing temperature control to within 0.1 K.

Photolysis was achieved with 3- and 8-ns pulses of a frequency-doubled Nd:YAG laser (532 nm, 120 mJ). Transient absorption changes were monitored with a stabilized tungsten lamp. A monochromator selected the desired wavelength, typically 436 nm. Laser stray light was removed using blue filters. A photomultiplier tube (Hamamatsu R928) coupled to a two-way amplifier system (100 and 1 MHz) recorded the kinetic response. Between 10 ns and 5 μs a digital oscilloscope (Techscope 2100, Gould) was used for data storage (8-bit resolution). From 1.2 μs to 300 s, a home-built recorder, operating on a logarithmic time base, averaged and stored the digitized data (12-bit resolution). These systems allow the recording of absorption changes over 10 decades in time with a dynamic range of 3.5 decades. In the double pulse experiments, a second Marx-Bank with a variable quartz-stabilized delay time (typically 100 μs) opened the Pockels cell for the second flash. The repetition time used ($\Delta t = 0.1$ s) was that of the laser flash lamp. The baseline was averaged over 3 ms before the first shot. 1–256 shots were averaged in each measurement, depending on temperature.

Single pulse determination of the beta-function

The normalized absorption change for horse-MbCO is reported as the survival probability in Fig. 1 *a*. The data was fitted on the log-log scale to an eighth order polynomial which was differentiated analytically to yield $B(t)$, Fig. 1 *b*. For all temperatures in the transition region (T = 150–210K), $B(t)$ shows the characteristic shape involving a maximum and minimum which we would like to identify as the "relaxation footprint."

To understand why this feature should not be confused with the solvent process, it is helpful to recall the peculiarities of horse Mb kinetics as compared with the traditional sperm-whale (SW) sample. The horse sample clearly shows *two* sets of maxima/minima in $B(t)$. Below 200K only the first set is seen, Fig. 1 *b*. This set is either absent or else appears as a slight change in slope of the beta-function of SW-MbCO (Post et al., 1993, Fig. 10). The difficulty of observing this feature in SW-MbCO, which in the past has been *the* Mb sample to study, is partly responsible for problems in identifying the Agmon-Hopfield relaxation in Mb.

The onset of the solvent process is signalled by the *second* set of maxima/minima in $B(t)$. In Fig. 1 *b*, the second maximum is seen only at 210K (around 10 ms), but is evident at all temperatures above 210K (see "Protein relaxation at higher temperatures" below). We suggest three arguments to justify its assignment as the onset of the solvent process. First, dependence on solvated CO concentration begins only *after* the second minimum in $B(t)$. The first set is [CO] independent and hence geminal. Second, the location of the second maximum in $B(t)$ of horse-MbCO agrees nicely with the onset of the solvent process previously observed in SW-MbCO (Steinbach et al., 1991). Finally, we have recently found (Leyser et al., manuscript in preparation) that the first set of extrema in $B(t)$ is independent of solvent viscosity, while the extremal times of the second set do scale with external viscosity.

In contrast to the difficulty in observing the "relaxation footprint" in SW-MbCO, the low T kinetics in both samples are quite similar. Hence one may discuss the geminate phase using the SW-MbCO parameters (Agmon and Hopfield, 1983, Table I). At 185K one obtains $ay_{eq} = 15$, which is appreciably larger than e. We therefore *expect* to find two solutions to Eq. 9 namely, type (ii) behavior. This is indeed observed experimentally in the horse-MbCO data of Fig. 1 *b*. More quantitatively, Eq. 9 gives $\tau_{max} = 0.07$ and $\tau_{min} = 4.1$, thus $\log(t_{min}/t_{max}) \approx 1.8$. In the data of Fig. 1 *b*, a value around 2 is indeed typical for the temporal extent of the "relaxation footprint." Given that the measurements span more than 8 orders of magnitude this sort of agreement, obtained from the potential surface without readjusting any parameter, lends support to the protein relaxation picture.

