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Conference Paper

Low Temperature Kinetics as a Probe of Protein Structure and Dynamics*

R. H. Austin

Max Planck Institute for Biophysical Chemistry, Göttingen-Nikolausberg, Federal Republic of Germany

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The recombination kinetics of flash-photolyzed carbon monoxy heme proteins has been studied as a function of temperature over the range of 2 K—350 K. Low temperature kinetics ($\ll$ 200 K) reveal that internal activation energy barriers to recombination (*a*) control the room temperature kinetics, (*b*) are of a distributed nature, forming an ensemble of activation energies, (*c*) are specific to the protein studied and are sensitive to the presence of substrates bound to the protein.

Cytochrome *P450* from camphor induced *Pseudomonas putida* reveals low temperature kinetics which are highly dependent on the presence or absence of the camphor substrate.

INTRODUCTION

The use of sub-physiological temperatures has become an important tool for studies of short-lived or unstable intermediate states in biochemistry. In our studies we have used a very wide temperature range, extending from 320 K to 2 K, to discover low activation energy barriers to ligand binding in heme proteins, barriers that are effectively hidden at room temperatures.

EXPERIMENTAL

The basic experimental method is simple. We work with heme proteins that bind the easily photodissociated carbonmonoxide (CO) ligand. The reduced protein is dissolved in a glycerol-water mixture and placed in a variable temperature helium cryostat. A pulsed dye laser fires a 100 millijoule photolyzing light pulse into the sample to dissociate the CO from the iron in the heme. The pulse width of the laser pulse, 1 microsecond, determines the time resolution of the apparatus. A monitoring beam passes through the sample at a right angle to the exciting beam. The change in the transmission of the beam at 435 nanometers as the CO molecule recombines with the heme is stored in a digital transient recorder and transfered to an on-line computer for analysis.

We have found that for many kinetic measurements a linear time base does not span a large enough range in time to store a suitably complete picture of the kinetics. A related problem is the burying in noise, in our case photon shot noise, of an exponentially decaying signal over long times due to the high frequency response necessary to record the faster parts of the signal. We have developed a digital transient recorder which attacks these problems by sweeping through the memory on a

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pseudo-logarithmic scale in order to greatly reduce the memory requirements of a storage range of 10^8 in time. The recorder simultaneously averages the signal over increasingly longer sample intervals during the sweep in order to remove unnecessary high frequency noise. The instrument spans 8 powers of 10 in time in a single sweep, from 2 microsecond to 300 seconds, using only 256 memory words but yet maintaining a resolution of 30 samples per decade of time.

MYOGLOBIN RESULTS

In order to appreciate the *P450* results it is easiest to first understand the results of experiments with myoglobin (Mb), which we have exhaustively studied. Let us view the recombination kinetics of CO with Mb as we descend in temperature from 300 K to 40 K (Figure 1). A more complete analysis of myoglobin can be found in ref. 1.

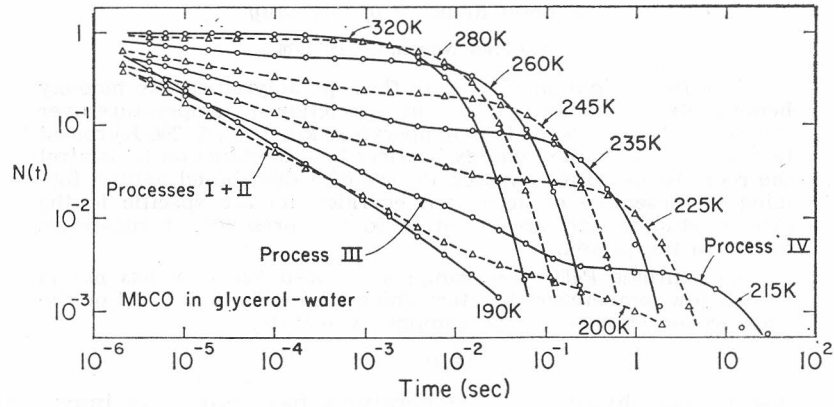

Figure 1. The high and transition region kinetics of Mb. N (*t*) is the normalized number of Mb molecules which have not yet rebound CO at time *t* after the laser pulse. The terms »process I«, *etc.* refer to terminology used in ref. 1.

The high temperature range. — At temperatures > 270 K we see a simple bimolecular reaction, exponential in time for [CO] $\gg$ [Mb]. The bimolecular rate constant in 75% glycerol-water is about $16^6\ M^{-1}\ s^{-1}$, less than the diffusion controlled value.

