Reports

Dynamics of Carbon Monoxide Binding by Heme Proteins

Abstract. *Rebinding of carbon monoxide to myoglobin and to cytochrome P-450 after removal by a light flash occurs down to 50°K for myoglobin and 25°K for cytochrome P-450 in glycerol-water solution. Above 240°K the reaction is second order; between 240° and 200°K the rebinding becomes exponential and independent of the carbon monoxide concentration. Below 150°K the reaction follows a power law and is approximately 10^3 times faster for cytochrome P-450 than for myoglobin.*

Many heme proteins in the ferrous state, particularly those that bind oxygen, form stable complexes with carbon monoxide. The CO binds as an axial ligand to the iron and the linkage can be broken in high quantum yield by irradiation with visible light (*1*, p. 27). The kinetics of CO binding to heme proteins has received much attention (*2*). Except for the studies of Chance and co-workers (*3*), nearly all the measurements have been made at physiological temperatures. For two heme proteins, myoglobin (Mb) and cytochrome P-450, we have extended the range of binding measurements from room temperature, 300°K, to the temperature of liquid helium, 4.2°K, by using the technique of flash photolysis. Both proteins show typical second-order kinetics at room temperature. As the temperature is lowered, however, three additional ranges of drastically different kinetic behavior occur. After describing the experiment, we discuss the four regions of kinetic behavior, which we denote as 1 to 4.

Dissociation of the CO from the heme proteins was accomplished by using a light pulse from a xenon flash tube; the $1/e$ duration of the pulse was 10 μsec and the input energy was 100 joules. The photodissociation and subsequent rebinding were observed optically since the Soret absorption maxima of the CO-bound and free ferrous heme proteins are easily distinguishable (*4*): Mb · CO, 423 nm; Mb, 434 nm; P-450 · CO, 447 nm; P-450, 411 nm. Upon photodissociation, the absorption changes rapidly to the value for the CO-free protein, and as the CO rebinds it returns to the original value with a time dependence that provides information about the binding kinetics. The absorption was monitored with a light beam with a bandwidth of less than 10 nm centered at the Soret peak of the CO-free species. The transmitted light was detected by a photomultiplier and recorded on a storage oscilloscope. Low levels of monitoring light were used to avoid disturbing the reaction kinetics. Afterglow from the xenon lamp obscured the signal for approximately 0.5 msec after the flash. The samples (1-cm optical path length) were placed in an Andonian optical cryostat capable of maintaining any temperature between 4.2°K and room temperature.

Myoglobin (sperm whale skeletal type 2) was used as purchased from the Sigma Chemical Company. The cytochrome P-450 was purified from camphor-induced *Pseudomonas putida* by published methods (*5*). The substrate D-camphor was removed by passage through a Sephadex G-25 column equilibrated at 5°C with 50 m*M* phosphate buffer, *p*H 7. The heme proteins at 200 m*M* in the same buffer were diluted by adding 0.4 ml to 7 ml of a glycerol-water mixture (volume ratio 5/1) to maintain a liquid state and form clear glasses at low temperatures. The iron was reduced to the ferrous heme state under an inert atmosphere by adding 0.1 ml of freshly prepared