Furthermore, we may use the parameterized data fit (Post et al., 1993) to compare with the predictions from the 2d

surface (Agmon and Hopfield, 1983). Let us take again 185K as a representing case for the intermediate temperature range. From (Post et al., 1993) $\Delta H^{\dagger}$(185K) = 18 kJ/mol and β(185K) = 0.62. Using these parameters in Eq. 14 we find two solutions, τ_{max} = 0.05 and τ_{min} = 6.4. These values are in remarkable agreement with the values 0.07 and 4.1 obtained above based on the potential energy surface alone. Also, contrary to previous assertions (Šrajer et al., 1988), the enthalpy shift of 18 kJ/mol is in reasonable agreement with the value obtained from the 2d surface, $\Delta U^{\dagger} = k_B T\, a y_{eq} = f y_{eq}^2/3$ = 23.5 kJ/mol. If recombination from the relaxed protein at 200K proceeds via the saddle point rather than along straight lines of constant y (as at short times and low temperatures), this value decreases to 22 kJ/mol (Agmon and Hopfield, 1983, Table II). Why this value decreases further at higher temperatures is discussed below.

The multipulse inhomogeneity test

Inhomogeneous kinetics means that each conformation reacts as if it was a distinct chemical species. It may be either bound or unbound but may not interconvert into another conformation. If a laser pulse photolyzes the whole sample, it necessarily regenerates the same conformational distribution in the unbound state. Thus $S(t)$ should be independent of the number of pulses, n. At low T this is the case for all times. At higher temperatures, we expect inhomogeneous behavior only at short times.

Fig. 4 presents multipulse data at two temperatures in the intermediate T regime. Panels a and c show the survival probabilities while panels b and d show their logarithmic derivatives. From the upper panels we infer that the kinetics is inhomogeneous up to the time when the curves start to diverge, t_{inhom}. If the end of the inhomogeneous phase is due to protein relaxation and protein relaxation begins at t_{max}, one expects that $t_{inhom} \approx t_{max}$. This is verified by the results shown in Fig. 4. Thus a likely explanation for "pumping" (Powers et al., 1987; Ansari et al., 1987) is partial relaxation along the protein coordinate.

Both characteristic times depend only weakly on the number of applied pulses: For large n values, t_{max} is somewhat shorter but so is t_{inhom} (*dashed line*, panels c and d). In addition, both times depend only weakly on the interpulse separation, Δt, as long as $\Delta t > t_{max}$. When $\Delta t < t_{max}$, the protein does not start relaxing and the whole time course after a second pulse is identical to that after the first pulse (Post et al., 1993).

The agreement between two independent experimental observables namely, the maximal slope of a single pulse decay curve and the divergence point in multipulse measurements, helps establish t_{max} as signaling the onset of protein relaxation.