The transition range. — A curious thing happens upon further cooling. While the bimolecular process slows as expected a concentration independent process appears at about 260 K and assumes dominance, in terms of the fraction of molecules recombining by this mode, by about 210 K. The temperatures quoted here apply only to 75% glycerol-water mixtures. If a less viscous solvent mixture is used then the concentration independent process appears at a lower temperature, while if water is used the transition occurs abruptly when the water freezes. Evidently we see the solvent becoming more rigid and locking the photolyzed CO molecule in an internal pocket where geminate recombination can occur. Note from Figure 1 that the kinetics in this region are non-exponential and that more than one process can be seen, that is, the curves are not smooth.

The low temperature region. — (See Figure 2.) Independent of the solvent system used, at roughly the same temperature we enter a region where the kinetics are CO and Mb independent and the kinetics are smooth, indicating

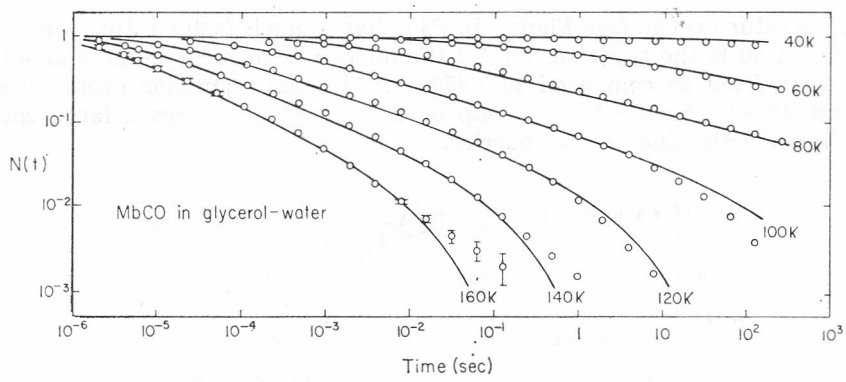

Figure 2. The low temperature kinetics of Mb.

a single process. This region consists of all temperatures below 170 K. The kinetics are unusual in that they are not exponential in time but instead fit well to a power law of the form:

$$N(t) = N(o)\ (1 + t/t_o)^{-n} \tag{1}$$

$$n = \alpha RT \tag{2}$$

This function (1) is a straight line on a log (N) *versus* log (*t*) plot (a log-log plot).

P450 RESULTS

We have also studied the protein *P450,* as isolated and purified by Dr. Gunsalus' group from the bacteria *Pseudomonas putida.* The normal substrate for this protein is camphor and I shall call the ferrous substrate free protein *P450* and the substrate bound ferrous protein *P450*CAM. *P450* is a water soluble, highly purified enzyme with well characterized states and thus provides an ideal subject for us to explore the relevance of our work to a real enzyme.

As we cool the *P450* or the *P450*CAM we see the presence of inner activation barriers as in Mb (see Figure 3. Data for *P450* are qualitatively similar.) The heights of the activation barriers seen in the transition region are different from those obtained in Mb. The greatest differences however are seen in the

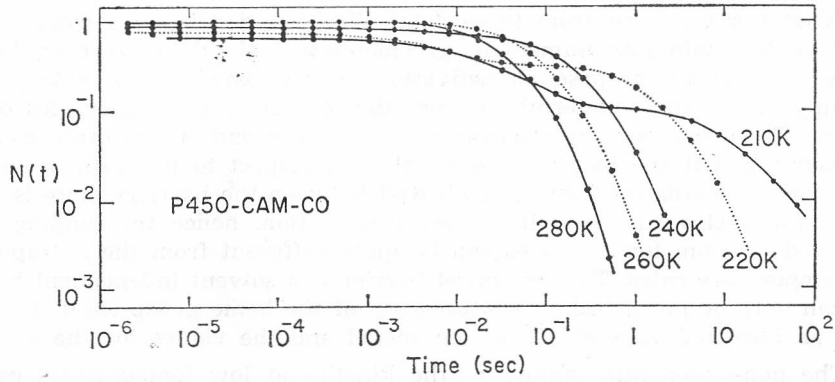

Figure 3. A sample of the high and transition kinetics of *P540*CAM.

low temperature range (see Figure 4). *P450* has a much faster return time than *P450*CAM; at 60 K the $t_{1/2}$ (time for 1/2 the molecules to recombine) is about 10^4 smaller for *P450* as compared to *P450*CAM. Although camphor probably does not bind directly to the heme group or the iron, it produces a large change of the inner activation energy barrier.