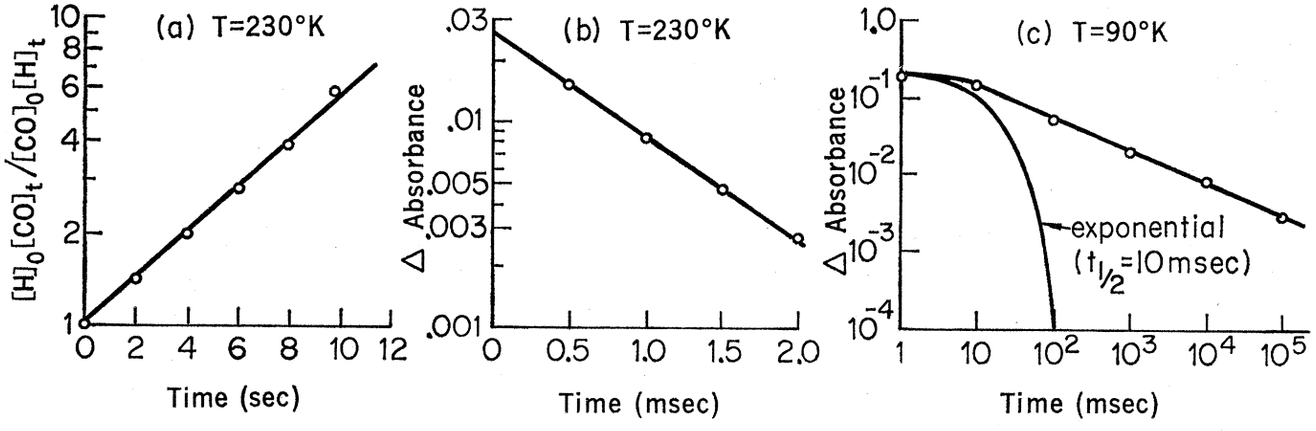

Fig. 1. Time dependence of carbon monoxide–myoglobin recombination. The kinetics for cytochrome P-450 are similar. The conditions were: heme protein (10 μM) in a glycerol-water mixture (volume ratio 3/1), saturated with (a and b) 1 percent CO in argon, total pressure 1 atm; (c) CO, 1 atm. Measurements were made with a 1-cm light path. For Mb the change in the millimolar extinction coefficient at 435 nm ($\Delta\epsilon_{mM}^{435nm}$) is 56; for P-450, $\Delta\epsilon_{mM}^{410nm}$ is 52. (a and b) At 230°K, two components of kinetic behavior are observed: (a) a slow component, which obeys the second-order rate equation, and (b) a fast one, which is exponential (first order). (c) At 90°K, in region 4, the logarithm of the change in absorbance is plotted against log t. The exponential $0.2 \exp [-(t \ln 2)/t_{\frac{1}{2}}]$, for $t_{\frac{1}{2}} = 10$ msec, is shown for comparison; the shape is independent of $t_{\frac{1}{2}}$. Changing $t_{\frac{1}{2}}$ merely shifts the curve on the time axis.

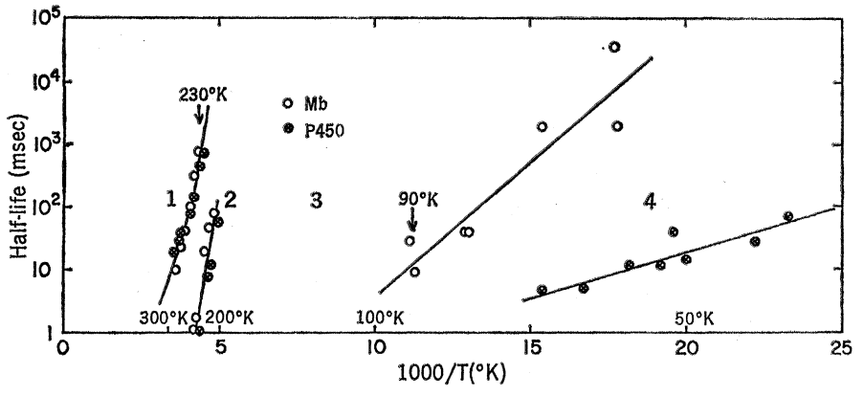

Fig. 2. Temperature dependence of CO binding to myoglobin (Mb) (open circles) and cytochrome P-450 (crossed circles). The conditions were: heme protein (10 μM) in a glycerol-water mixture (volume ratio 3/1); and CO, 1 atm. In region 1 the half-lives of CO rebinding depend on the CO concentration; in regions 2 and 4 the half-lives are independent of CO concentration.

100 mM sodium dithionite, and the CO adducts were formed by saturation with CO or 1 percent CO in argon at 21°C under a total pressure of 1 atm.