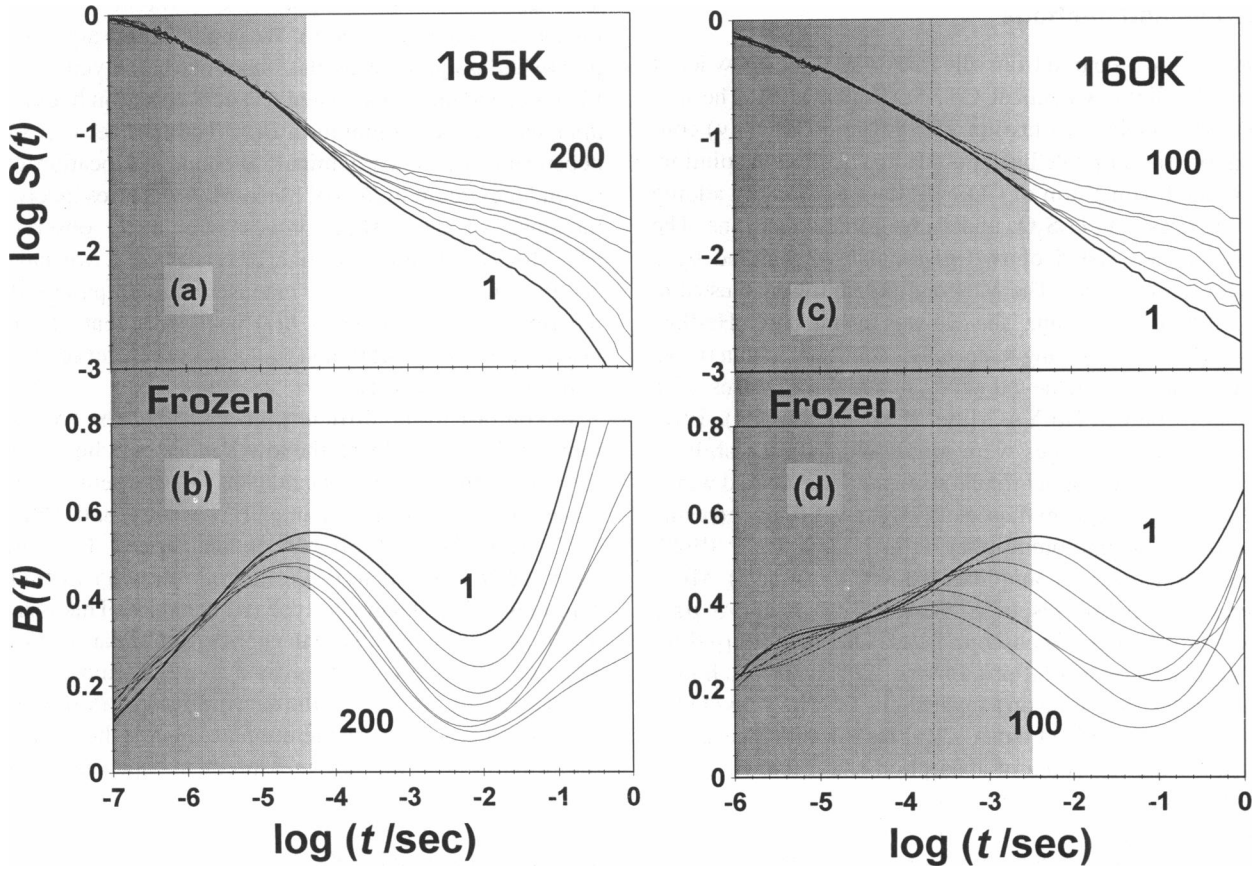

FIGURE 4 Multipulse measurements of CO binding to horse myoglobin at two temperatures. (a and b) 185K: n = 1, 2, 5, 10, 20, 50, 100 and 200 pulses; (c and d) 160K: n = 1, 2, 5, 10, 20, 50 and 100 pulses. The grey rectangle covers the time regime of an inhomogeneous protein.

The multipulse homogeneity test

In the opposite limit of homogeneous kinetics all the protein molecules are identical on the average, so that the rebound fraction behaves like the whole ensemble. Denoting the survival probability after the nth pulse by $S^{(n)}(t)$, we expect that (Post et al., 1993)

$$S^{(2)}(t) = S^{(1)}(t + \Delta t) + [1 - S^{(1)}(\Delta t)]S^{(1)}(t). \quad (15)$$

This differs from the inhomogeneous case where $S^{(2)}(t) = S^{(1)}(t)$. Whenever Eq. 15 holds, we will conclude that the protein behaves homogeneously for $t > \Delta t$. For $\Delta t = 0.1$ s it was shown (Post et al., 1993) that the multipulse data obeys Eq. 15 at 185K but not at 145K, suggesting that transition to homogeneity occurs in the range $T = 145$–185K. We have extended these measurements to additional temperatures in order to locate the transition temperature more precisely.

Fig. 5 shows that at 160K the homogeneity test still holds while it breaks down at 150K. If the minimum in $B(t)$ signals the "termination" of the relaxation process, we expect to see homogeneous behavior whenever $\Delta t > t_{min}$. At high temperatures the protein is homogeneous. With lowering temperature t_{min} increases, until at 150K we have $t_{min} \approx 0.3$ s $> \Delta t$, so that the protein becomes nonhomogeneous. At t_{min} the homogeneous protein still shows nonexponential kinetics (Post et al., 1993). This follows because t_{min} is the center of the second power-law phase. At longer times we do expect $S(t)$ to become exponential. Unfortunately, at such long times the signal becomes extremely small, so judgment on this issue is deferred pending more accurate measurements.

