Communications

Use of a Fluorescent Analogue of 2,3-Diphosphoglycerate as a Probe of Human Hemoglobin Conformation during Carbon Monoxide Binding*

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SUMMARY

The interaction of human hemoglobin (Hb) with 8-hydroxy-1,3,6-pyrenesulfonate (HPT) was studied at pH 6.0 and ionic strength 0.1, and it was found that the fluorescence of HPT is quenched upon interaction with Hb. HPT binds to deoxy-Hb with a stoichiometry of 1 mole per mole of Hb tetramer and with a higher affinity for the deoxy than the liganded form analogous to the binding of the physiologically important 2,3-diphosphoglycerate (DPG). Binding is prevented by either DPG or inositol hexaphosphate. A qualitative comparison of the time courses of HPT release and CO combination during the reaction of CO with deoxy-Hb indicates a marked lag in the release of HPT compared with CO binding. This led to the conclusion that during the course of CO binding, significant amounts of partially liganded Hb intermediates are formed and the release of HPT from the intermediates does not occur in proportion to the amount of CO bound. Quantitative analysis of the data, in the form of least squares fitting procedures, was carried out to examine the ability of some elementary models to describe the results. The best fit was obtained on the assumption that HPT is released after 3 CO molecules have bound to hemoglobin.

Although the crystal structures of the initial and final states of the hemoglobin molecule are now known, we still know very little about intermediates which may be formed during the course of ligand binding to deoxy-Hb. Numerous experiments have been performed in the attempt to examine their properties, but for the most part such experiments have been without success, and indeed until recently even the existence of intermediates was purely speculative.

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In 1956 Gibson (1) observed that if the CO was partially removed from HbCO by flash photolysis, the recombination of CO with the hemoglobin was rapid in comparison with the rate of combination of CO with deoxy-Hb as observed by either complete photodissociation or by rapid mixing experiments. In terms of the classical Adair formulation (2) (see Equation 1), this

\[
\text{Hb}_4(\text{CO})_{n-1} + \text{CO} \rightleftharpoons \text{Hb}_4(\text{CO})_n \quad n = 1 \text{to} 4
\]

result was taken to indicate that the rate of combination of the fourth ligand (\(l^4\)) was much larger than the rate of combination with the first (\(l^1\)). Presumably HbX4 has the quaternary conformation of fully liganded Hb, characterized by a high combination velocity with ligand and by high affinity and uncooperative binding. Muirhead and Greer (3), Bolton and Perutz (4), and Perutz (5) have determined the structural features of the molecule which are correlated with this transition from the slowly reacting deoxy conformation to the rapidly reacting liganded conformation of hemoglobin. Perutz (6) has also suggested an attractive and plausible mechanism for ligand binding. In his tentative scheme, the tertiary structure of the ligand-bound subunits changes from a deoxy to an oxy conformation as each molecule of ligand combines. After 2 ligand molecules have combined with the tetrameric protein, the quaternary transition from the deoxy to oxy-like form occurs, at which time bound organic phosphate is released and there is an increase in affinity of the 2 subunits which are still ligand-free. The relation between ligand binding and release of phosphate is thus of central significance in current theories of hemoglobin ligand interaction. Unfortunately, the optical properties of 2,3-diphosphoglycerate are unsuitable for following the step of phosphate release.

In this communication, we report on the use of a fluorescent anion, 8-hydroxy-1,3,6-pyrenesulfonate, which binds to deoxy-Hb competitively with DPG, and because its fluorescence is quenched upon binding to hemoglobin, its release during the course of CO binding may readily be observed.

Hemoglobin and carbon monoxide solutions were prepared as described previously (7) and the hemoglobin was freed of bound phosphates before use (8). The stopped flow and data collection systems have been described (9, 10). Fluorescence titrations were performed using a Baird Atomic model SF-1 fluorescence spectrophotometer. HEP, DPG, bis-tris, and HPT were obtained from Sigma, Calbiochem, Aldrich, and Eastman, respectively. All experiments were performed using 0.05 M bis-tris chloride, pH 6.0, as a buffer.

