Proc. Nat. Acad. Sci. USA
Vol. 70, No. 5, pp. 1550–1553, May 1973

Heme Proteins: Effect of an Intermediate on Photochemical Behavior

PAUL E. PHILLIPSON*, BRUCE J. ACKERSON*, AND JEFFRIES WYMAN†

* C.N.R. Center for Molecular Biology, University of Rome, Italy; Department of Physics and Astrophysics, University of Colorado, Boulder, Colo. 80302; and † C.N.R. Center for Molecular Biology, University of Rome and Istituto Regina Elena Rome, Italy

Contributed by Jeffries Wyman, March 19, 1973

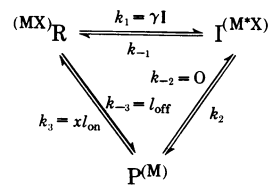
ABSTRACT **This paper analyzes, in terms of a triangular kinetic scheme, the possible effects of an intermediate in the photochemical behavior of simple heme proteins. Both steady-state and transient phenomena are analyzed. The magnitude of the quantum yield for a given system is determined by a competition between quenching and ligand detachment, measured by the two rate constants, k_{-1} and k_2, respectively; as a first approximation, it can be described by a single parameter, namely the ratio k_{-1}/k_2. The individual values of k_{-1} and k_2 can only be determined at light intensities so high that the measured quantum yield becomes itself a function of light intensity. Under these conditions the relaxation time for transient approach to the steady-state can be complex, corresponding to heavily damped chemical oscillations. It is pointed out that when the model is extended to include more than one intermediate, multiple oscillations are possible. In this more general case the system in the steady-state may also show pseudocooperativity or anticooperativity.**

The original observations of Haldane and Loraine-Smith (1) on the extreme photosensitivity of carbon monoxide hemoglobin have been followed by a large amount of experimental work by various investigators, extended to include other heme proteins and other ligands (2–11). The greater part of this work has been directed to the determination of quantum yields, which, for a given ligand, vary significantly from protein to protein, and, for a given protein, vary much more—indeed by several orders of magnitude—from one ligand to another. In all this work three methods for determining quantum yield have been used, based on: (*i*) measurements of relaxation times, of which the most direct are of those for the approach to the steady state when the light is turned on and the return to equilibrium when it is turned off; (*ii*) measurements of the displacement of the binding curve produced by the light; and (*iii*) measurements of the amount of ligand removed by a light pulse of known and variable intensity. In the several analyses which have so far been given, no explicit account appears to have been taken of the effect of a photoexcited intermediate, although the existence of such an intermediate would seem to be mandatory on general physical grounds. More specifically, it is suggested by the fact that the quanta involved are not all directly absorbed at the ligand-binding site but also by other, often remote parts of the macromolecule, particularly those involving tryptophan, as shown by Bücher and Kaspers (5)‡. In this paper we develop a model which brings out the role played by such an intermediate, not only in its effect on quantum yield but also on the character of relaxation processes.

‡ Bücher and Kaspers reported that for wavelengths 5460 Å, 3660 Å, 3340 Å, 3130 Å, and 2800 Å the quantum yields for CO-myoglobin are 0.91, 0.99, 1.09, 0.97, and 1.01, respectively. At 2800 Å almost half the light is absorbed by the aromatic amino acids, while at the other wavelengths the photons are absorbed by the heme group. This result indicates energy transfer from the protein to the prosthetic group where the ligand is detached. For hemoglobin one might expect that such energy transfer processes would also occur between subunits.

