Isotope Effect in Molecular Tunneling

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The intramolecular binding of carbon monoxide ($^{13}C^{16}O/^{12}C^{16}O$ and $^{12}C^{18}O/^{12}C^{16}O$ mixtures) to myoglobin at 20 and 60 K is observed with time-resolved Fourier-transform infrared spectroscopy. The binding rates (at ≈ 1 ks) for the molecules $^{12}C^{16}O$, $^{12}C^{18}O$, and $^{13}C^{16}O$ are in the ratio 1:0.84:0.65 at 20 K, 1:0.87:0.84 at 60 K. The large isotope effect confirms earlier evidence for molecular tunneling. The fact that $^{12}C^{18}O$ binds faster than $^{13}C^{16}O$ implies that structure effects are significant in molecular tunneling.

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Quantum-mechanical tunneling, introduced by Hund in 1927 to explain intramolecular rearrangements,[1] has been observed in systems from nuclei to biomolecules.[2,3] The rate coefficient k for tunneling through a barrier of height H can be written as

$$k = A \exp\{-\gamma[2M(H-E)]^{1/2} l/\hbar\}, \quad (1)$$

where γ is a constant of order unity, M the tunneling mass, E the excitation energy of the initial state, and l the corresponding barrier width as shown in Fig. 1(a).[4] The preexponential A becomes temperature independent below a certain temperature. Tunneling is characterized by this temperature independence and an exponential mass dependence. In contrast, the classical over-the-barrier process has an Arrhenius temperature dependence and a small mass dependence. In previous papers we have found that the binding of carbon monoxide (CO) to heme proteins is essentially temperature independent below about 10 K and consequently most easily explained by intramolecular tunneling.[5–7] We confirm tunneling by showing that the binding rate also exhibits a pronounced mass effect.

We study the binding of CO to myoglobin (Mb) by flash photolysis. Mb is a globular protein with a diameter of about 4 nm and a molecular weight of 17.2 kDa. Embedded in the protein in a pocket is heme, an organic molecule with a central iron atom. CO binds covalently to the iron [state A in Fig. 1(b)]; the heme is planar and the iron lies in the heme plane. The bond between Fe and CO can be broken by light; after photodissociation the heme group buckles and the iron moves out of the mean heme plane [state B in Fig. 1(c)]. CO ultimately rebinds and the system returns to state A. Three results from our previous studies[8,9] are essential for the present work: (i) Below about 160 K, CO does not leave the pocket after photodissociation but rebinds directly from state B. (ii) At low temperatures the transitions $B \rightarrow A$ is not exponential in time, but must be described by a distribution of rate coefficients. We interpret this behavior by postulat-

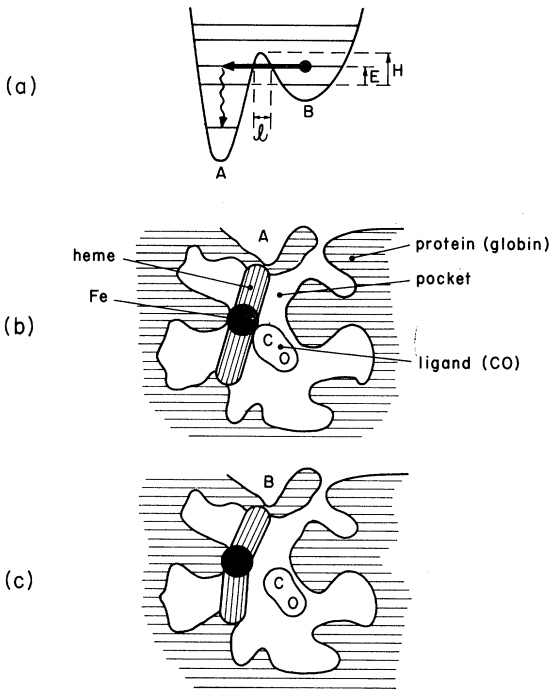

FIG. 1. The states involved in the tunneling of CO within myoglobin. (a) Tunneling occurs from the "free" state B to the bound state A. (b) In state A, the CO is bound to the heme iron, the heme group is planar, and the iron lies in the heme plane. (c) After photodissociation, the Fe–CO bond is broken, the CO molecule has moved away from the iron into the pocket, the iron has moved out of the heme plane, and the heme is domed.