Kinetic region 1 occurs at physiological temperatures, where the observed time and concentration dependence of CO rebinding after photodissociation obeys the second-order rate law (*6*)

$$\frac{1}{[CO]_0 - [H]_0} \ln \frac{[H]_0 [CO]_t}{[CO]_0 [H]_t} = k^b t \quad (1)$$

Here, $[CO]_t$ and $[H]_t$ are the concentrations of free CO and CO-free heme protein in solution at time t, and k^b is the bimolecular rate constant, whose value depends on the heme protein. Figure 1a is a plot of the logarithmic term in Eq. 1 against t for a particular Mb measurement; Eq. 1 is well satisfied. For large CO concentrations ($[CO]_0 \gg [H]_0$), Eq. 1 reduces to a pseudo first order law

$$[H]_t = [H]_0 \exp(-t \ln 2/t_1) \quad (2)$$

where t_1, the half-life of the reaction in region 1, is given by

$$t_1 = \frac{\ln 2}{k^b [CO]_0} \quad (3)$$

For a solution saturated with pure CO at 1 atm, our data are well described by Eq. 2. We interpret the data in region 1 by assuming that, upon photodissociation, CO diffuses into the solvent and all CO molecules in the solvent then compete for the vacant binding sites. If the CO molecule has to surmount an energy barrier to bind to the protein, the bimolecular rate constant k^b and the half-life t_1 should show a temperature dependence given by the Arrhenius equation

$$t_1(T) = Ce^{E/kT} \quad (4)$$

where T is the temperature, k is the Boltzmann constant, and E is the activation energy. The measured values of $\ln t_1(T)$ for Mb and P-450 show similar behavior. The data for solutions saturated with CO at 1 atm are plotted in Fig. 2 as a function of $1/T$. Equation 4 is satisfied separately above and below 240°K, but a break occurs near this temperature.

Region 2 is characterized by a fast component in CO rebinding that appears when the temperature is lowered to about 240°K and becomes the dominant process within 20°K. For this component $[H]_t$ satisfies a first-order rate law

$$[H]_t = [H]_0 \exp(-t \ln 2/t_2) \quad (5)$$

as shown in Fig. 1b. The half-life t_2 is independent of the CO concentration. In a temperature range where both components are present, and with the solution saturated with 1 percent CO in argon at 1 atm, t_2 is about 10^4 smaller than t_1. For both Mb and P-450 the values of $t_2(T)$ plotted in Fig. 2 satisfy an equation of the form Eq. 4, with an activation energy of about 0.6 ev (14 kcal/mole). The independence of t_2 of the CO concentration suggests that each photodissociated CO molecule returns to the heme protein from which it was released. The CO could not have diffused far into the solvent, but must have remained near the molecule from which it dissociated. The onset of region 2 and the break in the curve of t_1 occur at the same temperature (see Fig. 2).

In region 3 the signal appears to become small. This region extends from about 150° to 200°K for Mb, and from 70° to 200°K for P-450. The solvent freezes into a glass approximately in the middle of this region, without apparent effect on the CO binding. It is not yet clear whether the small signal is caused by a small quantum yield or the recombination is too fast to be observed with our present equipment.

In region 4 photodissociation of CO unexpectedly returns or again becomes measurable. The time dependence of $[H]_t$ for CO recombination at temperatures below 150°K for Mb, or 70°K for P-450, is indicated in Fig. 1c, where $[H]_t$ is plotted against log t, rather than t, to accommodate the data in which the recombination takes place over many orders of magnitude in time. The concentration of CO-free heme protein does not follow a first- or second-order rate law. The behavior is as follows:: $[H]_t$ is nearly constant over a range of t, then breaks and follows a power law. Although we have not yet fit the data with a simple expression, to extract some information we denote the time at which $[H]_t$ falls to half the initial value as t_4. For $t > t_4$, $[H]_t$ satisfies

$$[H]_t = \text{constant} \times t^{-n} \qquad n \simeq 0.1\text{–}0.5 \quad (6)$$

