Proc. Nat. Acad. Sci. USA
Vol. 69, No. 4, pp. 868–871, April 1972

Carbon Monoxide Binding by Hemoglobin and Myoglobin under Photodissociating Conditions

(heme proteins/ligand binding/protein photochemistry)

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Contributed by Jeffries Wyman, January 26, 1972

ABSTRACT **Carbon monoxide binding by myoglobin and hemoglobin has been studied under conditions of constant illumination. For hemoglobin, the homotropic heme–heme interaction (cooperativity) and the heterotropic Bohr effect are invariant with light intensity over a 1000-fold change of $c_{1/2}$. The dissociation constant, measured as $c_{1/2}$, increases linearly with light intensity, indicating that photodissociation is a one-quantum process. At sufficiently high illumination the apparent enthalpy of ligand binding becomes positive, although in the absence of light it is known to be negative. This finding indicates that light acts primarily by increasing the "off" constants by an additive factor. The invariance of both cooperativity and Bohr effect raises a perplexing issue. It would appear to demand either that the "off" constants for the various elementary steps are all alike (which is contrary to current ideas) or that the additive factor is in each case proportional to the particular "off" constant to which it is added (a seemingly improbable alternative).**

It is abundantly clear that hemoglobin, along with many enzymes and other working proteins, is an allosteric molecule whose operation depends on ligand-linked conformational changes. Within this general framework, however, many questions remain to be settled. For instance, is only one major conformational change involved in ligand binding or are there several? Does the heterotropic Bohr effect stem from the same conformational change (or changes) as the homotropic cooperativity, or does it perhaps originate as an intrachain effect due to a ligand-linked conformational change within the monomers? From a kinetic point of view, how much of the cooperativity of ligand binding arises from the "off" constants and how much from the "on" constants? Finally, which of the current allosteric models comes nearest to meeting the facts?

It was discovered more than 75 years ago by Haldane and Lorrain-Smith (1) that carbon monoxide hemoglobin is extremely photosensitive, the binding of CO by the protein being significantly reduced even at very moderate illumination. The same effect, though greatly reduced, is observed in the case of oxygen and other ligands. Since its discovery the phenomenon has been studied in much greater detail, primarily in terms of quantum yield, by several investigators (2–4) using both myoglobin and hemoglobin. For myoglobin the quantum yield is constant and close to unity; for hemoglobin it is 0.25–0.70 and changes with pH and other variables. The effect clearly raises important issues regarding energy partition and flow in a macromolecule; apart from this, the photosensitivity may be used to provide an interesting example of the pseudoequilibrium (or steady state) induced in a complex macromolecule by the constant absorption of energy, as distinct from the true, unperturbed equilibrium realized in the absence of light. The study reported in this paper makes use of the phenomenon to provide evidence, of a novel type, bearing on the general question of the behavior of hemoglobin as an allosteric protein.

MATERIALS AND METHODS

Hemoglobin was prepared from whole, fresh human blood by the ammonium sulphate procedure and freed from organic and inorganic ions by passage of the hemoglobin solutions that had been dialyzed against water through a mixed-bed ion-exchange column (5).

Sperm whale myoglobin from Seravac was purified by fractional precipitation with ammonium sulphate. Protein concentrations were determined spectrophotometrically from published extinction coefficients (5).