The stoichiometry of binding and affinity of HPT for deoxy-Hb were determined by titration of deoxy-Hb with HPT and measuring the fluorescence quenching. For the calculations, we assumed on the basis of the large (42 A) quenching radii of the hemes (11) that HPT was completely quenched when bound. Fig. 1 illustrates data from a typical titration replotted in a double reciprocal manner. The intercept on the ordinate indicates a maximum stoichiometry of 1.1 moles of HPT bound per mole of deoxy-Hb tetramer. The dissociation constant of the deoxy-Hb-HPT complex is 8 \(\mu M\). Accurate titration of liganded hemoglobin was technically more difficult because HPT has a lower affinity for HbCO than for deoxy-Hb. From the amount
of fluorescence in the presence and absence of IHP, the dissociation constants were also determined during the course of stopped flow experiments and values of approximately 7 and 19 μM were determined for dissociation from deoxy-Hb and HbCO, respectively. In the presence of either 100 μM IHP or 200 μM DPG, no fluorescence quenching of HPT was observed. These results strongly suggest that HPT interacts with hemoglobin in a manner qualitatively similar to that of the physiologically important phosphates which also show a stoichiometry of 1 mole bound per mole of deoxy-Hb tetramer and which show a lower affinity for liganded Hb than for deoxy-Hb (12). Also, like DPG and IHP, HPT lowers the oxygen affinity of hemoglobin but to a lesser extent; 0.2 mM HPT caused a 4-mm increase in the O2 pressure required for half-saturation, whereas DPG at 0.2 mM caused a 10-mm increase at pH 6.

To measure the time course of HPT release from liganded Hb during the course of CO binding, a solution of deoxy-Hb containing a small amount of HPT was mixed with a solution of CO. Changes in both the absorbance, due to CO combination, and the fluorescence, due to HPT release, were measured. Consideration of the optical properties of the system suggested suitable wave lengths for excitation and for observation of fluorescence. Possible optical artifacts due to absorbance changes were checked for by repeating each experiment in the presence of IHP. This prevents HPT binding to hemoglobin but does not alter the amplitude of the absorbance changes.

Fig. 2A shows typical fluorescence and absorbance time courses. Clearly, the increase in fluorescence greatly lags behind the absorbance change. Neither process was limited by a first order reaction since increasing the CO concentration resulted in an increased rate of fluorescence and absorbance change. Fig. 2B shows the data replotted as the percentage of HPT released versus the percentage of saturation with CO. It is obvious that the release of HPT from the intermediates (partially liganded hemoglobin) does not occur in proportion to the amount of CO bound. An important, model-independent, qualitative conclusion follows: during the course of CO binding, stoichiometrically significant amounts of intermediates are formed. A similar phenomenon was observed by Gibson and Parkhurst (7) in a study of the reaction of CO with hemoglobin using a combination of stopped flow and flash photolysis. They observed a lag in the occurrence of rapidly reacting material.

A more complete formulation of the course of CO binding was obtained by quantitative analysis of the data. We have examined three simple models for the Hb ligand-binding reaction and tested their ability to fit both the observed optical and fluorescence changes. At the outset it should be noted that only those
Adair scheme in which phosphate is released after the 3rd molecule of ligand binds, as proposed by Forbes and Roughton (13) would correspond to an intrinsically equivalent condition. Fortunately, this latter condition is satisfied by the liganded conformation of Hb, which reacts rapidly under the conditions of the experiment. If we assume that for phosphates, the equation for the Adair II model, $l_1' = 0.337$, $l_2' = 0.283$, $l_3' = 1.4$; $l_4'$ was set at 6.0 $\mu M$; for the MWC model, $L = 1083$, $c = 0.069$. $l'$ (T state) was set at 6.0 $\mu M$ sec$^{-1}$. Since for these latter two models the residuals are nonrandomly distributed, it is not possible to calculate meaningful values for the standard deviations of the parameters.

models based on the tetramer as the functional unit are easily reconciled with the results since only 1 mole of HPT is bound per tetramer. Thus models based on the dimer are inappropriate. That is to say, the dihedral symmetry, implicitly assumed for the structural unit in such models, is absent in phosphate-bound hemoglobin.

