p-Mercuribenzoate as an Indicator of Conformation Change in Hemoglobin*

QUENTIN H. GIBSON

From the Section of Biochemistry and Molecular Biology, Wing Hall, Cornell University, Ithaca, New York 14850

SUMMARY

When deoxyhemoglobin is mixed simultaneously with carbon monoxide and p-mercuribenzoate, the reaction of the —SH groups at $\beta_\alpha$ with the mercurial lags behind the binding of carbon monoxide to the heme. Analysis of the data shows that intermediate compounds are formed which contain carbon monoxide but retain the low reactivity toward p-mercuribenzoate characteristic of deoxyhemoglobin. It is suggested that the rate of reaction with p-mercuribenzoate depends on the transition of the tetramer from the deoxy to the liganded form. Experiments with n-butyl isocyanide confirm that the rate of reaction with p-mercuribenzoate is not determined solely by the presence of ligand on the $\beta$ chains, as suggested by Antonini and Brunori ((1969) J. Biol. Chem. 244, 3909).

Although the idea that hemoglobin may exist in at least two conformations is an old one, explicitly stated, for example, by Forbes and Roughton (1) and by Haurowitz (2), it is only relatively recently that functional means of visualizing these conformers have become available. Among the first experiments were those of Antonini et al. (3) who showed that bromthymol blue bound more rapidly to deoxy than to oxyhemoglobin, and those of Gibson (4) who followed the absorbance change in the Soret region as the conformation of deoxyhemoglobin changed. Antonini and Brunori (5) then investigated the reaction of p-mercuribenzoate with oxy- and deoxyhemoglobins, finding that the rates differed by a large multiple. They sought to use this difference to follow the appearance of the liganded conformation during binding of carbon monoxide, but found that there was a very close parallel between the $p$-MB	extsuperscript{2} reaction and carbon monoxide binding. This was interpreted to show that the rate of the $p$-MB reaction is fast if the $\beta$ chains are liganded and slow if they are free. The rate was not thought to be influenced by the over-all (quaternary) conformation of the hemoglobin tetramer. In unpublished experiments in this laboratory, H. F. Bunn and Q. H. Gibson, working with the mutant hemoglobin Kempsey found a close correlation between the $p$-MB reaction and the hemoglobin-haptoglobin reaction, which is thought to depend on the conformation of the tetramer. The reaction between $p$-MB and hemoglobin has therefore been reinvestigated with results which suggest that the conclusion of Antonini and Brunori (5) was not justified by their experiments, and that the rate of the $p$-MB reaction is primarily determined by the conformation of the hemoglobin tetramer.

EXPERIMENTAL PROCEDURE

Materials

Hemoglobin was prepared and phosphates removed as described by Olson and Gibson (6). Concentrations were determined as before, with the extinction coefficients of Banerjee et al. (7). Inositol hexaphosphate and $p$-MB were obtained from Sigma. Solutions of $p$-MB were prepared by weighing out the required quantity of the solid, dissolving with the minimum quantity of dilute soda, and at once diluting with buffer. n-Butyl isocyanide was obtained from Aldrich Chemical Co. Solutions were prepared as described by Olson and Gibson (8). Solutions of carbon monoxide were prepared by bubbling with the pure gas (The Matheson Co., East Rutherford, N. J.) and making dilutions with oxygen-free buffers as required.

Methods

The stopped flow apparatus and data collection system were as previously described (9, 10) except that the tungsten lamp was replaced by a deuterium lamp operated from a stabilized power supply.

RESULTS

Many of the experiments of Antonini and Brunori (5) were repeated, and gave results in good agreement with theirs. These results included the rates of reaction of $p$-MB with deoxy- and carboxyhemoglobins, the nonlinearity between $p$-MB concentration and rate of reaction, the amplitude of absorbance change at 255 nm, and the correlation between the progress of carbon monoxide binding as followed at 255 nm and at other wave lengths, specifically at 372 nm in the present instance. As this work was all confirmatory, it is not described further. In experiments in which deoxyhemoglobin was mixed simultaneously with $p$-MB and carbon monoxide, the pH and the concentration of carbon monoxide were varied, thus going beyond Antonini and Brunori (5) who reported results for a single concentration of carbon monoxide and a single pH value.

* This work was supported by Grant GM 14276-08 from the United States Public Health Service.

1 The abbreviation used is: $p$-MB p-mercuribenzoate.
The effect of varying carbon monoxide concentration was examined at pH 6, 7, 7.6, and 9.0. The results for pH 6 are shown in Figs. 1 and 2. The results for all pH values are summarized in Table I. The effect of pH on the rate of the reaction of p-MB with deoxyhemoglobin and on the rate of binding of carbon monoxide were compared, limiting the CO-binding reaction to the first 10% of the binding sites available. The rate so observed gives a rough estimate of $k_1$ (where $k_1$ and $k_2$ are the rate constants for binding of the 1st and 2nd molecules of CO, respectively). These results are shown in Fig. 3. Their interpretation is complicated by the large effect of change in buffer anion at pH 7.6.