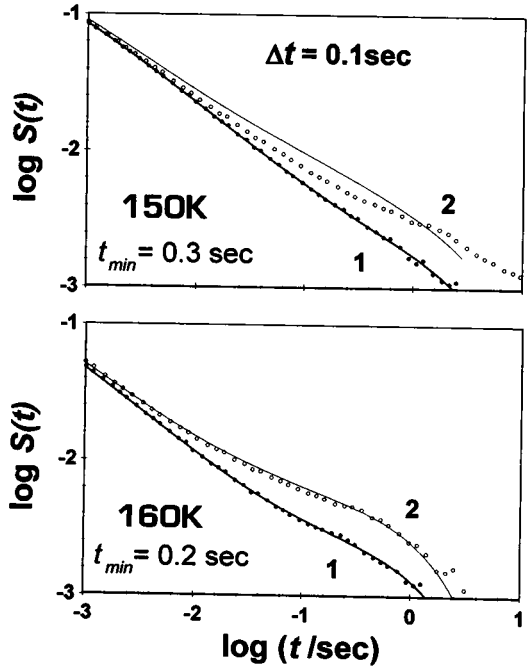

FIGURE 5 Two-pulse measurements of CO binding to horse myoglobin at two temperatures. Full and open circles are measured data for $n = 1$ and 2. Bold lines are polynomial fits to the $n = 1$ data set, $S^{(1)}(t)$. Thin lines are the prediction for the double-pulse experiment, $S^2(t)$, assuming homogeneous kinetics and using Eq. 15.

Comparison with CW measurements

Ansari et al. (Ansari et al., 1987, Fig. 16) have measured CO rebinding following CW illumination of SW-MbCO. In the low T regime they found only a small degree of slowing down "pumping" (see also Powers et al., 1987). At 140K this pumping effect was already substantial. After 100 s of continuous illumination, the decay curve diverged from the unilluminated data at about 10 ms. Since SW and horse Mb behave similarly at low T values, we consult Fig. 1 and find indeed t_{max} (150K) = 10 ms. Our conclusions concerning the onset of relaxation seem to be independent on whether repeated photolysis is achieved by a pulse-train or via continuous illumination.

Comparison with B-state kinetics

Hong et al. have measured the transient stretching band of bound (A-band) and flashed-off CO (B-band) during recombination (Hong et al., 1991). Below 170K the B-state loss tracks the A-state gain as expected for a simple two state system. However above 170K the B-band decays faster than the growth of the A-state population. The A-state kinetics in this range coincides roughly with our data obtained in the Soret region (Fig. 1). We interpret the enhanced B state decay as reflecting the relaxational process. To a first approximation, both effects occur on a similar time scale: For $T = 200$–220K, we determine the "onset" of relaxation as $t_{max} = 2$–6 μs, while the enhanced B-state decay begins at about $t_B = 0.5$ μs (Hong et al., 1991, Fig. 1 *d*).

From the point of relaxational models (Agmon and Hopfield, 1983; Steinbach et al., 1991) the excess kinetics of the B-state is surprising: The relaxation of the heme group alone should not drastically alter the environment of the dissociated ligand in the distal pocket. Consequently Hong et al. suggest diffusion of the ligand out of the pocket into the hydration shell but not all the way into the bulk solvent. This explanation is compatible with our model only if the migration process is not rate limiting to rebinding namely, it occurs after protein relaxation. However, we have found no solvent viscosity dependence of structural relaxation (e.g., of t_{max}) and internal ligand binding, whereas the ligand escape rates do scale nicely with the external viscosity (Leyser et al., manuscript in preparation). These findings suggest that structural relaxation and internal rebinding are not coupled to solvent controlled motions on the protein's surface.

Alternatively one could consider enhanced pocket-structure fluctuations once the deoxy state is reached. In this case, the similarity in the two timescales points, above all, to distal-proximal coupling. Indeed, comparison of the x-ray structures of liganded and unliganded hemes reveals structural changes on *both* sides of the heme (Perutz et al., 1987). Both the proximal histidine (F8) and the distal histidine (E7) and valine (E11) show large movements upon ligand binding. This suggests a possible rationale for the observation that

$t_B < t_{max}$. In the language of Frauenfelder (Ansari et al., 1985), this may reflect a hierarchal "protein quake" in which side chains relax before the backbone. Thus His-E7 and Val-E11 relax first (affecting the B-state kinetics) and subsequently the F-helix moves and with it the proximal His-F8 and the iron (as reflected by the rebinding kinetics). While we concede that this last interpretation is speculative, it serves to demonstrate the potential in comparing the two kinds of kinetic measures.