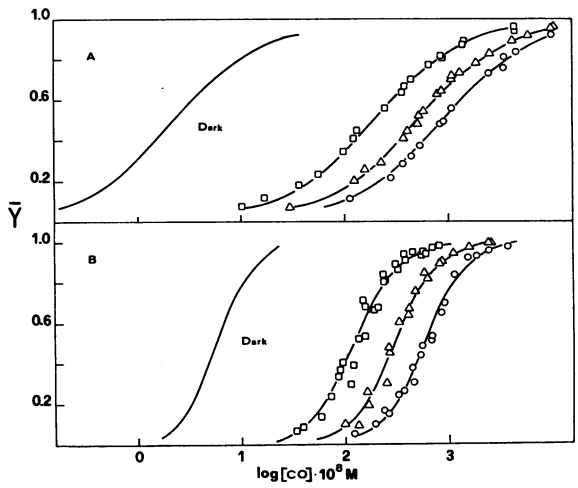

FIG. 1. Carbon monoxide binding curves under photodissociating conditions for sperm whale myoglobin (*A*) and human hemoglobin (*B*) in 0.2 M phosphate (pH 7) at 20°. *Solid lines* correspond to theoretical binding curves with $n = 1$ (sperm whale myoglobin) and $n = 2.2$ (human hemoglobin). *Dark curves* correspond to published (5, 6) binding curves. *Data points* are composites from several experiments with myoglobin and hemoglobin, at concentrations ranging from 1.9 to 4.9 μM and 1.2 to 4.8 μM in heme, respectively. O, full actinic light (relative intensity, 1.0); △, relative intensity, 0.50; □, relative intensity, 0.21.

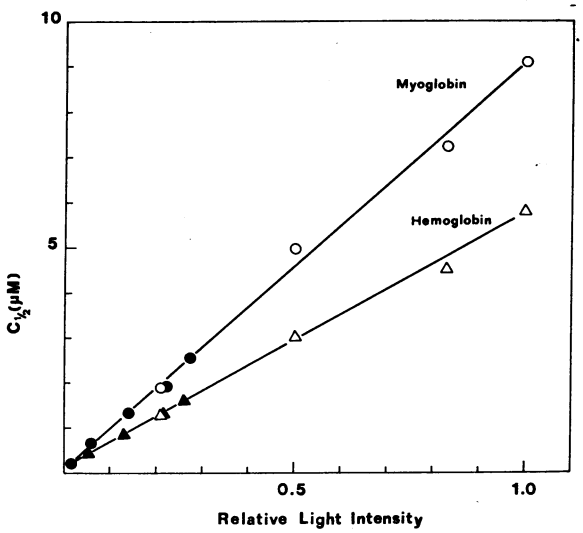

FIG. 2. Dependence of the dissociation constant ($c_{1/2}$) on the relative light intensity for sperm whale myoglobin (*circles*) and human hemoglobin (*triangles*) in 0.2 M phosphate (pH 7) at 20°. *Closed symbols* correspond to experiments in which only a portion of the actinic beam ($\lambda > 490$ nm) was used.

Carbon monoxide binding curves under photodissociating conditions were obtained with a cross-beam illumination apparatus. The shift in the fractional saturation with the ligand induced by shining light from a dc-operated xenon arc (150 W) was measured spectroscopically at 436 nm. By repetition of this same operation for a whole series of solutions containing different CO concentrations, a complete ligand binding curve in the presence of light of various intensities could be determined. The different light intensities were achieved by use of calibrated neutral filters.

The characteristics of the apparatus and the experimental procedure used will be described in greater detail in a forthcoming paper.

RESULTS

Under photodissociating conditions the carbon monoxide binding curve of sperm whale myoglobin maintains its simple shape ($n = 1$), as shown in Fig. 1*A*. The displacement of the curve parallel to itself corresponds to an increase of the apparent dissociation constant nearly 1000-fold from "dark" to full illumination.

Similar results obtained with hemoglobin are shown in Fig. 1*B*. As with myoglobin, the effect of the light is to shift the ligand binding curve to the right, without affecting its shape. A least-squares fit of the results gives a value of the Hill coefficient of 2.2 for hemoglobin* (as compared with 1 for myoglobin). These values of n are independent of the relative light intensity over a 100-fold range.

The dependence of ligand affinity given by $c_{1/2}$ is linear in light intensity for both myoglobin and hemoglobin, as shown by Fig. 2.

