Partial Restoration of Normal Functional Properties in Carboxypeptidase A–Digested Hemoglobin  
(heme proteins/ligand binding/inositol hexaphosphate/enzyme modifications)

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ABSTRACT In the absence of organic phosphates human hemoglobin A digested with carboxypeptidase A (des His, Tyr β) has high ligand affinity, a greatly reduced Bohr effect, and no heme–heme interaction. Under these conditions, it shows the simple, homogeneous ligand-binding kinetics characteristic of noncooperative heme proteins in which the high combination velocity for both O₂ and CO accounts, to a large extent, for the increased affinity for both these ligands.

Addition of inositol hexaphosphate dramatically alters the functional properties of this digested hemoglobin. The Bohr effect is greatly increased, and at neutral pH the protein shows significant, though still reduced, heme–heme interaction, together with a 5-fold decrease in affinity. In the presence of saturating amounts of the organic phosphate, the value of n is pH dependent, dropping from 1.9 at pH 5.8 to 1.3 at pH 8.6. After inositol hexaphosphate addition, the combination of the deoxy form of the digested hemoglobin with CO is 10-times slower than that observed in the absence of the inorganic phosphate; also the combination with CO after flash photolysis is biphasic and is similar, in many respects, to that observed for unmodified hemoglobin. Besides these functional changes, addition of inositol hexaphosphate to the modified deoxy-hemoglobin results in an increase in the extinction coefficient at 430 nm similar to that observed on mixing the isolated α and β chains of normal hemoglobin. The results are consistent with the idea that inositol hexaphosphate shifts an equilibrium between high- and low-affinity forms of the protein.

Understanding regulatory phenomena in biological systems is one of the fundamental objectives of molecular biology. In the past few years it has become clear that control of enzymatic activity is often accomplished by means of conformational changes, and several allosteric models have been proposed to explain the role of such changes in determining the functional behavior of proteins. The results presented here are relevant to the problem of how conformational changes in hemoglobin, a prototype of an allosteric protein, can affect its functional properties.

Digestion of human hemoglobin A by carboxypeptidase A removes the C-terminal residues of the β chains (histidine 146 and tyrosine 145), with dramatic effects on the functional and structural properties of the molecule (1). The resulting hemoglobin, HbCPA, has a markedly reduced oxygen Bohr effect and the heme–heme interactions are abolished (1). The large increase in ligand affinity, which makes HbCPA similar to the isolated chains, is independent of the nature of the ligand, the partition coefficient between oxygen and other ligands (e.g., CO) being similar to that of normal hemoglobin (2, 3). Digestion with carboxypeptidase A also produces large changes in the redox equilibrium, the oxidation Bohr effect being reduced in the lower pH range, and heme–heme interaction being absent (4). Changes in far-ultraviolet optical rotatory dispersion (ORD) accompany ligand binding (5), but crystals of HbCPA in the oxygenated and deoxygenated states are isomorphous (M. F. Perutz and L. Mazzarella, personal communication). The modified hemoglobin, moreover, shows no changes in reactivity toward bromthymol blue upon ligand binding (6). On the basis of these properties, HbCPA is considered as a type case of the close correlation between conformational changes and cooperativity of ligand binding.

The present investigation was undertaken with the object of studying, in greater detail, the equilibrium and kinetics of ligand binding by HbCPA. In the course of the work, a dramatic and unexpected effect of IP₆ on the properties of the protein was discovered. Several of the characteristics of normal hemoglobin, which are lost upon removal of the histidine and tyrosine residues, partially reappear in the presence of the organic phosphate. Thus upon addition of IP₆, HbCPA exhibits a large Bohr effect and significant heme–heme interaction. At neutral pH the overall affinity is decreased 5-fold, and a slow phase in ligand-binding kinetics appears. Although direct evidence is lacking, the results are consistent with the idea that the IP₆ effect consists in the shift of a conformational equilibrium between high- and low-affinity forms of the protein.