ing that each Mb molecule is frozen into a different conformational substate with different barrier height H. We denote with $g(H)dH$ the probability of finding barriers with heights between H and $H+dH$. The fraction $N(t)$ of Mb molecules without bound CO at time t after photodissociation is given by

$$N(t)=\int dH\, g(H)\exp[-k(H)t]. \quad (2)$$

(iii) Between 80 and 160 K, all data can be fitted with a unique $g(H)$ and with $k(H)$ satisfying the Arrhenius relation $k(H)=A'\exp(-H/k_BT)$. Below 80 K, binding faster than predicted by the Arrhenius relation suggest tunneling. Comparison of the experimental data with extrapolation of Eq. (2) indicates that 1 ks the ratio of tunneling to Arrhenius transitions is greater than 10^8 at 20 K and may be as large as 0.5 at 60 K.

A convincing experiment to study the isotope effect must be designed so that two isotopes can be observed simultaneously in the same sample. Infrared spectroscopy makes such an approach feasible[10]: The stretching frequency of the free CO molecule (2143 cm^{-1} for $^{12}C^{16}O$) differs from the Mb-bound one; in the Mb-bound state, $^{12}C^{16}O$ (1945 cm^{-1}) can be distinguished from either $^{13}C^{16}O$ (1901 cm^{-1} or $^{12}C^{18}O$ (1902 cm^{-1}). CO rebinding after photodissociation is monitored simultaneously for two isotopes by measuring the growth of the absorption spectra at the Mb-bound CO stretching frequencies.

The Mb-CO samples contain about 16 mM protein in 99% glycerol. The ligand is a mixture of $^{12}C^{16}O$ with either $^{13}C^{16}O$ or $^{12}C^{18}O$. Interferograms are taken with a Digilab FTS-14 Fourier-transform spectrometer at 60 K where the Arrhenius process dominates and 20 K where tunneling prevails. Each sample is cooled to the selected temperatures in the dark and the transmitted intensity $I_A(\nu)$ determined as function of the frequency ν. The subscript A denotes the bound state A in Figs. 1(a) and 1(b). The sample is illuminated for about 90 min. Within a few minutes equilibrium between photodissociation and rebinding is reached; $I_{eq}(\nu)$ is then measured. The photolyzing light is switched off and kinetic data $[I(t;\nu)]$ are collected and averaged logarithmically with the number of scans doubled for each successive interferogram. Between experiments the sample is warmed to 200 K to allow complete relaxation to state A. Absorption spectra are computed as $\Delta\mathfrak{A}_A=\log[I_{eq}(\nu)/I_A(\nu)]$ and $\Delta\mathfrak{A}(t;\nu)=\log[I_{eq}(\nu)/I(t;\nu)]$. The fraction $N(t)$ of Mb molecules without bound CO at time t after illumination is given by

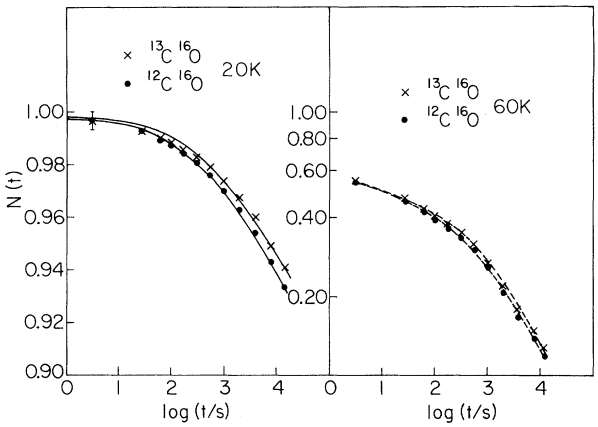

FIG. 2. Rebinding of $^{12}C^{16}O$ and $^{13}C^{16}O$ to Mb after photodissociation at 20 and 60 K. $N(t)$ denotes the fraction of Mb molecules that have not rebound CO at time t after the end of the steady-state illumination. The solid curves at 20 K are fits to the data as described in the text. The dashed curves at 60 K are drawn to guide the eye. For 20 K, errors at the earliest times are shown; by 10^3 s, the errors are smaller than the size of the data points. For 60 K the errors are smaller than the size of the data points.