THE MODEL

The model we propose is the following:



(Scheme **A**)

Here M represents a heme protein, X a ligand, x its activity, and $\mathbf{I}$ the light intensity. The other quantities are constants, of which k_3 corresponds to the "on" constant l_{on} and k_{-3} to the "off" constant l_{off}, measured in the absence of light. γ is the constant which applies to the absorption of light to produce a photoexcited intermediate I. The fact that $\mathbf{I}$ occurs to the first power implies that the creation of the intermediate is a "one-hit" process. k_2 is a measure of the speed of breakdown of I into the photoproducts and would be expected to vary most strongly with the nature of the ligand for a given protein. k_{-1}, on the other hand, is a measure of the back-rate of conversion of I into reactant R and is, thus, a measure of quenching in all its aspects; for a given ligand, k_{-1} would be expected to vary most strongly with the nature of the protein. Setting $k_{-2} = 0$ is based on the assumption that in the absence of light $\mathbf{I} = 0$, so that the triangular scheme degenerates into the simpler linear scheme

$$R \underset{xl_{no}}{\overset{l_{off}}{\rightleftharpoons}} P$$

It should be noted that the triangular model is a special case of a more general square model, which includes an M^* form.

The equations governing this model are the following:

$$\frac{dR}{dt} = -(\gamma \mathbf{I} + l_{off})R + k_{-1}I + xl_{on}P$$

$$\frac{dI}{dt} = (\gamma \mathbf{I})R - (k_2 + k_{-1})I \qquad [1]$$

$$\frac{dP}{dt} = l_{off}R + k_2 I - x l_{on} P$$

THE STEADY STATE

The steady-state concentrations are found by setting the left sides of Eq. 1 equal to zero. The ratios of these concentrations, distinguished by subscript s, are

$$R_s : I_s : P_s = [x l_{on}(k_2 + k_{-1})] : [\gamma \mathbf{I}(x l_{on})] : [(\gamma \mathbf{I})k_2 + l_{off}(k_2 + k_{-1})] \qquad [2]$$

It follows that the expression for the apparent, or pseudo, equilibrium between the liganded and unliganded forms of the macromolecule is

$$\frac{P_s}{R_s + I_s} = \frac{(k_2 + k_{-1})l_{off} + (\gamma \mathbf{I})k_2}{x l_{on}[(k_2 + k_{-1}) + \gamma \mathbf{I}]} \equiv \frac{K_s}{x} \qquad [3]$$

Thus the apparent, or pseudo, equilibrium constant K_s may be written as

$$K_s = \frac{k_{off}}{k_{on}} \qquad [4]$$

with

$$k_{off} = \frac{(k_2 + k_{-1})l_{off} + (\gamma \mathbf{I})k_2}{(k_2 + k_{-1}) + \gamma \mathbf{I}}, \quad k_{on} = l_{on} \qquad [5]$$

When $\mathbf{I} \to 0$, K_s of course approaches the true equilibrium value $K_{eq} = l_{off}/l_{on}$; on the other hand, as $\mathbf{I} \to \infty$ K_s approaches k_2/l_{on}.

It will be seen from Eq. 5 that for all values of $\mathbf{I}$ the binding curve (fractional saturation versus ln x) will have the same invariant form as that of a simple titration curve characterized by a Hill coefficient $n = 1$. It should be noted, however, that this very simple behavior, involving strict invariance of shape of the binding curves in the presence of light, results from setting $k_{-2} = 0$ and cannot, in general, be expected. Even for a one-site molecule, whenever the reaction scheme involves a closed pathway containing one or more intermediates, as in scheme A, the binding curve for the steady state will only be invariant in shape in very exceptional circumstances, and departures from the simple form, $n = 1$, will correspond to pseudocooperativity or anticooperativity.