Olson and Gibson (6) have shown that n-butyl isocyanide reacts much more rapidly with the $\beta$ chains in deoxyhemoglobin than with the $\alpha$ chains. On the scheme of Antonini and Brunori (5) there should be a correspondingly rapid reaction with p-MB as the $\beta$ chains become liganded. The results of an experiment with n-butyl isocyanide are shown, in part, in Fig. 4. It is clear that the p-MB reaction, far from leading the over-all ligand-binding reaction, shows a marked lag, lacking any representation of the rapid binding of n-butyl isocyanide to the $\beta$ chains.

**DISCUSSION**

The p-MB reaction is conveniently discussed with the aid of Figs. 1 and 2. In each panel of Fig. 1 the progress of CO binding is shown with filled symbols (○) and the progress of the p-MB reaction with empty symbols (●). Since the reaction of p-MB with liganded hemoglobin, although rapid, is not instantaneous, it is first necessary to allow for the lag in the p-MB reaction due to the consecutive reactions of hemoglobin with CO and p-MB. The simplest method is to treat hemoglobin as though it were myoglobin, combining with carbon monoxide in a single second order reaction. It is assumed that p-MB reacts with deoxyhemoglobin and carboxyhemoglobin at the experimentally determined rates. The drawback to this procedure is that combination of carbon monoxide with hemoglobin is not second order, and the calculated curves do not fit the observed points very closely. Nonetheless, the results give a clear indication that the lag in p-MB binding as compared with CO binding is greater than can be accounted for by the trivial explanation that consecutive reactions are involved. This is true even for the most unfavorable case examined, with 230 $\mu$M CO, as illustrated in Fig. 1A. A more satisfactory picture of the lag can be obtained by replacing the simple second order reaction used to represent the reaction of carbon monoxide with hemoglobin with the consecutive reactions in the upper line of Fig. 5, giving the result shown in Fig. 1B. Computations carried out for lower concentrations of carbon monoxide show, as would be expected, that the progress curve for the p-MB-binding reaction comes closer to that for carbon monoxide binding, and because of the contribution from the p-MB-deoxyhemoglobin reaction, crosses over it at a point which moves to an earlier stage in the reaction as the concentration of carbon monoxide is reduced. Experimentally, a point is reached at which there is little difference between the time course of the two reactions (Fig. 1C), although

**FIG. 1.** Reaction of deoxyhemoglobin (46 $\mu$M, heme basis) with 0.1 mM p-MB and carbon monoxide, 0.05 M phosphate buffer, pH 6.0, 25°C, 255 nm, 4-mm light path. ○, course of the p-MB reaction; ●, course of the reaction with carbon monoxide. Results normalized to make the absorbance excursion with carbon monoxide equal to that due to the p-MB reaction. The results for the p-MB reaction have been corrected for the contribution due to CO binding at 255 nm. Panel A, 230 $\mu$M CO, lines calculated to show lag to be expected due to consecutive reactions with a 1-unit scheme. Panel B, as Panel A but lines calculated with the scheme of Fig. 5 but assuming all liganded sites to react at the rate given by $k_2$, all unliganded sites at the rate given by $k_1$. Panel C, CO concentration 57.5 $\mu$M, observed results, lines drawn through points. Panel D, CO concentration 57.5 $\mu$M, calculated time course for 1-unit scheme, continuous line, CO binding, dashed line, p-MB reaction.

**FIG. 2.** Fitting observed time course of absorbance changes at 255 nm to scheme of Fig. 5. Conditions as for Fig. 1. Panel A, CO = 230 $\mu$M; Panel B, CO = 115 $\mu$M; Panel C, CO = 57.5 $\mu$M; Panel D, CO = 29 $\mu$M. The points are observed, the lines calculated. Ordinates give the absorbance excursion directly as measured, the upper line in each panel is for the reaction with CO and p-MB, the lower line for CO alone.
The reaction was followed with light of 255 nm using a path of 4 mm at 20°. Data were collected with carbon monoxide concentrations of 230, 115, 57.5, and 29 μM after mixing, in the presence and in the absence of 0.1 mM p-MB. The data were fitted to the scheme of Fig. 5: values for k₁ and k₃ were obtained in separate experiments with deoxyhemoglobin without carbon monoxide, and carbon monoxide hemoglobin. The hemoglobin concentration was 50 μM on a heme basis, varying slightly in the different experiments. The rate constants are all intrinsic constants in units of μm⁻¹ s⁻¹, ψ was set at 6.0.

