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Heme Proteins: Quantum Yield Determined by the Pulse Method

(photodissociation/sperm-whale myoglobin/trout hemoglobin I)

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ABSTRACT **We report results of the application of the "pulse method" to the study of photodissociation of various ligands from several heme proteins. By use of this technique, which allows the determination of the quantum yield of photodissociation accurately and rapidly, several ligands (CO, O_2, isocyanides) have been investigated for sperm-whale myoglobin and trout hemoglobin I. In agreement with previous results, the new data lead to the conclusion that no simple relationship exists between the quantum yield and the affinity constant in the ground state. For trout hemoglobin I, the experiments were extended to measure the quantum yield of the CO photodissociation as a function of the initial degree of saturation, from fully saturated down to the initial values of about 1.5%. The results yield additional information to that obtained by the "steady-state" method, and in particular exclude the idea that the photochemical yield is in any way dependent on the fractional saturation of the molecule with carbon monoxide.**

The photosensitivity of complexes of ferrous heme proteins with different ligands is well known. After the original observation of Haldane and Lorrain-Smith (1), many experiments bearing on various aspects of the phenomenon were performed. Most of these have been directed towards measurement of the quantum yield and elucidation of the various factors on which it depends (2–8). They have shown that the quantum yield varies greatly with the type of ligand, being maximal for carbon monoxide, and that for the same ligand it also varies with the protein moiety.

Recently the photosensitivity of the CO complex was used to investigate the relative roles of the various kinetic constants in determining the cooperativity of ligand binding shown by hemoglobin. The experiments, performed with a continuous illumination and therefore under steady-state conditions, revealed a remarkable invariance in the ligand-binding properties of hemoglobin under photodissociating conditions; thus both homotropic and heterotropic interactions are maintained in the presence of strong light flux (7). These results have raised several questions related both to the mechanism of ligand binding and to the photochemical events occurring in complex molecules that exist in different conformational states (8–10).

In general, three methods have been used in the photochemical experiments: (*i*) the displacement of the ligand-binding curve under the influence of light, (*ii*) the relaxation times involved in dark-to-light and light-to-dark transitions, and (*iii*) the breakdown of the complex by a short light pulse of variable (known) intensity (9). For a simple monomeric heme protein, like myoglobin, the three methods should all give the same information, as they are found to do. In the more complex case represented by hemoglobin, this is not necessarily true.

This paper reports results of the application of the "pulse" method to the study of photodissociation of various ligands from myoglobin and hemoglobin. For hemoglobin, the data yield additional information to that obtained by the "steady-state" (displacement) method (7), and in particular exclude the idea that the photochemical yield is in any way dependent on the fractional saturation of the molecule with carbon monoxide.

MATERIALS AND METHODS

Myoglobin from sperm whale was obtained from Seravac and repurified by ammonium sulfate precipitation. Hemoglobin from *Chironomus thummi thummi* was kindly provided by Dr. H. Formanek. Horseradish peroxidase, type VI, was obtained from Sigma. Carboxymethylated cytochrome *c* (11) *Aplysia* myoglobin (12), and trout hemoglobin I (13) were prepared as reported.

All experiments were performed with a modified version of the flash photolysis apparatus described (14). The duration of the flash was about 150 μ sec. Its intensity was reduced by known amounts by using sets of neutral filters calibrated directly in the flash apparatus. The thermostated cell had a 2-cm light path for observation and a total volume of 2 cm^3.

RESULTS

A short pulse of light absorbed by the heme-protein complex results in a dissociation of CO, according to the scheme

$$\mathrm{MbCO} \rightarrow \mathrm{Mb} + \mathrm{CO} \qquad [1]$$

If the pulse is short enough, recombination of the free partners is negligible during the duration of the flash; at the same time the amount of ligand photodissociated is proportional to the total energy delivered by the pulse and, consequently, to the light flux I. From these two considerations it follows that

$$\frac{d(\mathrm{MbCO})}{dI} = -\omega \cdot (\mathrm{MbCO}) \qquad [2]$$

where the constant ω is proportional to the quantum yield. Thus, the quantum yield can be determined by measuring the fractional amount of ligand dissociated from the complex as a function of I.