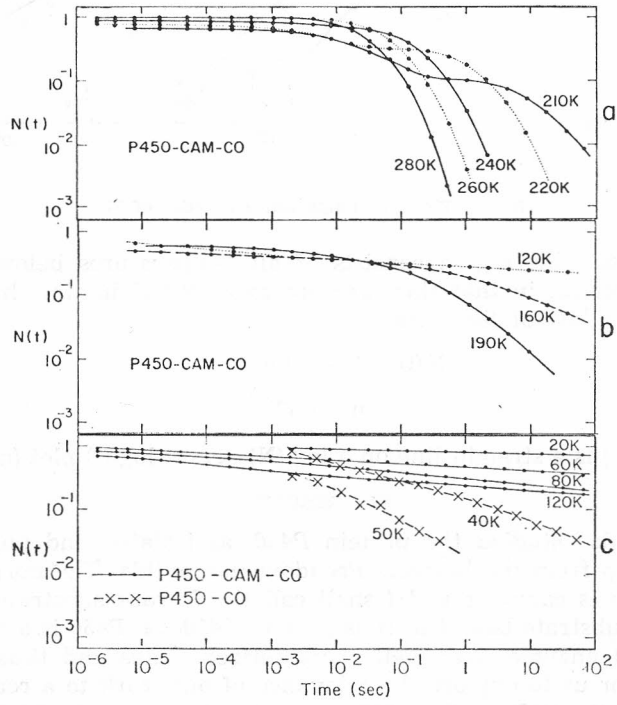

Figure 4. Top: High temperature kinetics of *P450*CAM. Middle: Low temperature kinetics of *P450*CAM. Bottom: Comparison of *P450* and *P450*CAM low temperature kinetics. Note the time axis is logarithmic.

INTERPRETATION OF RESULTS

What can we learn from these data? We have developed a model which allows us to obtain good numerical agreements with the data. For each distinct kinetic process we propose an activation energy barrier with entropy and enthalpy values to be determined from the temperature dependencies of the kinetics. The progression of processes seen as we descend in temperature can fit by assuming that the barriers are serial with respect to the path of the CO as it moves towards the iron. At each well between the barriers there is probability for backwards as well as forwards motion, hence the binding rates measured at room temperatures can be quite different from the extrapolated low temperature rates. The innermost barrier is a solvent independent barrier and can only be perturbed by perturbation of the heme group itself. Figure 5 gives an idealized view of Mb in our model and the values for the barriers.

The non-exponential nature of the kinetics at low temperatures can be fit by assuming that an ensemble of protein molecules exists as a function of

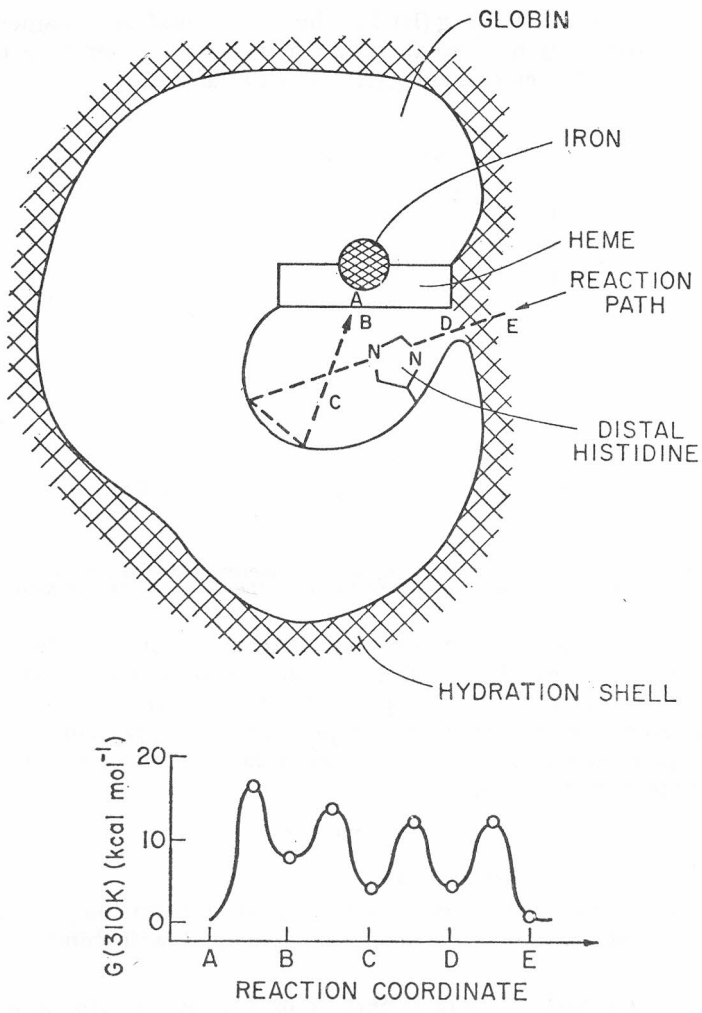

Figure 5. Top: Idealized picture of Mb, showing possible sources for the inner activation barriers. Bottom: Values for the Gibbs energies of the barriers in Mb.