The rate of recombination is independent of the CO concentration. The exponent in Eq. 6 is the same for Mb and P-450, but the parameter t_4 is different. At a particular temperature, CO rebinding occurs 10^2 to 10^3 times faster for P-450 than for Mb. Figure 2 shows that for both heme proteins the activation energy is small, in the range of 0.03 to 0.1 ev. At temperatures below 50°K for Mb or 25°K for P-450, the rebinding becomes very slow (*7*). The positions of the Soret absorption maxima of the low-temperature photodissociated species and the CO-free ferrous heme protein are indistinguishable. At the low temperatures characteristic of region 4, diffusion into the frozen solvent should be very slow and thus the observed rebinding of the CO occurs only if the CO molecule does not leave the protein. The absence of a CO concentration dependence also favors such a hypothesis. A detailed explanation of the observed time dependence for $[H]_t$ and of the activation energy will require more study, possibly including investigation of other heme proteins and model compounds.

R. H. Austin, K. Beeson
L. Eisenstein, H. Frauenfelder
I. C. Gunsalus, V. P. Marshall
Department of Physics and
Department of Biochemistry,
University of Illinois, Urbana 61801

References and Notes

1. E. Antonini and M. Brunori, *Hemoglobin and Myoglobin in Their Reactions with Ligands* (North-Holland, Amsterdam, 1971), pp. 13–39.
2. G. A. Millikan, *Proc. Roy. Soc. Ser. B* **120**, 366 (1936); Q. H. Gibson, *J. Physiol. London* **134**, 112 (1956); T. Omura, R. Sato, D. Y. Cooper, O. Rosenthal, R. W. Estabrook, *Fed. Proc.* **24**, 1181 (1965); I. C. Gunsalus and J. D. Lipscomb, in *The Molecular Basis of Electron Transport*, J. Schultz and B. F. Cameron, Eds. (Academic Press, New York, 1972), p. 179; J. A. Peterson and B. W. Griffin, *Arch. Biochem. Biophys.* **151**, 427 (1972).
3. B. Chance, B. Schoener, T. Yonetani, in *Oxidases and Related Redox Systems*, T. E. King, H. S. Mason, M. Morrison, Eds. (Wiley, New York, 1965), pp. 609–614.
4. K. D. Hardman, E. H. Eylar, D. K. Ray, L. S. Banaszak, F. R. N. Gurd, *J. Biol. Chem.* **241**, 432 (1966); I. C. Gunsalus, J. D. Lipscomb, V. Marshall, H. Frauenfelder, E. Greenbaum, E. Münck, in *Biological Hydroxylation Mechanisms*, G. S. Boyd and R. M. S. Smellie, Eds. (Academic Press, New York, 1972), pp. 135–157.
5. M. Katagiri, B. N. Ganguli, I. C. Gunsalus, *J. Biol. Chem.* **243**, 3543 (1968); C. A. Yu and I. C. Gunsalus, *Biochem. Biophys. Res. Commun.* **40**, 1431 (1970).
6. K. J. Laidler, *Theories of Chemical Reaction Rates* (McGraw-Hill, New York, 1969).
7. This effect has been seen for cytochrome a_3 below 160°K [see (*3*)].

8. We thank Drs. T. L. Brown, D. Hendrickson, and M. Duggan for a generous loan of the Andonian cryostat, and Drs. R. Marcus, P. Debrunner, E. Münck, J. Dow, D. Lazarus, and C. P. Slichter for many useful discussions. Supported in part by NSF grants GP 26282 and GH 36966, HEW grants GM 18051 and AM 00562, and NIH postdoctoral fellowship 5 F02 HD 41376.