A plot based on Eq. 2 for the CO complex of sperm-whale myoglobin is shown in Fig. 1. In this simple case ω is independent of both CO concentration (from 5–500 μM) and protein concentration (from 0.75–7.5 μM heme), as predicted. Since

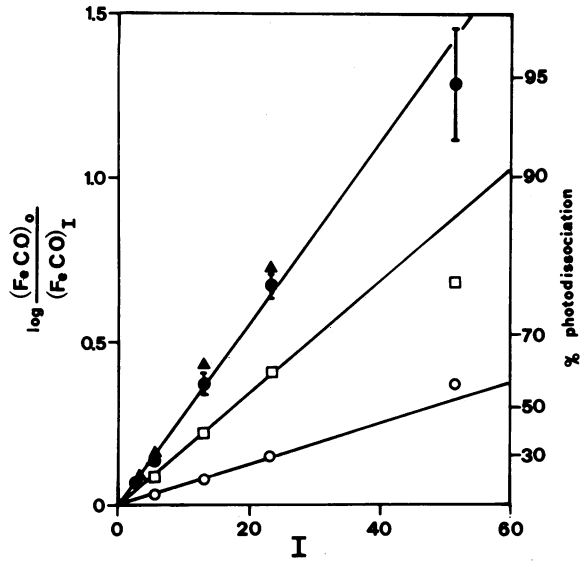

FIG. 1. Photodissociation of the CO derivative of: (●) sperm-whale myoglobin (showing SD); (▲) *Aplysia* myoglobin; (□) horseradish peroxidase; (○) carboxymethylated cytochrome *c*. Dependence on relative light intensity (*I*) of log $(FeCO)_0/(FeCO)_I$, where: $(FeCO)_0$ is the concentration of the CO complex of any one of the heme proteins at I = 0, and $(FeGO)_I$ is the corresponding value at various light intensities. Conditions: pH 7, 0.2 M phosphate buffer, and 20°. Protein concentration is generally around 4 μM (heme) in all cases. (For sperm-whale myoglobin, concentration was varied from 0.75–7.5 μM with identical results.)

in this case the quantum yield is known to be equal to 1 (2, 3), the corresponding value of ω is taken as a reference.

As a further test of the method, we determined the quantum yield for the photodissociation of CO from several other single-chain heme proteins for which independent information is

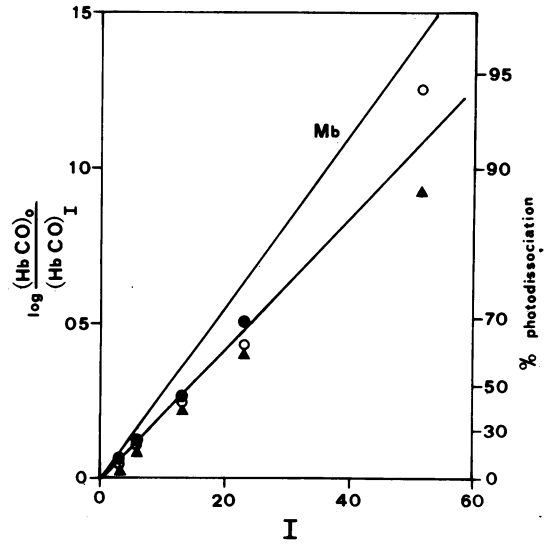

FIG. 2. Dependence of photodissociation of the CO complex of trout hemoglobin I (*HbCO*) on relative light intensity (*I*). Initial saturation with CO corresponds to: (●) = 100%; (○) = 13%; (▲) = about 1.5%. Conditions: pH 7.0, 0.2 M phosphate buffer, and 20°. Hemoglobin concentration 5 μM (heme). CO concentration from 0.075–25 μM. *Mb*, curve for myoglobin.