MATERIALS AND METHODS

Hemoglobin A was prepared from fresh acid citrate–dextrose blood by the ammonium sulphate procedure, and freed of ions by passage through a column of mixed-bed ion exchangers (2). HbCPA was prepared by two sequential digestions (1) of oxyhemoglobin A with carboxypeptidase A (Worthington, diisopropylfluorophosphate-treated). The homogeneity of the preparation was determined by amino-acid analysis, starch gel electrophoresis at pH 8.6, and isoelectric focusing through a pH gradient from 6 to 8. A further check of the homogeneity came from analysis of the kinetics of...
ligand binding in the absence of organic phosphates. P$_2$Glr solutions were prepared by dissolving the pentacyclohexyl-ammonium salt of 2,3-diphasphoglyceric acid (Sigma) in water, followed by conversion of the salt to the free acid with Dowex 50-X8 and neutralization of the acid with 4 N NaOH. Inositol hexaphosphate was prepared by dissolving the sodium salt of phytic acid (Sigma) in water and neutralizing the solution with concentrated phosphoric acid. Bis-Tris buffers were prepared by pH adjustment of 0.1 M N,N-bis(2-hydroxyethyl)-imino-tris(hydroxymethyl)methane (General Biochemicals) with 1 N HCl. Oxygen equilibria were performed by the spectrophotometric method of Rossi-Fanelli and Antonini (7). Oxygen kinetics by temperature jump were performed according to established procedures (8). Rapid mixing experiments were performed with a Gibson-Durrum stopped-flow apparatus (9) and flash photolysis experiments were with an apparatus previously described (10). Sodium dithionite (about 0.5%) was used to deoxygenate hemoglobin in kinetic experiments.

RESULTS

The HbCPA used in this work was judged to be pure on the following grounds: (a) amino-acid analysis showed that after the first digestion equal amounts of His and Tyr had been released. Only very small quantities of His and Tyr were released after further exposure to CPA, an indication that the reaction had gone to completion. (b) Starch gel electrophoresis of the digested material at pH 8.6 gave a single band, with the same mobility as HbA, as expected for des His Tyr Hb at this pH. (c) Isoelectric focusing of the digested Hb through a pH gradient from 6 to 8 gave a major band, with an isoelectric point of 6.95, and a very minor one (< 5%), probably corresponding to undigested HbA (isoelectric point 7.2). The functional behavior of HbCPA eluted from the electrofocusing column was, in all respects, identical to that of the material before electrofocusing. (d) Kinetic analysis provided further evidence of the purity of the preparation. Combination velocity measurements after flash photolysis in Bis-Tris gave no trace of the slow rate characteristic of unmodified hemoglobin.

Oxygen equilibria

Fig. 1 shows that in Bis-Tris, HbCPA is noncooperative ($n = 1$), and has a very high ligand affinity ($pK_r = 0.42$ mm Hg at 30° and pH 7). The Bohr effect, although present, is only about 1/3 of its normal magnitude, as shown in Fig. 2. On addition of IP$_6$, the affinity at neutral pH decreases ($pK_r = 1.85$ mm Hg at 30° and pH 7), and the value of $n$ becomes significantly greater than one near neutrality ($n = 1.6$), as shown in Fig. 1. At the same time, the Bohr effect is increased (see Fig. 2), and indeed becomes similar in magnitude and position to that of unmodified hemoglobin as observed in inorganic phosphate (given by dashed lines in Fig. 2). It is, however, much smaller than that shown by HbA in the presence of IP$_6$ (see Fig. 2). Fig. 3 shows the relationship between pH and $n$ for HbCPA with and without IP$_6$, as well as values of $n$ for HbA with IP$_6$. The experiments reported here were performed with saturating concentrations of IP$_6$.

P$_2$Glr in 10-fold excess over tetramer concentration also produces a decrease in the oxygen affinity of HbCPA ($pK_r = 0.81$ at 30° and pH 7.2), without, however, affecting $n$, which remains equal to 1. This confirms previous results of Chanutin and Curnish (11). In contrast to human Hb, mouse hemoglobin digested with carboxypeptidase A has been reported to be devoid of P$_2$Glr effect (12).