activation energy. Thus, the number of protein molecules not bound by CO as a function of time can be written:

$$N(t) = N(o) \int g(H)\, e^{-kt}\, dH \tag{3}$$

$$k = Ae^{-H/RT} \tag{4}$$

Formally equation 3 has the same form as a Laplace transform and it is theoretically possible by an inverse Laplace transform to determine from the data a g (H) that will fit the data over the entire low temperature range. The data obtained for Mb match quite well.

A similiar g (H) can be calculated for the *P450* data. The *P450*CAM low temperature kinetics slow much more rapidly with temperature than those for Mb, so we expect the *P450*CAM g (H) to be at higher values than that for Mb.

The *P450* data show that the g (H) for this state must lie considerably lower than the Mb distribution since it shows a weaker temperature dependence. Figure 6 compares the calculated g (H) for *P450* and Mb.

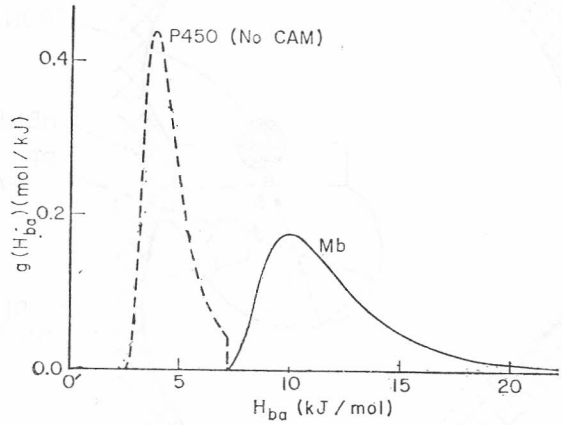

Figure 6. The experimentally found values for the innermost activation barriers for Mb and *P450*. The *P450*CAM is expected to lie to the right of the Mb g(H).

We believe the g(H) originates from different folding of the protein, also known as conformers in which each conformer has a unique activation enthalpy for CO recombination. At low temperatures the distribution of conformers is time independent, while at high temperatures the protein rapidly moves through all possible conformations, »rapidly« here meaning rapid with respect to the average recombination time.

CONCLUSION

To summarize, we have learned:

1) The activation energy barriers measured at room temperatures are not necessarily the actual activation energies associated with binding at the iron site.

2) The distributed barriers observed at low temperatures are sensitive to modifications of the heme environment.

3) The model developed possesses enough variables to allow good numerical agreements with the data but does not yet afford a molecular understanding of how the activation energies are structurally related to the protein.

We feel that the following areas should be explored:

1) Do the same barriers also control the binding of the substrate, camphor?

2) The distributed barriers are observed for CO recombination. We feel that we have good evidence that the electronic states of the protein are distributed and in dynamic equilibrium at room temperature. However, we must confirm this model through the use of independent physical techniques that are also sensitive to the electronic states of the heme, such as EPR or the Mössbauer effect, for example. When such a correlation is found, then a link will exist between a well defined physical parameter and activation energy in an enzyme.

Much of the model that we have developed is of a speculative nature and still requires further experimental support. We hope that our work will not be just an experimental curiosity but will lead to a firmer understanding of the influence of the protein on the active site conformation in both a static and especially a dynamic sense.

REFERENCES

1. R. Austin, K. Beeson, L. Eisenstein, H. Frauenfelder, and I. C. Gunsalus, *Biochemistry* **14** (1975) 5355. (This work contains a complete account of the Mb results and further references.)

DISCUSSION

S. Maričić:

How would you compare myoglobin and cytochrome *P450* in quite general terms as to their »flexibility«, or »plasticity«?

R. H. Austin:

It is rather difficult to interpret our results directly in terms of protein flexibility. It is true that we see a very large change in our g(H) for camphor binding, implying a high sensitivity at the iron site to non-heme substrate binding. However, we do not know if this is due to a direct electronic interaction of the camphor, or if the structure of the protein is particularly adapt at transmitting perturbations to the heme site.

H. Schleyer:

(1) Is there a pH-dependence in the case of myoglobin? (2) Do your results depend on the cooling rate? (3) Would you give us a brief description of your »Translog« instrumentation?