TABLE 1. *Quantum yield for the photodissociation of various derivatives of sperm-whale myoglobin (Mb), trout hemoglobin (Hb) I. and human hemoglobin A (HbA) (at pH 7, 0.2 M phosphate, 20°)*

Ligand	Mb	Trout Hb I	HbA
CO	1.0	0.75	~0.40*
O_2	0.03*	0.045	0.008*
EIC	0.04	0.20	0.05*
n-PIC†	0.18	0.13	—
i-PIC†	0.08	0.13	—
n-BIC†	0.32	0.20	—

* Data from Noble *et al.* (4) or from Ainsworth and Gibson (6).

† *n-PIC* = *n*-propylisocyanide; *i-PIC* = *i*-propylisocyanide; *n-BIC* = *n*-butylisocyanide.

available. The values of ω = 1.0 for *Aplysia* myoglobin, ω = 0.70 for horseradish peroxidase, and ω = 0.25 for carboxymethylated cytochrome *c* obtained from the data included in Fig. 1 are indeed in good agreement with those obtained by the steady-state method (8, 11), taking into account the fact that the total absorbance over the wave-length range used is the same.

The quantum yield for different ligands may likewise be easily determined by reference to the quantum yield for CO myoglobin, used as a standard, provided possible differences in absorbtion coefficients are taken into account. The results for various ligands, given in Table 1, lead to two conclusions: (*i*) in general, whenever a comparison is possible, the quantum yield for trout hemoglobin I is higher than for human hemoglobin; and (*ii*) there is no simple relationship between the quantum yield for various ligands and their affinity constants in the ground state (see also refs. 5 and 6).

Trout hemoglobin I was examined in special detail. This molecule displays a pH-independent cooperative ligand binding (*n* for O_2 about 2.5), is tetrameric under most of the accessible experimental conditions, and shows no quickly reacting form on complete photodissociation. The last fact, in particular, makes it especially useful for flash photolysis studies, and there seems to be every reason to believe that the same basic phenomena are operative in both human and trout-I hemoglobins (15).

As a final but important feature of the work, we determined the quantum yield for CO photodissociation from trout hemoglobin I as a function of the degree of ligand saturation. As shown by the data given in Fig. 2: (*i*) the logarithm of the percentage breakdown is linear in the relative light intensity; and (*ii*) the slope of the curve is independent of the initial saturation with CO, i.e., the same value of ω is obtained starting from fully saturated hemoglobin down to initial saturation values of about 1.5%.

DISCUSSION

As already mentioned, for a simple one-site molecule like myoglobin it is predicted that all three methods of measuring the quantum yield (steady-state, relaxation, and pulse methods) give the same information and the same results over a very wide range of conditions (9). All relevant experimental results, including those described here, do indeed confirm this expectation as far as they go (8). For an allosteric molecule like hemoglobin, which contains several sites for the ligand

and exists in at least two different conformations, the situation is much more complicated. Here the steady-state method and the pulse method (the one used in the present study) provide different kinds of information, although the two are closely related and complement one another in a powerful way.

The steady-state experiments on hemoglobin, which show that the effect of the light is simply to shift the binding curves ($\bar{X}$ against ln x observed in the dark along the ln x axis, without changing their shape), might be simply explained if the quantum yield of a site in a given conformation is proportional to the dark off constant, l_{off}, corresponding to that conformation (7, 10). However, nothing can be deduced from this shape invariance *alone* regarding the equality or inequality of the values of either ω or l_{off} for the different conformations.