### Table I

<table>
<thead>
<tr>
<th>Buffer</th>
<th>k₁ ± k₂</th>
<th>k₃ ± k₄</th>
<th>k₅ ± k₆</th>
<th>k₇ ± k₈</th>
<th>k₉ ± k₁₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>KP, 6.0</td>
<td>0.10 ± 0.01</td>
<td>0.55 ± 0.13</td>
<td>0.13 ± 0.01</td>
<td>0.01</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>KP, 7.0</td>
<td>0.11 ± 0.01</td>
<td>1.00 ± 0.45</td>
<td>0.11 ± 0.01</td>
<td>0.01</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>KP, 7.5</td>
<td>0.11 ± 0.01</td>
<td>0.57 ± 0.17</td>
<td>0.10 ± 0.02</td>
<td>0.01</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>Tris 7.6</td>
<td>0.29 ± 0.01</td>
<td>1.08 ± 0.83</td>
<td>0.20 ± 0.01</td>
<td>0.01</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>Borate 9.0</td>
<td>0.35 ± 0.11</td>
<td>1.05 ± 1.01</td>
<td>0.23 ± 0.01</td>
<td>0.23</td>
<td>0.32 ± 0.05</td>
</tr>
</tbody>
</table>

**Fig. 3.** The effect of pH on the rate of the reaction of deoxyhemoglobin with CO and with p-MB. Δ, CO reaction; O, p-MB reaction. The hemoglobin concentration was 50 μM; p-MB concentration 50 μM; CO concentration, 5 μM. The left ordinate gives the intrinsic rate constant for the p-MB reaction (Δ and ○), μm⁻¹ s⁻¹, the right ordinate gives that for the CO reaction in the same units (Δ and ○). Buffers were 0.05 M phosphate from pH 6 to 7.6, ○ and Δ; borate from pH 7.4 to 9.0, ○ and Δ.

Some lag in the p-MB reaction remains as compared with the calculated course using the 1-unit model (Fig. 1D). The results of Fig. 1, C and D, provide an explanation of the results of Antonini and Brunori (5) who used only one concentration of carbon monoxide, 50 μM, or about that used in the experiment of Panel C. Under these conditions the time courses of ligand binding and of the p-MB reaction are indeed roughly similar, and without the clues provided by experiments performed over a wider range of conditions the difference between CO binding and the p-MB reaction would readily be missed, especially without detailed analysis of the expected time courses of the two reactions.

Since the lag in the p-MB reaction is clearly greater than can be accounted for by simple consecutive reactions it seems necessary to suppose that intermediates are present which contain some carbon monoxide but which do not have the high reactivity associated with the liganded form. The results can be well represented by an extension of the Adair (11) scheme in which each intermediate exists in two states, free from, and reacted with p-MB. This scheme, shown in Fig. 5 is an oversimplification neglecting αβ differences and possible kinetic differences.

**Fig. 5.** Scheme for reaction of hemoglobin with CO and p-MB.

1 R. Gray and Q. H. Gibson (12) were unable to demonstrate αβ chain differences in the reaction of deoxyhemoglobin with carbon monoxide at high pH, or in buffers of low ionic strength around neutrality. Differences were seen in phosphate buffers, and these
between species having 1 and 2 molecules of p-MB. Measurements at wave lengths longer than those at which p-MB absorbs measurably have failed to show any differences when the reaction deoxyhemoglobin + CO was compared with the reaction deoxyhemoglobin + (CO and p-MB). It may be assumed therefore that the rate constants $k'$ and $p$ ($p'\alpha$) (Fig. 5) must be similar, at least for small values of $n$, or that the rates described by $k_o$ for small values of $n$ are small compared with the rates of carbon monoxide binding to the same intermediates. The first assumption has been adopted in describing the experimental results obtained under various conditions. In using the scheme of Fig. 5 the rate constants $k$, $k_o$, and $k_2$ were fixed at their known and observed values, and the additional assumption made that p-MB reacts at the same rate with Hb$_4$(CO)$_2$ and Hb$_4$(CO)$_4$, so fixing $k_2 = k_4$. The values of $l'$, $l'_2$, $l'_3$, $k_o$, and $k_2$ were allowed to vary freely, using as initial values for the CO-binding rates results drawn from the experience of MacQuarrie and Gibson (14) in fitting similar curves. The data were fitted directly as observed at 255 nm during the reaction of carbon monoxide with and without p-MB, considering four different CO concentrations simultaneously. Good solutions (Fig. 2) were obtained with the values of the rate constants given in Table I. The standard errors given there have comparative rather than absolute value because fixed values were assigned to $k_1$ and $k_2$ although these rates are subject to experimental error. Examination of Table I shows that in every case except that of borate at pH 9 the rate of reaction of p-MB with Hb$_4$(CO) was given by a small (but poorly defined) number, while the rate of reaction with Hb$_4$(CO)$_2$ ($k_o$) was intermediate between the rates of reaction of deoxy- and liganded hemoglobins and was well defined. This contrasts with the behavior of $l'_2$ and $l'_3$, the corresponding rates for CO binding. Here $l'_2$ was larger and $l'_3$ smaller; as already noted by MacQuarrie and Gibson (14), $l'_4$ does not show much variation with pH. These results suggest a close parallel between the binding of 8-hydroxy-1,3,6-pyrenetrisulfonate to hemoglobin intermediates and their reaction with p-MB, MacQuarrie and Gibson having found Hb$_4$CO and Hb$_4$ similar in affinity, and Hb$_4$(CO)$_2$ intermediate between deoxy- and liganded hemoglobins. It seems reasonable to suggest that the changes in p-MB reactivity and in pyrenetrisulfonate binding have a common origin in the change of hemoglobin conformation occurring after several molecules of CO have been bound. The results are incompatible with a simple Monod-Wyman-Changeux two-state model because there is no parallel between the rate of CO binding and the rate of p-MB binding, i.e. between $k_o$ and $l'_2$.