2 April 1973

Radio Noise from Towns: Measured from an Airplane

Abstract. *Measurements of broadband radio noise in the range 73 to 440 megahertz were made over several small Illinois cities during August, September, and December 1972. Results for cities with a population larger than 25,000 are presented as brightness temperatures between 2400 and 9600 degrees Kelvin. Even the smallest villages produce significant noise pollution. There is considerable diurnal variation and some evidence for seasonal variation.*

Users of the radio-frequency spectrum have long been aware that the electromagnetic noise environments of cities and towns are not conducive to sensitive measurements of natural electromagnetic phenomena or to communication with minimum power. The problem is perhaps most apparent to radio astronomers, who must seek remote, interference-free sites for their instruments. However, communication transmitters routinely use more power than would be dictated by natural noise alone, in order to overcome incidental man-made noise. Similarly, broadcasting transmitters produce extremely high intensities in urban areas, in part to overcome the ambient man-made noise. If lower noise levels prevailed, transmitter powers could be reduced. This, in turn, would reduce pollution of the electromagnetic spectrum by spurious emissions from transmitters.

The increasing pollution of the electromagnetic environment is a largely unappreciated but nonetheless important social problem. The question of incidental man-made noise has received some attention from radio engineers, and some experimental data taken from the air appear in the literature (*1–3*). These data refer mainly to very large cities, and, even for them, present only a tentative sampling of the radio noise environment.

A brief series of measurements was made from the air in the fall and winter of 1972 for a preliminary assessment of the electromagnetic noise generated by small cities and towns in the very-high-frequency and ultrahigh-frequency bands. The noise under consideration is unintentional and might have been generated by electrical appliances, power distribution facilities, automobiles, and other electrical equipment.

The measurements were made from the air, with use of a light airplane, on frequencies of 74, 148, 222, and 440 Mhz. On the 74 Mhz frequency, the receiving antenna was a half-wavelength coaxial dipole towed horizontally behind the airplane; on 148 and 222 Mhz, a three-element Yagi-Uda array (driven dipole and two directors) was mounted vertically beneath the metal wing, which acted as a reflector; and on 440 Mhz, a five-element Yagi-Uda array, including one reflector element, was mounted beneath the wing. The Yagi-Uda antennas were directed vertically downward. Measurements were made one frequency at a time (it was necessary to land while changing antennas). The antenna patterns were not measured; however, their behavior when we were flying over unresolved radio sources indicated they had normal directivity patterns for antennas of their respective types. A typical antenna installation is shown in Fig. 1.

Fig. 1. The 222-Mhz antenna installed on a Cessna 150 airplane.

The receiving equipment consisted of a transistor preamplifier and crystal-controlled frequency-converter for each radio frequency, followed by a standard triple-detection superheterodyne communication receiver tuned to the output frequency of the converter. The overall predetection bandwidth was 4.8 khz, and the detector was followed by a resistance-capacitance integrator with a time constant of 1.4 seconds. The output was recorded graphically. The noise figures of the receivers were measured with an Airborne Instrument Laboratories noise generator, type 07005. Noise figures and other parameters used in calculating the characteristics of radiation sources are listed in Table 1.

The ignition systems of light airplanes, even though shielded, typically produce strong, impulsive noise in the frequency range under investigation. Thus, it was necessary to equip the receiver with a "noise-blanking" system that cancels the receiver output during a strong noise impulse of short duration. This system was highly effective and reduced the influence of the airplane's engine to a negligible level. To test the validity of measurements made

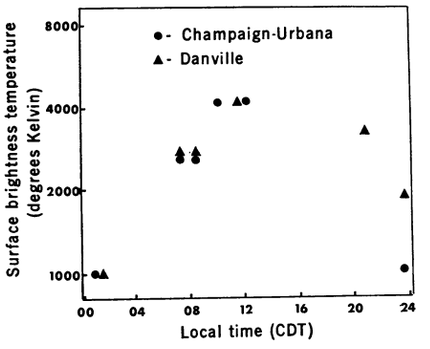

Fig. 2. Diurnal variation of surface brightness temperature of cities in August and September 1972, on a frequency of 222 Mhz. No distinction is made as to day of week.