On the other hand, from the fact that the quantum yield is the same when measured at all degrees of saturation (Fig. 2), it follows that it is the same for a site in all the different conformations that it may assume. The argument here is very simple. The binding polynominal of a macromolecule, expressed in its most general form, in terms of Adair constants, is

$$1 + k_1x + k_2x^2 + \ldots k_rx^r \qquad [3]$$

where r denotes the total number of sites for the ligand (16). Consequently, if S denotes the rate at which the ligand molecules are being driven off during the flash, the quantum yield measured by the pulse method is proportional to:

$$\sum_{i=1}^{r} ik_ix^i\bar{S}_i \Big/ \sum_{i=1}^{r} ik_ix^i, \qquad [4]$$

where $\bar{S}_i$ is the value of S averaged over all the sites of the macromolecule when it is in the equilibrium state where i sites are occupied.* The condition that the quantum yield be independent of x and therefore of the degree of saturation, $\bar{X}$, as observed, is clearly that the $\bar{S}$ be all the same. But at the very high light intensities used in the pulse experiments, each S will be proportional to the corresponding quantum yield ω multiplied by the light intensity I. It follows that the quantum yield of any site must be the same for all conformations. In the special case of the Monod–Wyman–Changeux model (17), where only two conformations, T and R, are involved, this means that $\omega_T = \omega_R$.†

Although these pulse experiments by themselves say nothing as to the values of the l_{off}, since the l_{off} do not appreciably affect the values of the S at the very high light intensities used, nevertheless, taken in connection with the shape invariance of the steady-state binding curves in the presence of light (7), they lead to an important conclusion. Thus, if, for example, we adopt the Monod–Wyman–Changeux model, from the invariance property it follows that:

$$(l_{off})_R/\omega_R = (l_{off})_T/\omega_T \qquad [5]$$

and therefore, the fact that $\omega_R = \omega_T$ implies that $(l_{off})_R = (l_{off})_T$.

This simple but significant conclusion seems hard to reconcile with deductions drawn from kinetic experiments according to which a large part of the cooperativity has its origin in the "off" reaction (18, 19). At the present time there seems to be no unique answer to this apparent conflict between two well-established bodies of data; there are, however, two possibilities that should be borne in mind. In the first place it should be realized that the photochemical experiments have so far been limited mainly to CO. On the other hand, the most convincing kinetic experiments have been performed with oxygen. Perhaps, therefore, the trouble arises from the fact that we are trying to relate the opposite sides, not of the same, but of two different pennies. With the availability of better light sources it may be possible before long to extend the photochemical work to oxygen.

Another possible way of explaining the contradiction would be to invoke the "induced fit" version of the allosteric model in which it is assumed that *all* the occupied sites are in the same high-affinity form at every degree of saturation, the number of occupied low-affinity sites being always negligible (20). However, the introduction of this model leads to other problems of its own in connection with the kinetic observations.

We shall not attempt to go into this matter further at this point. Rather we content ourselves with stressing the main fact brought out by the pulse experiments, namely that the quantum yield of a site is the same in all conformations and that it is independent of the presence or absence of the ligand (CO) at other sites, at least in the tetrameric molecule of trout hemoglobin I.

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* Application of this expression for large values of I presupposes that during the brief period of the flash (about 150 μsec) there is no significant conformational change. If there is such a change, the expression is only valid for values of I for which the fractional amount of ligand driven off is very small. However, in the present case this poses no problem, since (Fig. 2) the data fall on a straight line (ω = constant) within experimental error up to the highest values of I used.

† For the Monod–Wyman–Changeux model as applied to the 4-site hemoglobin molecule this becomes

$$\frac{4x(\nu_T k_T s_T + \nu_R k_R s_R) + 12x^2(\nu_T k^2{}_T s_T + \nu_R k^2{}_R s_R) + 12x^3 (\nu_T k^3{}_T s_T + \nu_R k^3{}_R s_R) + 4(\nu_T k^4{}_T s_T + \nu_R k^4{}_R s_R)}{4x(\nu_T k_T + \nu_R k_R) + 12x^2(\nu_T k^2{}_T + \nu_R k^2{}_R) + \ldots}$$

where ν gives the mole fractions of the T and R forms in the absence of ligand.

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