Protein relaxation at higher temperatures

In Fig. 6 we show the beta-function for $T > 210$ K. There are now *two* sets of maxima and minima, the earlier set being our "relaxation footprint." Fig. 7 shows the power $\alpha_{max} \equiv B(t_{max})$ as a function of temperature. Above about 190K it decreases drastically until at 260K it disappears in the noise. Numerical propagations on the 2d MbCO potential (Rabinovich and Agmon, 1993, Fig. 2) showed that α_{max} decreases rapidly with η once $\eta > 1$. We tentatively conclude that at high temperatures protein relaxation becomes comparable or faster than ligand motion. In this limit, the approximation leading to the 1d Smoluchowski equation, Eq. 1, breaks down and one needs to analyze the full 2d model.

It is interesting to consider the kinetics expected for $\eta \geq 1$. When $\eta = 1$ (isotropic diffusion), the system spends equal times exploring the x and y directions, so it can locate the best gateway from reactants to products, which is the saddle point (SP). On the 2d potential, Fig. 2, the SP energy is already somewhat lower than the barrier along x from the equilibrated deoxy conformation ($y = 0$).

A more spectacular effect occurs as η is increased above unity: The effective barrier height becomes *lower* than the SP. In the limit $\eta \to \infty$, one may integrate over the fast motion, y, and obtain the effective one dimensional potential (Klosek-Dygas et al., 1989)

$$\exp\left[-\frac{U_{eff}(x)}{k_B T}\right] = \int \exp\left[-\frac{U(x,y)}{k_B T}\right] dy. \quad (16)$$

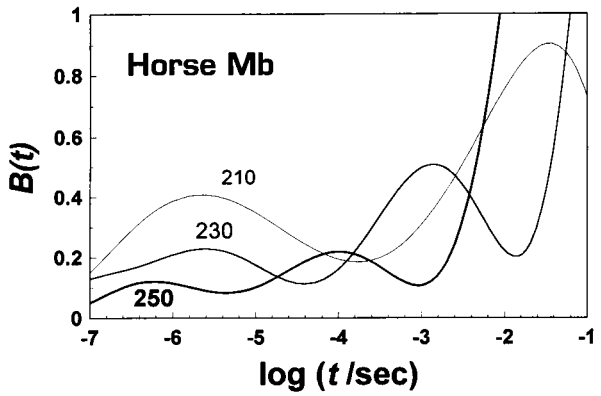

FIGURE 6 The beta-function at elevated temperatures. Line thickness corresponds to increasing temperature.

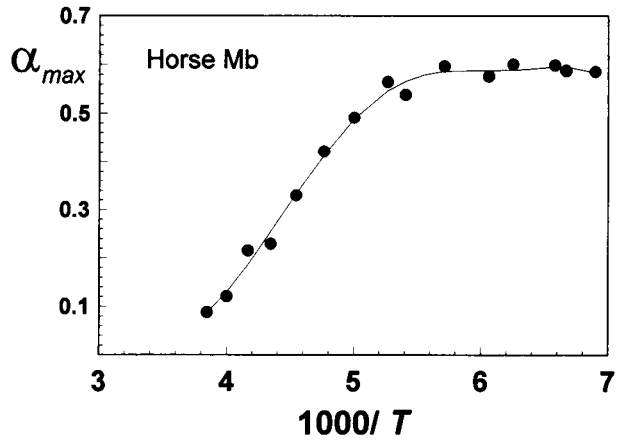

FIGURE 7 The temperature dependence of the first peak height in the beta-function obtained from horse-Mb kinetics. Line drawn to guide the eye.

Since $U(x, y)$ is a double well potential for any cut along x, so is $U_{eff}(x)$. The SW-MbCO parameters give $U^{\dagger}_{eff} = 20$ kJ/mol as compared with a SP energy of 32 kJ/mol. This corresponds to a relaxational barrier height shift of only 10 kJ/mol (Agmon and Rabinovich, 1992), in agreement with an observed room temperature value, $\Delta H^{\dagger} = 12$ kJ/mol (Steinbach et al., 1991). For horse-Mb, from a parameterized data fit to Eq. 13 one finds that $\Delta H^{\dagger}$ decreases from 18 kJ/mol (its low T limit) to 14 kJ/mol at 260K (Post et al., 1993, Fig. 3).