For simplicity, we have made the assumption that hemoglobin exists in only two forms, one with a high affinity (deoxy form) and one with a lower affinity (liganded form) for phosphates or HPT. A more exact description of the binding reactions will only be possible when the affinity of each of the intermediates for phosphates is known. The first model we shall consider quantitatively is the four-step Adair scheme of Equation 1. A structural formulation of this mathematical scheme is one in which the CO binds sequentially to the four hemes, all of which are intrinsically equivalent. Fortunately, this latter condition is fulfilled since the $\alpha$ and $\beta$ chains react with CO at the same rate under the conditions of the experiment. If we assume that the liganded conformation of Hb is one that reacts rapidly with CO and which binds phosphates with low affinity, then the proposal of Forbes and Roughton (13) would correspond to an Adair scheme in which phosphate is released after the 3rd molecule of ligand is bound. We have designated this the Roughton model. The absorbance and fluorescence data (each given equal weight) were fitted simultaneously to such a model by a standard least squares procedure in which $l'$, in accord with previous conclusions (14), was held constant at about 20 times the observed rate. The fitting is little dependent upon the value of $l_4'$ under these conditions (14). The best fitting values of $l_1'$, $l_2'$, and $l_3'$ were thus determined and used to generate the curves in Fig. 2. Obviously, the model describes the data very well: the average residual (absolute value of the observed minus calculated absorbance) was less than 1% of the signal change. Two other models were considered. The first, suggested recently by Perutz (6) and described above, mathematically reduces to the Adair scheme (Equation 1) with the quaternary transition occurring after the 2nd ligand molecule binds; that is, HPT is released after 2 CO molecules bind. Perutz has not proposed it as a unique representation of the events accompanying binding, and he has indicated that the transition may occur at other points in the binding sequence depending on factors such as pH and the presence of organic phosphates. We have designated this the Adair II model and fitted the data to it in the same manner as in the Roughton model except with the constraint that after 2 CO molecules bind, HPT is released.

The other model considered is a kinetic analogue of that proposed by Monod, Wyman, and Changeux (15) (MWC model). This was fitted by varying $l$ (the equilibrium constant between the $T$ and $R$ forms of the protein), $c$ (the ratio of affinities of the two forms for CO), and the rate constant for reaction of CO with the $T$ form. The rate constant for reaction with the $R$ form was fixed as above for $l_4'$. Thus, all three models were fitted to the data by varying three constants. The residuals are shown in Fig. 3. Both the Adair II and MWC models give significantly poorer fit to the data than the Roughton model and, moreover, the residuals are nonrandomly distributed.

We have also compared CO binding and HPT release using the following modified hemoglobins: PMB-Hb, NEM-Hb, HB Chesapeake, HB Hiroshima, and des-His HB (native HB lacking the C-terminal histidines of the $\beta$ chains). All of these hemoglobins show kinetic behavior different from native HB and in most cases the lag in HPT release is decreased.

In this study HPT has been employed as both an analogue of the physiologically important phosphates and as a probe of the conformational state of the hemoglobin. The HPT was used at low concentrations relative to the hemoglobin in order to maximize the fluorescence change during CO binding as well as not to perturb the functional properties of the hemoglobin. Control experiments showed that the CO binding rate was virtually uninfluenced by the presence of HPT even at high concentrations. Thus, we conclude that the results obtained are applicable to phosphate-free hemoglobin and that under these conditions, and of the three models considered, only that proposed by Forbes and Roughton satisfactorily accounts for the kinetics.

REFERENCES


If the rate of interconversion of the $R$ and $T$ forms is rapid in comparison with the rate of ligand binding, the MWC model reduces to a special case of the Adair scheme in which the four successive rate constants increase monotonically. However, the observed time course of CO binding is best fitted by a combination of rate constants which do not increase monotonically, and it is for this reason that a systematic misfit is found for the basic MWC model. As a further result, the values of $L$ and $c$ are poorly determined in the fitting procedure so that many combinations of $L$ and $c$ values other than those used (Fig. 3) give similarly unsatisfactory fits.
Low Molecular Weight Deoxyribonucleic Acid Polymerase in Mammalian Cells*

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SUMMARY

Extracts of mammalian cells contain a 3.3 S deoxyribonucleotidyltransferase activity in addition to the 6 to 8 S DNA polymerase. The 3.3 S enzyme extracted from rabbit bone marrow uses template information for nucleotide selection and is therefore a DNA polymerase and not a terminal deoxynucleotidyltransferase. The 3.3 S enzyme also differs from the 6 to 8 S DNA polymerase in its behavior on phosphocellulose and its optimum activity at alkaline pH.