The effect of pH on log $c_{1/2}$ is reported in Fig. 3. The results obtained at different light intensities, together with previous

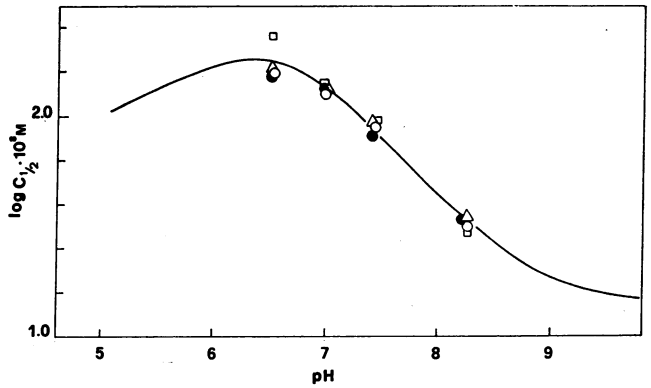

FIG. 3. Bohr effect for binding of CO to human hemoglobin under photodissociating conditions. *Solid line* corresponds to the CO Bohr effect in the "dark," as determined from differential titrations (from ref. 5). Log $c_{1/2}$ values at different light intensities are shifted along the vertical axis in order to normalize them to the value obtained with full actinic illumination. Symbols are for various light intensities from 1.0 (○) to 0.05 (□).

data obtained in the dark by differential titration, show that, within the accuracy of the experiments, the Bohr effect is unaltered under photodissociating conditions.

For both myoglobin and hemoglobin the apparent enthalpy of binding to carbon monoxide determined from a van't Hoff plot of log $c_{1/2}$ against $1/T$, changes from a negative value in the dark to a positive value in the presence of strong light. The results on hemoglobin shown in Fig. 4 correspond to a value of $\Delta H = -11.5 \pm 0.5$ kcal/mol in the dark (5), and a value of $\Delta H \cong 7$ kcal/mol at the highest light intensities used. For myoglobin in the presence of strong light the value of $\Delta H \cong +7$ kcal/mol is very similar to the activation heat for the "on" process, as determined directly from parallel kinetic experiments to be published in the near future.

In the case of hemoglobin, which dissociates at high dilution, experiments were performed at different concentrations from 1.2 to 4.8 μM in heme. No effect of concentration was observed, although at the lowest concentrations the ligand-bound form of hemoglobin is more than half dissociated into dimers.

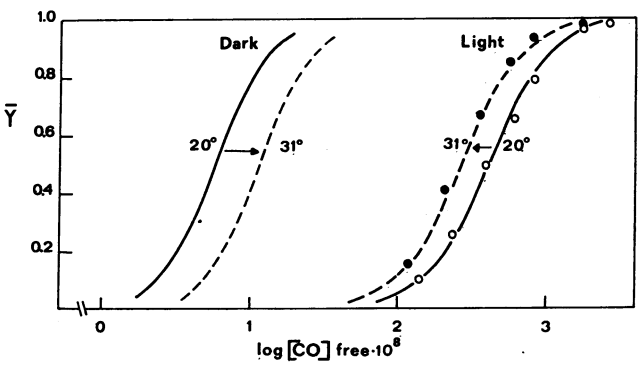

FIG. 4. Temperature dependence of CO binding to human hemoglobin in the dark and under photodissociating conditions at 20° or 31°, as shown. The curves correspond to $n = 2.3$ in the dark and $n = 2.2$ in the light. The shift in the "dark" equilibrium was calculated on the basis of a $\Delta H = -11.5$ kcal/mol (5).

* The value of n for the CO binding curve of hemoglobin at protein concentrations comparable to those used here is 2.3 (6), and not 2.8–3.0 as observed with whole blood (7).