TIME-DEPENDENT BEHAVIOR

In order to obtain a general solution of Eq. 1, we assume that the system is buffered in the sense that the activity x remains constant, as in most of the experiments. The equations then become linear and may be dealt with in the usual way by assuming solutions of the form $B_i \exp(-t/\tau)$, $i = 1, 2, 3$, substituting them into the rate equation, and setting the determinant of the coefficients B_i equal to zero. This results in two roots corresponding to two relaxation times that may be written as

$$\frac{1}{\tau_\pm} = \lambda \pm i\omega, \qquad [6]$$

where

$$\lambda = {}^1/_2[\gamma \mathbf{I} + k_2 + x l_{on} + l_{off} + k_{-1}]$$

$$\omega = {}^1/_2\{-(\gamma \mathbf{I})^2 + 2\gamma \mathbf{I}[(k_2 + x l_{on}) - (l_{off} + k_{-1})] - [(k_2 + k_{-1}) - (x l_{on} + l_{off})]^2\}^{1/2} \qquad [7]$$

Thus, when ω is real (the τ_s' are complex), the approach to the steady state will be a damped oscillatory process of frequency ω and damping constant λ. In the opposite case (ω imaginary) the process will be characterized by two ordinary (real) relaxation times. In either case, as the steady state is approached the concentrations of the three forms pass from their initial values to the steady-state values given by Eq. 2, which were obtained without any special assumption about buffering of ligand.

QUANTUM YIELD

It was pointed out that there are three operational methods of measuring quantum yield. Let us see how each of these is described by our model. Consider first the method based on the displacement of the binding curve in the presence of light. By Eq. 3,

$$d \ln\left(\frac{P_s}{R_s + I_s}\right) = \frac{d \ln K_s}{d\mathbf{I}} d\mathbf{I} - d \ln x = \frac{d \ln k_{off}}{d\mathbf{I}} d\mathbf{I} - d \ln x \qquad [8]$$

Consequently, by setting $d \ln [P_s/(R_s + I_s)] = 0$,

$$\left(\frac{\partial x}{\partial \mathbf{I}}\right)_{\bar{X}} = \frac{\bar{X}}{k_{off}}\left(\frac{\partial k_{off}}{\partial \mathbf{I}}\right)_{\bar{X}} = \left(\frac{\bar{X}}{1 - \bar{X}}\right)\frac{1}{l_{on}}\frac{dk_{off}}{d\mathbf{I}} \qquad [9]$$

where $\bar{X}$ denotes fractional saturation of the macromolecule with ligand. This result shows that the value of x required to produce a given saturation $\bar{X}$ will be linear in $\mathbf{I}$ if, and only if, k_{off} is also linear in $\mathbf{I}$; by Eq. 5 this will be true whenever $\gamma \mathbf{I} \ll k_2 + k_{-1}$ and not otherwise. Recent measurements (10) show that in the case of myoglobin and several other one-site heme proteins linearity prevails over a wide range of light intensities, in fact up to the highest intensities used in the steady-state experiments. This finding provides confirmation of two basic assumptions of the model, (*a*) that $\mathbf{I}$ occurs to the first power and (*b*) that l_{on} is a true constant unaffected by light. Assumption (*b*) is also supported by other, more-direct measurements (11). If the binding curves were *not* invariant in shape with $n = 1$, no such simple behavior would be predicted.

Consider next the method based on relaxation measurements. Here the situation is more complicated, since it is necessary to invoke the full solution of the equations. We shall not attempt to give the analysis in detail, but content ourselves with presenting only the results. They say that provided certain conditions are satisfied both relaxation times are real and that

$$\frac{1}{\tau_+} = k_2 + k_{-1}$$

$$\frac{1}{\tau_-} = (x l_{on} + l_{off}) + \gamma \mathbf{I}\left(\frac{k_2}{k_2 + k_{-1}}\right) \qquad [10]$$

The conditions for this result, which are both necessary and sufficient, are the following:

$$k_2 \gg \gamma \mathbf{I},\ k_2 \gg l_{off},\ (k_2 + k_{-1}) \gg (x l_{on} + l_{off}) \qquad [11]$$

They impose limits to the values of x and $\mathbf{I}$ and make it clear that $\tau_+ \ll \tau_-$. This prediction agrees with the fact that in experiments covering a wide range of values of x and $\mathbf{I}$ only one relaxation time is observed and that this is linear in $\mathbf{I}$. When $\mathbf{I} \to 0$, $1/\tau_-$, which then becomes equal to $xl_{on} + l_{off}$, describes relaxation to equilibrium. Thus, the difference between the reciprocal of the relaxation time for the approach to the steady state when the light is turned on and that of the relaxation time for the return to equilibrium when it is turned off is given by $\gamma\mathbf{I}[k_2/(k_2 + k_{-1})]$ and is proportional to $\mathbf{I}$. This also is just what is observed.