R. H. Austin:

(1) We have observed no great pH effect in the case of myoglobin. Studies on the *P450* protein have not been done as yet. (2) In the case of myoglobin we also did not notice a dependence on the rate of cooling of the g(H) obtained at low temperature. (3) The instrument is entirely digital. An ADC samples continuously at 500 kHz. A clock sums up conversions over time intervals which increase by a factor of 2 in duration after 10 regularly spaced intervals. The summed up conversions are averaged after the end of the intervals and stored in a memory for later output to a computer.

I. Blanck:

(1) The separation of the distinct reaction barriers in myoglobin has been well performed. Did you also succeed in resolving distinct steps with *P450* in the respective temperature ranges? (2) Have you any interpretation of the different behaviour of myoglobin and *P450* at low temperatures?

R. H. Austin:

(1) Yes, the *P450* data also display a series of internal barriers as in myoglobin. I do not have a plot of the actual barrier heigths, since this work is still in progress. (2) Since the *P450* rather resembles the low temperature kinetics in heme alone, we could suggest that the heme in the *P450* without substrate is rather open to the solvent. In the camphor bound state the slowness of the kinetics could suggest a very tightly closed pocket, as in myoglobin. — Moreover, I must stress that we do not understand the origins of the g(H) enough at this point to make any definitive statements. The g(H) seems to be affected by many things.

D. L. Williams-Smith:

Can you detect excited states of the heme or CO extremely short times after the flash at low temperatures? Could the transfer of excited state energy generate the conformers?

R. H. Austin:
In myoglobin there is no sign of an excited state, however, for other proteins there is definite evidence for such an excited state of a very short lifetime and of very low activation energy. If the g(H) is due to conformers of the protein, it is very unlikely that we could alter the structure of the protein at low temperature by absorbing light in the heme. Furthermore, multiple flashing indicates that g(H) is stable under repeated illumination, and one would perhaps expect an evolution of the conformers with time under illumination if it were light-induced.

T. G. Traylor:
We have found a very fast CO independent rate of recombination to our imidazole-heme model compounds dissolved in a polystyrene matrix. This seems to be similar to your low temperature process. It is also satisfying that you have found several processes in the proteins which slow down rebinding. The model compounds bind with a K_{CO} of about 3×10^8 M^{-1} and this increased binding appears to depend upon on-rates rather than off-rates; is this consistent?

R. H. Austin:
In answer to the first remark, we have also found that putting the protein in a rigid matrix, in our case of poly-vinyl-alcohol, a CO-concentration independent kinetics ensues. In regard to the second question, I agree this is completely consistent with our findings, in fact we find that the effective $k_{on-rate}$ of CO is slowed 100-fold at room temperature because of internal barriers. Thus, a major part of the protein control on binding constants is accomplished by altering the on-rate by these protein-associated internal barriers.

S. Maričić:
(1) Do you still hold that the structural interpretation of your published myoglobin data is correct? (2) What would you expect from other techniques for the clarification of the structural picture you have derived thus far?

R. H. Austin:
(1) We have postulated possible causes for the internal barriers, however, we cannot say to have proved the features indicated actually **do** cause the barriers. Clearly, something within the protein is providing activation barriers in addition to the heme itself. (2) We see a distribution in activation energies, and internal barriers. Perhaps the g values in EPR or Mössbauer lines also posses a »spread« in values, which would lead to broadening of the lines. On the other hand in the case of PMR, perhaps because the solvent cage closes access to the interior of the protein at lower temperatures the water relaxation rates change. Perhaps it is even possible to use relaxation techniques to observe the onset of the proposed »conformational breathing« of the protein molecule.

SAŽETAK

Kinetika na niskim temperaturama za proučavanje strukture i dinamike proteina

R. H. Austin

Rekombinacijska kinetika ugljikova monoksida i hemoproteina proučavana je laserskom fotolizom od 2—350 K unutar osam vremenskih dekada, od 2 μs do 300 s. Pri niskim temperaturama, ispod 200 K, kinetika pokazuje interne barijere za aktivaciju rekombinacije koje (*a*) u stvari određuju aktivacijsku energiju mjerenu na sobnoj temperaturi, te da (*b*) te aktivacijske barijere čine raspodjelu aktivacijskih energija koje su (*c*) specifične za mioglobin i citokrom *P450*, kao i zavisne o vezivanju supstrata na enzim.

MAX PLANCK INSTITUTE
FOR BIOPHYSICAL CHEMISTRY
GÖTTINGEN-NIKOLAUSBERG,
FRG

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