DISCUSSION

The results on myoglobin (Mb) present no problem; indeed, they are just what would be expected for a simple one-site molecule that undergoes no conformational change on ligand binding, has no Bohr effect, and, of course, no cooperativity, and whose reaction with CO under all conditions can be described as a simple one-step process:

$$\mathrm{Mb} + \mathrm{CO} \underset{l}{\overset{l'}{\rightleftharpoons}} \mathrm{MbCO}; \; L = \frac{l'}{l} = \frac{1}{c_{1/2}} \qquad [1]$$

The fact that $c_{1/2}$ *increases* with light intensity is in agreement with interpretations given to previous photochemical experiments, where it has been assumed that the total dissociation in the light is the sum of the dark "off" constant (l) and a photochemical term (ωI) (2). The idea that the light acts only on the "off" constant, which would be expected on any basis, has been confirmed by recent unpublished kinetic experiments showing that there is no effect of light on the "on" constant. It is further substantiated by the present observation that the apparent enthalpy changes from its "dark" negative value to a positive value characteristic of the heat of activation of the "on" process. This result makes it clear that the effect of the light is to increase the dark "off" constant not by a *multiplicative* factor, but by *addition* of a term that represents the amount of photodissociation. In fact, the photodissociation process may be supposed to be essentially independent of temperature; when this term becomes dominant, at high light intensities, the contribution to the observed heat arising from the dark "off" constant becomes negligible. The fact that the increase of $c_{1/2}$ with light intensity is linear means that the amount of photodissociation is itself proportional to light intensity and, hence, is a single-quantum process.

The interpretation of the results on hemoglobin is far less simple. The principal facts may be summarized as follows: (*a*) The shape of the (cooperative) binding curves of CO ($\bar{Y}$ vs. log c) remains unchanged in the presence of light, i.e., the shape of the curve is invariant with light intensity. (*b*) The Bohr effect likewise remains unchanged in the presence of light, i.e., it too is invariant with light intensity. (*c*) Just as in the case of myoglobin, when the light intensity increases, the apparent heat of liganding, as given by the van't Hoff relation

$$\frac{\Delta H}{R} = -\frac{d \ln c_{1/2}}{d(1/T)} \qquad [2]$$

changes from its negative value in the dark reaction to a positive value, which we may identify as the heat of activation of the "on" process and which is similar to that for myoglobin. (*d*) Also, just as in the case of myoglobin, the increase of $c_{1/2}$ light intensity is linear, showing that a one-quantum process is involved. The slope of the line, however, is less than for myoglobin, a finding in accord with the smaller quantum yield observed in earlier experiments (2–4).

It should be pointed out that a really careful study of the shape invariance of the binding curves with light was limited to the pH range from 6.5 to 7.4. Moreover, since the Bohr effect was studied in terms of the variation of log c_m (or, in practice, $c_{1/2}$) with pH, no very exact exploration of the variation of the shape of the curves with pH in the presence of light was attempted outside of this pH range. Nevertheless, as far as they go, and in connection with the known invariance with pH of the curves in the absence of light (5, 8), the results indicate that invariance of shape holds for *all* values of light intensity and pH, and extends over a domain where $c_{1/2}$ changes by a factor of at least 1000.

In any attempt to rationalize these observations, there are several principles that should be kept in mind. The equilibrium of any macromolecule, existing in any number of conformations, with a ligand X can always be expressed in terms of a binding potential Π of the form

$$\Pi = RT \ln (1 + K_1X + \ldots + K_rX^r), \qquad [3]$$

where X is ligand activity, r is the number of binding sites, and K_i is the overall macroscopic equilibrium constant for the reaction of the macromolecule with i molecules of ligand (9). It can be shown that K_i is always an i-order homogeneous function of the individual equilibrium constants for the various elementary binding steps involved in the reaction, and consequently is homogeneous and of i-order both in the "on" velocity constants and in the "off" velocity constants that describe these steps.

When the system is *not* in true thermodynamic equilibrium, but only in a steady state (as under the influence of light in the present experiments), the concept of the binding potential is no longer applicable, and, in general, the binding of ligand cannot be expressed in terms of a simple binding polynomial as given by the expression in parenthesis in Eq. [3] (9, 10).