Finally, consider the pulse method. This is based on the determination of the amount of ligand driven off by a very brief flash of very intense light. The intensity of the light, though variable, is kept always so high that under steady-state conditions it would drive off essentially all the ligand. Analysis shows that subject to exactly the same conditions as those given for the relaxation method plus the condition that

$$xl_{on} + l_{off} \ll \gamma\mathbf{I}\left(\frac{k_2}{k_2 + k_{-1}}\right) \qquad [12]$$

the logarithm of the fraction of bound ligand which is driven off by the flash is given by

$$\ln\left|1 - \frac{\Delta\bar{X}}{\bar{X}}\right| \propto \gamma\mathbf{I}\left(\frac{k_2}{k_2 + k_{-1}}\right) \qquad [13]$$

This result agrees exactly with what has been observed. (In practice, the fraction driven off is usually so small as to be identifiable with the logarithm.) It should be mentioned that a similar assumption to Eq. **12** is required to account for the results even when no intermediate is postulated.

It will be seen that in all those methods the key quantity is the product $\gamma k_2/(k_2 + k_{-1})$. In the case of the displacement method this is proportional to $[(1 - \bar{X})/\bar{X}]l_{on}(\partial x/\partial\mathbf{I})_{\bar{x}}$; in the case of the relaxation method to $\tau_s^{-1} - \tau_{eq}^{-1}$, where subscripts e and s denote equilibrium and steady state, respectively; in the case of the pulse method to $d(\Delta\bar{X}/\bar{X})/d\mathbf{I}$. If we divide each of these quantities by the molar extinction coefficient appropriate to the light source, we have, therefore, a quantity proportional to the quantum yield, ϕ, and by comparing such quantities we get relative values of quantum yield. In all but a few measurements, namely those of Warburg and collaborators, only relative values of quantum yield have been determined; on the basis of Warburg's absolute value $\phi = 1$ for carbon monoxide myoglobin, we can assign absolute values of ϕ to other molecules to which the model is applicable.

OSCILLATION

Under all the special conditions introduced in the last section to provide for linearity of measured quantities, the relaxation times are real. However, in the absence of special conditions there is always the possibility that in an "open" system approaching a steady state the relaxation times are complex, corresponding to a damped oscillatory process. This possibility *could* be realized in experiments of the type we have been considering under conditions where linearity breaks down. Oscillations require a real positive value of ω as defined in Eq. **7**. Since ω^2 is negative at both $\mathbf{I} = 0$ and $\mathbf{I} \to \infty$, and passes through a maximum for intermediate values, everything hinges on whether or not the value of this maximum is positive or negative. The maximum is obtained by the condition $\partial\omega/\partial\mathbf{I} = 0$, and on the basis of Eq **7** occurs at the value of $\mathbf{I}$ given by

$$\mathbf{I}_{max} = (xl_{on} + k_2) - (k_{-1} + l_{off}) \qquad [14]$$

The corresponding value of ω is

$$\omega_{max} = [(xl_{on} - k_{-1})(k_2 - l_{off})]^{1/2} \qquad [15]$$

Since $\mathbf{I}_{max}$ must, of course, be positive, these two equations lead to the following conditions for oscillation

$$xl_{on} > k_{-1},\ k_2 > l_{off} \qquad [16]$$

The first of these conditions is inconsistent with the linearity observed in the various experiments. It is evident, therefore, that linearity is inconsistent with oscillation. However, nonlinearity, though a necessity, is not a sufficient condition for oscillations.