DNA polymerase is the familiar name for a class of deoxyribonucleotide-polymerizing enzymes that require template information for nucleotide selection. A survey of mammalian tissue extracts (1) showed that most enzymes of this kind sediment at about 6 to 8 S on sucrose gradients, corresponding to a molecular weight of around 100,000. Some other deoxynucleotide-polymerizing enzymes have been shown to have appreciably lower molecular weight, notably the terminal deoxynucleotidyltransferase from calf thymus gland having a molecular weight of 32,460 (2), but this particular enzyme does not use template information for nucleotide selection and seems to be unique to thymus (1).

This communication shows that a low molecular weight deoxynucleotide-polymerizing activity is present in tissues that do not contain terminal transferase. Studies on partially purified material from rabbit bone marrow with several templates show that the enzyme uses template information for nucleotide selection and that it should, therefore, be classified as a DNA polymerase.

EXPERIMENTAL PROCEDURE

Rabbit tissues collected from New Zealand rabbits and stored at -20° or purchased (frozen) from Pel-Freeze Biologicals, Inc., Rogers, Arkansas, were used as the source of enzyme. From samples were thawed and extracted with 2 volumes of Buffer A (40 mM KPi, pH 7.4, 40 mM NaCl, and 1 mM mercaptoethanol) by homogenization in a glass-Teflon pestle homogenizer. The homogenate was centrifuged for 10 min at 7,000 × g followed by 1 hour at 105,000 × g and the supernatant fraction was designated "soluble fraction." The 7,000 × g pellet was dispersed in 8 volumes of Buffer A with a glass-Teflon pestle homogenizer, adjusted to 1 M NaCl, and allowed to extract for 6 hours. The nuclear suspension was then centrifuged at 105,000 × g for 2 hours. The nuclear supernatant fraction was dialyzed against Buffer B (0.1 M KPi, pH 7.5, and 1 mM mercaptoethanol) and was designated "nuclear fraction." When concentrated fractions were desired for sucrose gradients or gel filtration, the soluble and nuclear fractions were salted out at 70% saturation with (NH₄)₂SO₄ and dialyzed against Buffer B. Sedimentation values were computed from the relative position of activity in the sucrose gradient (3).

Activated DNA (prepared by limited digestion of calf thymus DNA with crystalline pancreatic DNAse) was prepared as described by Aposhian and Kornberg (4). Homopolymer templates, oligodeoxyribonucleotides (5), and dNTPs (6) were synthesized in this laboratory. The use of oligodeoxynucleotide-initiated homopolymer templates for DNA polymerase has been described in earlier work (7, 8). Radioactive dNTPs were purchased from Schwartz-Mann or Amersham-Searle. All assays were worked up on glass fiber discs essentially as previously described (9). The improvement in H⁺-efficiency on glass fiber discs has been noted by Furlong (10).

RESULTS

Sucrose gradient analysis of soluble and nuclear extracts of rabbit spleen are shown in Fig. 1. This sample shows the high molecular weight DNA polymerase at 6 to 8 S and a small amount of activity at 3.3 S (tubes 4 to 6) corresponding to a molecular weight of approximately 30 to 40,000. The 3.3 S activity is clearly shown by its response to dC₃.dG₃ template. A number of other tissues have a pattern similar to Fig. 1, but we continued this set of experiments with bone marrow because it seems to have a lower level of 6 to 8 S DNA polymerase. To demonstrate that the low sedimentation rate is not due to association with low density material, nuclear and soluble extracts of rabbit bone marrow were subjected to gel filtration on Sephadex G-100. The results in Fig. 2 show that both extracts contain the low molecular weight activity. Repeating the low molecular weight nuclear fraction from G-100 on sucrose gradients again shows only one activity maximum at 3.3 S. The tentative identity of the nuclear and soluble activities was examined further by fractionation on phosphocellulose. Fig. 3 shows that both nuclear and soluble extracts contain this DNA polymerase activity (eluting in Fractions 90 to 100) quite separate from the usual DNA polymerase (eluting in Fractions 60 to 80).

The reader is advised that the authors realize that the terms soluble and nuclear are not meaningful terms in the sense of cell fractionation studies when salt extraction is used. For this reason, and for reasons concerning the rates of utilization of different templates, the quantitative relationships between the two polymerases in the extracts used in Figs. 1 to 3 are not relevant to the argument in the experimental part of this paper. The experimental argument concerns only the existence of a low molecular weight DNA polymerase activity. Preliminary results on cell fractionation studies are noted under "Discussion."
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