These conclusions, drawn from quantitative examination of the reaction with carbon monoxide, are supported qualitatively by the results with n-butyl isocyanide as ligand. As shown in Fig. 3 there is no rapid reaction with p-MB corresponding to the rapid binding of n-butyl isocyanide to the $\beta$ chains. The scheme recently advanced by Olson and Gibson (6) to describe the hemoglobin n butyl isocyanido reaction suggests that the intermediates most populated are successively, $\beta, \beta, \beta, \beta, \alpha, \beta, \beta, \alpha, \alpha$, where the letters identify the chains to which ligand is bound. The main observable rate-determining steps are associated with the formation of $\beta$ and $\beta, \beta$, which contribute the fast phase of the reaction, and of $\beta, \beta, \alpha$, which is formed relatively slowly. The main conformation change is believed to occur as $\beta, \beta, \alpha$ is formed and so should occur rather slowly. The behavior illustrated in Fig. 3 is in good agreement with this scheme if it is assumed that the rate of reaction with p-MB does not increase much until the conformation change occurs. As shown in Fig. 3B, the p-MB reaction, which at first lags substantially behind the ligand reaction, later overtakes it, due to reaction of p-MB with unliganded and partially liganded hemoglobin. Numerical analysis would require that 20 species be taken into account in a scheme analogous to that of Fig. 5, and the reaction with n-butyl isocyanide was not, therefore, pursued further, especially since such a scheme would itself be incomplete.

Taken together, the results with carbon monoxide and n-butyl isocyanide seem to show that the p-MB reaction does not depend on the presence of ligand on individual chains, but on the conformation of the molecule as a whole. The contrary conclusion of Antonini and Brunori (5), although founded on experiments which can be reproduced, derived from study of an insufficient range of experimental data.

Although not a principal object of this paper, the effect of pH on the p-MB reaction deserves brief comment. The effect is much larger on the reaction with deoxyhemoglobin than on that with carboxyhemoglobin, 10 times increase as against 2 times, on going from pH 6 to 9 (with a change of buffers). When the effect of pH on $l'_4$, the rate of binding CO, was examined there was a large effect of changing buffers from phosphate to either borate or Tris at pH 7.6. With a series of borate buffers, $l'_4$ was pH independent from pH 7.6 to 0.0, and was substantially greater than in phosphate pH 7.6. With p-MB and deoxyhemoglobin there was no sharp break on changing buffers, the rates increasing gradually from pH 7.2 onward. The lack of correlation between the effects of pH and buffer on $l'_4$ and on the deoxyhemoglobin-p-MB reaction argues qualitatively against a model in which the effects of pH and buffer on $l'_4$ and on the deoxyhemoglobin-p-MB reaction are represented as disturbing an equilibrium between two forms of the protein, as in the Monod-Wyman-Changeux model, since such a model would require precise correlation between the rate of the p-MB reaction and $l'_4$. The effect is probably not to be explained by an effect of pH on p-MB itself, since the p-MB reaction with liganded hemoglobin was little affected.

REFERENCES

11. Aida, G. S. (1925) J. Biol. Chem. 65, 529
p-Mercrubenzoate as an Indicator of Conformation Change in Hemoglobin
Quentin H. Gibson


Access the most updated version of this article at http://www.jbc.org/content/248/4/1281

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/248/4/1281.full.html#ref-list-1