It is worth noting that in the simple system we have been considering, oscillations, even if they were to exist in the nonlinear range, would be so heavily damped as to be hardly observable. On the other hand, in the case of a more complex system where there are several intermediates, there is always the possibility of less-heavily damped and more persistent oscillations.

CONCLUSION

We have seen that in accordance with our model the quantum yield may be identified with the quantity $(\gamma/\epsilon)[k_2/(k_2 + k_{-1})]$, where ϵ is the molar extinction coefficient for the light. If γ were the same as ϵ, as might perhaps be expected, this expression would, of course, reduce to $k_2/(k_2 + k_{-1})$ and differences of quantum yield would stem exclusively from values of k_{-1}/k_2, that is, from a competition between protein detachment and quenching. Even if they were not the same, however, provided that γ/ϵ were roughly constant from one case to another, differences of quantum yield would still result largely from differences in k_{-1}/k_2. This is what we suggest, and it is noteworthy, and perhaps surprising, that this control exerted by the intermediate can be effective even when the intermediate is present in only vanishingly small amounts.

For the triangular model shown in Scheme **A**, where $k_{-2} = 0$, the steady-state binding curve is necessarily of the form of a simple titration curve with a Hill coefficient $n = 1$. However, in the general case n need not, and usually will not, be equal to 1 under steady-state conditions, even for a one-site molecule. Thus, there is the possibility of pseudocooperativity or anticooperativity. For a multisite molecule like hemoglobin, such a possibility is even more likely.

In the case of a functioning enzyme a steady state can result from a flow of matter through the macromolecular system and, when the system is allosteric and capable of combining with several ligands, all the effects we have been considering—oscillatory relaxations, pseudocooperativity, and anticooperativity—can occur in an enhanced form. It is to be noted that the relaxation oscillations considered here are quite distinct from the sustained oscillations that arise in nonlinear systems to which the "predator–prey" equation is applicable (12). As a sequel to this work, we plan to investigate in detail the possible effects that can appear in the absence of chemical equilibrium in an allosteric molecule with several sites for several different kinds of ligand.

We thank Prof. M. Brunori for many valuable discussions during the writing of this paper. P.E.P. thanks the European Molecular Biology Organization as holder of a senior research fellowship, B.J.A. acknowledges a predoctoral fellowship from the National Science Foundation, and J.W. was supported by a research grant from the National Science Foundation.

1. Haldane, J. & Lorrain-Smith, J. (1895) *J. Physiol.* **20**, 497–520.
2. Warburg, O. & Negelein, E. (1928) *Biochem. Z.* **202**, 202–228.
3. Warburg, O., Negelein, E. & Christian, W. (1929) *Biochem. Z.* **214**, 26–63.
4. Bücher, T. & Negelein, E. (1942) *Biochem. Z.* **311**, 163–187.
5. Bücher, T. & Kaspers, J. (1947) *Biochem. & Biophys. Acta* **1**, 21–34.
6. Warburg, O. (1949) *Heavy Metal Prosthetic Groups and Enzyme Action* (Clarendon Press, Oxford, England).
7. Ainsworth, S. & Gibson, O. H. (1957) *Nature* **180**, 1416–1417.
8. Noble, R., Brunori, M., Wyman, J. & Antonini, E. (1967) *Biochemistry* **6**, 1216–1222.
9. Antonini, B. & Brunori, M. (1971) *Hemoglobin and Myoglobin in their Reactions with Ligands* (North-Holland Publ. Co., Amsterdam).
10. Brunori, M. Bonaventura, J., Bonaventura, C., Antonini, B. & Wyman, J. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 868–871.
11. Bonaventura, C., Bonaventura, J. Antonini, B., Brunori, M. & Wyman, J. (1973) *Biochemistry*, in press.
12. Glansdorff, P. & Prigogine, I. (1971) *Thermodynamic Theory of Structure, Stability and Fluctuations* (Wiley-Interscience Press, New York).