$$N(t)=N_{eq}[1-F(t)/F_A]. \quad (3)$$

Here $F(t)=\int d\nu\,\Delta\mathfrak{A}(t;\nu)$ and $F_A=\int d\nu\,\Delta\mathfrak{A}_A$ are the areas under the absorption curves and N_{eq} is the equilibrium fraction of Mb without bound CO during illumination. The experiment determines $N(t)/N_{eq}$. To obtain N_{eq}, we compare $N(t)/N_{eq}$ with $N(t)$ determined in our previous flash-photolysis experiments at long times. With N_{eq} known, $N(t)$ is found; curves of $N(t)$ for $^{12}C^{16}O$ and $^{13}C^{16}O$ at 20 and 60 K are given in Fig. 2. At both temperatures, the heavy isotope rebinds more slowly.

In order to understand the data, we write the explicit mass dependence of the tunneling rates with Eq. (1) as

$$\ln(k_l/k_h)=\ln(A/k_l)[M_h^{1/2}-M_l^{1/2}]M_l^{-1/2}$$
$$\approx\ln(A/k_l)(\Delta M/2M), \quad (4)$$

where the subscripts l and h refer to light and heavy isotopes, $\Delta M=M_h-M_l$, and $M=(M_h+M_l)/2$. If binding were exponential in time, k would be single-valued at a given temperature and k_l/k_h would be time independent. However, binding is not exponential in time and the binding rates k vary over an enormous range.[8,9] Binding at time t is dominated by molecules having rate coefficients $k=1/t$. Equation (4) therefore implies that

TABLE I. Values of k_l/k_h at ≈ 1 ks and values of $\Delta M/M$, the relative mass change, in the binding of carbon monoxide to myoglobin. The model calculations refer to point particles moving in fixed potentials.

Quantity		$^{12}C^{16}O$ vs $^{13}C^{16}O$	$^{12}C^{16}O$ vs $^{12}C^{18}O$
Experimental: k_l/k_h	60 K	1.20 ± 0.05	1.15 ± 0.05
	20 K	1.53 ± 0.05	1.20 ± 0.05
$\Delta M/M$	(20 K)	0.040 ± 0.015	0.019 ± 0.007
Model: $\Delta M/M$			
M is the mass of	CO	0.035	0.069
	C	0.080	0
	O	0	0.118
M is the reduced mass of	CO-Fe	0.023	0.045
	C-Fe	0.066	0
	O-Fe	0	0.090

k_l/k_h increases with t: $k_l/k_h \approx (At)^{\Delta M/2M}$. Since, to a good approximation, the distribution function $g(H)$ in Eq. (2) is expected to be the same for the various isotopes the ratio k_l/k_h is found from Fig. 2 as ratio of the times t_h and t_l at which the two isotopes have rebound the same amount: $k_l/k_h = t_h/t_l$. Values of k_l/k_h at 1 ks are listed in Table I.

In principle $\Delta M/M$ and A can be extracted from values of k_l/k_h measured over a wide range of time. In practice, the data are not yet accurate enough to determine both unknowns. We obtain A independently according to the method used earlier[6,7] and find $A = 10^{6\pm2}\ s^{-1}$ at 20 K, where the large error is caused by uncertainties in the model. Equation (4) and the data for k_l/k_h in Table I together then yield $\Delta M/M$. Better values of $\Delta M/M$ are obtained by fitting the data with

$$\frac{N(t)}{N_{eq}} = \frac{\int dH\, g(H)\{k_{eq}/(k_{eq}+k)\}\exp(-kt)}{\int dH\, g(H)\{k_{eq}/(k_{eq}+k)\}}, \qquad (5)$$

where k_{eq} is the rate coefficient that characterizes the approach to equilibrium during illumination. The tunneling rate, Eq. (1), is parametrized in the form[6,7] $k = A\exp(-aH^{3/2})$. The mass ratio follows from the fitting parameters as $M_h/M_l = (a_h/a_l)^2$. The resulting values of $\Delta M/M$ are given in Table I. The large error in $\Delta M/M$ comes from the uncertainty in the preexponential A. Since A is nearly the same for all isotopes, the relative error for the ratio $\Delta M(^{12}C \rightarrow {}^{13}C)/\Delta M(^{16}O \rightarrow {}^{18}O)$ is much smaller than for $\Delta M/M$. The data in Table I lead to three main conclusions:

(1) The rate coefficient k for binding of CO to Mb shows an unambiguous mass dependence at 60 K and below.