The above result may first seem counterintuitive, since the SP corresponds to the barrier along the minimum energy path connecting reactants and products. How can the effective barrier be lower than that? Mathematically, the integration in Eq. 16 extends on *both* sides of the ridgeline. Thus configurations on the low energy product side contribute to lower the value of the effective barrier. Physically, ligand binding for $\eta > 1$ becomes *protein-assisted*: One may envision the iron as fluctuating very fast in and out of the porphyrin plane with the CO waiting for the right opportunity to snap on. This leads to the interesting possibility that the geminate process is ligand-controlled at low temperatures and protein-controlled at high temperatures.

The disappearance of the relaxation footprint above 260K makes it difficult to determine the onset of protein relaxation at room temperature. One may try extrapolating via an Arrhenius plot for t_{max}. The plot can be interpreted using two activation energies (about 40 kJ/mol below 200K and 20 kJ/mol above 200K) or as a nonlinear Arrhenius dependence. Extrapolation to room temperature gives $t_{max}(300K) = 50$–150 ns.

Finally, consider the second set of maximum and minimum that appears at longer times, Fig. 6. Let us denote these by t'_{max} and t'_{min}, respectively. t'_{max} agrees with the onset of the solvent process as previously observed in SW-MbCO samples. For example, at 240K the solvent process begins around 0.1–1 ms (Steinbach et al. (1991), Fig. 12). This is also the value of t'_{max} in the temperature range 230–250K, see Fig. 6. In addition, we find that the kinetics depend on external CO-concentration for $t > t'_{min}$. Therefore we attribute

the second set of extrema to the solvent process. One may again extrapolate to room temperature using an Arrhenius plot. We find that $t'_{max}(300K) \approx 800$ ns, in agreement with a ligand escape time of about 1 μs determined for SW-MbCO (Steinbach et al. (1991), Fig. 10).

CONCLUSION

Measured survival probabilities for ligand binding to myoglobin span a huge range of temperatures and times. At low temperatures $S(t)$ decays smoothly, in an almost power-law fashion. At higher temperatures it shows "kinks" and "bends." We have analyzed these features with the help of a two-dimensional model whose parameters were adjusted to the low temperature kinetics.

Evidently, our model has several limitations. The potential constructed in Fig. 2 reflects the overall recombination, without discriminating between the major bound-state distributions A_0, A_1, and A_3. These isomers differ in the angle which the CO bond assumes with respect to the heme normal in the bound state and gives rise to three CO-stretching bands. We cannot exclude that conformational relaxation depends on the initial state. In particular, substate A_3 with the largest off-axis angle and peak activation enthalpy (Ansari et al., 1987) is likely the most constrained structure and hence a good candidate for strong relaxational effects. We plan to construct potential surfaces accounting for individual A states to clarify this question.

The model addresses only the geminate phase and does not include the solvent process explicitly. Above 200K we find a second maximum in $B(t)$ which we tentatively attribute to the onset of the solvent process (t'_{max}). Presently, it makes sense to apply the model only for $t < t'_{max}$. Below 200K this spans the whole accessible time regime. Yet another problem is that the protein relaxation rate is not directly measurable. To address this problem, we have used the model to predict characteristic features of the relaxation process which are independent of the value of the relaxation rate coefficient:

- $B(t)$ for MbCO shows a maximum and a minimum displaced by nearly two logarithmic units;
- The protein is inhomogeneous up to t_{max};
- The protein is homogeneous as of t_{min}.

These features were difficult to detect in traditional SW-MbCO samples, but are clearly demonstrated by our horse Mb measurements.

While a kinetic mechanism can never be proven, we feel that this demonstrated agreement strongly supports the idea that protein relaxation affects the ligand binding kinetics. Following ligand dissociation at ambient temperatures, tertiary relaxation slows down ligand rebinding. The protein catalyzes ligand release by "closing the door" behind it. At later times it "opens the door" for escape into solution.

The role of protein conformational change in enzymatic activity has been discussed by simple models such as Koshland's "induced fit" model (Koshland and Neet, 1968). The wealth of data obtainable from heme protein measurements provides a rigorous testing ground for such ideas.

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