The only exception to this statement, apart from the obvious case of a one-site molecule existing in only one conformation, is when the disturbing agent (e.g., the light) acts on the system in such a way as to preserve exactly all the microscopic conditions of equilibrium. These conditions are that the product of the equilibrium constants taken around any closed path in the reaction scheme be unity. In the simple case illustrated in the accompanying Scheme **1**, where the small arrows denote the direction in terms of which the constants are defined, this means

$$\begin{array}{ccc} M_1 & \underset{}{\overset{L_1\rightarrow}{\rightleftharpoons}} & M_2 \\ {\scriptstyle K_2}\downarrow\uparrow & & \downarrow\uparrow{\scriptstyle K_1} \\ M_1X & \underset{L_2\rightarrow}{\rightleftharpoons} & M_2X \end{array} \qquad L_1K_1 = L_2K_2$$

(Scheme 1)

If we write each constant as the quotient of a forward and a backward velocity constant, this becomes

$$\frac{l_1'k_1'}{l_1\,k_1} = \frac{l_2'k_2'}{l_2\,k_2}. \qquad [4]$$

We see, therefore, that if, and only if, the disturbing agent (e.g., the light) acts on the "off" constants, e.g., k_1 and k_2, in such a way as to increase them by the same *fractional* amount will the form of the equilibrium condition be maintained. Otherwise the very condition for equilibrium will be destroyed, and although a steady state will always ensue, it will not, as was the true equilibrium, be describable by the simple mass law equation for a one-site molecule. There will be a circulation around the square (see Scheme **1**) and the Hill coefficient (n) will no longer be equal to unity. The same principle holds, of course, for any more complex reaction scheme applicable to the tetrameric hemoglobin molecule (10).

The observed invariance of the binding curve with changes

in light intensity, i.e., the fact that in the presence of the light the equilibrium, or rather the pseudoequilibrium, can be described by the same binding polynomial (except for a scale factor) as in the absence of light demands, therefore, *in so far as it is exact,* that the effect of the light is to increase every "off" constant by the same fractional amount. Since we know from the heat data that light increases the "off" constant by an additive, and not by a multiplicative, factor, this means either that the "off" constants are all alike, or that the additive factor is in each case proportional to the constant to which it is added. The latter alternative, involving such exact compensation, seems improbable, and is inconsistent with the results on ΔH; on the other hand, the former alternative appears to be in conflict with the current idea, based on evidence from various sources, that a difference between the "off" constants accounts, to a greater or lesser extent, for a significant part of the cooperativity† (5, 11, 12).

A possible basis for establishing whether or not more than one "off" constant is involved would be to study the exact form of the curve showing the transition in the heat of liganding from its dark to its light value as a function of light intensity. It can be deduced that if it were indeed true that only one "off" constant was involved, this curve should be a rectangular hyperbola.

The similar and equally striking invariance of the Bohr effect with changes of light intensity would, on the other hand, find a reasonable explanation on the assumption that the Bohr effect is a *local* phenomenon involving an *intrachain* ligand-linked conformational change (provided such a change were essentially complete under all conditions). This interpretation of the Bohr effect as an intrachain allosteric phenomenon is indicated by a number of observations (5), in particular those of Perutz (13) on the structure of hemoglobin crystals. The idea will be further elucidated in connection with later experiments to be published elsewhere.

Although these issues cannot be resolved on the basis of the information now available, it is clear that any ultimate resolution, which obviously calls for further experiments, is bound to give a deeper insight into the whole question of the mechanism by which hemoglobin binds ligands.

It is a pleasure to express our appreciation to Drs. W. Blumberg, Q. H. Gibson, and E. Whitehead for fruitful discussions. A grant from the National Science Foundation to J. W. is gratefully acknowledged. J. B. was supported by American Cancer Society Grant PF-627, C. B. by an EMBO Long Term Fellowship.

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† Among the most convincing of these are the experiments of Gibson, Q. H., personal communication, on mixtures of HbO_2 and HbCO, in which the hemoglobin is fully saturated with ligand. When such a mixture is reacted with dithionite in a stopped-flow apparatus, the deoxygenation is a slow monomolecular process. When, just prior to the reaction, the CO is driven off by a brief flash of light, the initial phase of the reaction is fast, presumably due to the high value of the "off" constant of the forms containing less than four molecules of oxygen.