(2) The most natural explanation for the isotope effect is quantum-mechanical tunneling. The isotope effect confirms the conclusion that we have previously drawn from the temperature independence of the rate below about 10 K.[5-7] The effect at 60 K is about one-half as big as at 20 K, in agreement with the estimate given above that at 60 K the ratio of tunneling to Arrhenius transitions can be as large as 0.5.

(3) The structure of CO affects tunneling. If CO moved as a point particle, the value of $\Delta M/M$ would be about twice as large for the replacement $^{16}O \rightarrow {}^{18}O$ than for $^{12}C \rightarrow {}^{13}C$. The data in Table I show the opposite behavior: $^{12}C^{18}O$ binds faster than $^{13}C^{16}O$ and $\Delta M(^{12}C \rightarrow {}^{13}C)/\Delta M(^{16}O \rightarrow {}^{18}O) = 2.06 \pm 0.03$. Predictions for some simple models are also listed in Table I. It is sobering to note that the value of $\Delta M/M$ obtained in the experiment on $^{12}C^{16}O$ vs $^{13}C^{16}O$ is close to that predicted for a point CO molecule tunneling through a fixed potential. Had we only studied one isotope pair, we would have noted excellent agreement with the simplest model! Both isotope pairs together, however, exclude any explanation not involving the structure of the CO molecule. A quantitative explanation of the tunneling rates may require features such as rotational motion around an axis perpendicular to the C-O vector, excitation of the CO molecule, and participation of other protein constituents such as the iron atom or part of the heme group. Molecular tunneling in heme proteins is even more complex than in paraelastic relaxation.[11]

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Position-Dependent Viscosity Effects on Rate Coefficients

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Kramers's modeling of a chemical reaction by barrier-mediated diffusion is generalized to include a possible spatial variation of viscosity along the reaction coordinate. Expressions are derived for the rate coefficient k. k is influenced by the viscosity at the location of the barrier top, which is partially affected by the solvent viscosity η. A predicted law $\delta(\ln k) = \epsilon\,\delta(\ln\eta)$ for $0 \leq \epsilon \leq 1$ is compatible with recent kinetic measurements in proteins.

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The viscosity dependence of rate coefficients is of great interest as it serves as a probe for the dynamic nature of the processes involved. Kramers[1,2] modeled a chemical reaction by a Brownian motion of a particle along the reaction coordinate in the presence of a potential-energy barrier. He derived an expression for the rate coefficient k_{AB} of the reaction $A \rightarrow B$ under initial rate conditions, i.e., in the presence of an excess of reactants and a small concentration of products, and similarly for k_{BA}. He found $k_{AB} \propto 1/\eta$ at a constant temperature where η is the medium viscosity. This relation holds in the so-called high-damping limit.[1-3] The proportionality factor contains the temperature and some geometric features of the potential-energy profile. The validity of Kramers's law has been investigated with computer simulations[4] and has been confirmed experimentally for polymer solutions,[5] for enzyme-catalyzed reactions,[6] and in some cases of ligand binding in proteins.[7] Its application to biochemical reactions and barrier-mediated transport processes through membranes seems to correlate parameters that govern structural fluctuations, with biochemical functions.[8]

Deviations from Kramers's $1/\eta$ law have been recently observed[7] for small-ligand binding kinetics in proteins; i.e., the quantity $\epsilon = -\delta(\ln k)/\delta(\ln\eta)$ was found to vary in the range $0 < \epsilon \leq 1$, depending on the reaction, the barrier number, and the viscosity (at high enough values). Since the location of some of the above reaction sites seems to be in the protein interior, where a direct contact of the ligand with the solution is highly improbable, the observed solvent viscosity effects imply that *variations of the solvent viscosity are partially transferred to the reaction sites. The resulting changes in the local viscosity influence the kinetic coefficients.*

The concept of local viscosity is in accordance