Carbon monoxide kinetics

Rapid mixing experiments performed under different conditions are shown in Fig. 4. In the absence of organic phos-
Inorganic phosphate (i.e., in Bis-Tris) the combination of HbCPA with CO follows simple second-order kinetics, with a very high "on" constant similar to that of isolated chains (2) (Table 1). When deoxy HbCPA is pre-equilibrated with IP₆, the subsequent combination with CO is almost 100% slow, as in the case of unmodified hemoglobin, although the HbCPA shows a slightly higher second-order rate constant (Fig. 4 and Table 1). As with HbA, the time course of the combination is independent of protein concentration (from 0.16 to 3 μM in heme). In these experiments the IP₆ concentration was in 10-fold excess over tetramer concentration. If IP₆ is not pre-equilibrated with the protein, but is added only to the syringe containing CO, the combination with CO is biphasic, as shown in Fig. 4, and does not change even when the IP₆ concentration is greatly increased. However, the relative magnitudes of the two phases vary with protein concentration;

\[
\text{Flash photolysis (M}^{-1} \text{sec}^{-1}) \quad \text{Rapid mixing (M}^{-1} \text{sec}^{-1})
\]

<table>
<thead>
<tr>
<th>Solvent</th>
<th>1' slow</th>
<th>1' fast</th>
<th>1' slow</th>
<th>1' fast</th>
</tr>
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<tbody>
<tr>
<td>Bis-Tris</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bis-Tris + IP₆</td>
<td>3.8 × 10⁶</td>
<td>3.8 × 10⁶</td>
<td>4.3 × 10⁶</td>
<td>4 × 10⁴</td>
</tr>
</tbody>
</table>

Temperature = 20°C, pH 7.0.

at high protein concentration (above 10 μM in heme), the slow component predominates.

The time course of recombination of HbCPA with CO after flash photolysis for three solvent conditions, i.e., in inorganic phosphate, in Bis-Tris, and in Bis-Tris plus IP₆, is reported in Fig. 5. In Bis-Tris or inorganic phosphate without IP₆, a single, fast bimolecular reaction is observed, and, in Bis-Tris at least, this is true even in solutions where the hemoglobin, whether liganded or unliganded, shows the presence of substantial amounts of both tetramers and dimers. The second-order rate constant, reckoned from the CO concentration dependence, is very similar to that obtained under the same conditions by flow (Table 1).

On the other hand, in the presence of IP₆ the recombination of HbCPA with CO after flash photolysis is biphasic, and, as in normal hemoglobin, the observed fraction of quickly reacting material increases with dilution of the protein and is dependent on the wavelength at which the observation is made (13). This was confirmed by the occurrence of a spectral change in the Soret region upon mixing deoxy HbCPA in Bis-Tris with a solution containing IP₆ (unpublished data).

**Oxygen kinetics**

In inorganic phosphate (0.2 M, pH 7), the dissociation of oxygen from HbCPA, as measured by the dithionite method, is a homogeneous process that follows first-order kinetics. The "off" constant is almost independent of pH and, contrary to what is observed with HbA (14), there is no effect of IP₆ on the "off" constant at either pH 7 or 9 (Table 2). In addition, the same rate constants were obtained by mixing oxy HbCPA with a dithionite solution containing 0.5 mM CO, both in the presence and absence of IP₆.

Temperature-jump measurements with HbCPA in 0.2 M phosphate (pH 7), showed a single relaxation time, both in the presence and absence of IP₆. The relaxation time (τ) is dependent on oxygen concentration, and the slope of the curve 1/τ against reactants concentration yields an apparent combination velocity constant of \( k_{\text{on}} = 4.5 \times 10^9 \text{M}^{-1} \text{sec}^{-1} \). However, the intercept of the curve with the ordinate axis, which on a simple basis should yield \( k_{\text{on}} \), is higher than that observed in the dithionite experiments. The reason for this discrepancy is not clear.

**DISCUSSION**

The results presented here on the kinetics and equilibria of ligand binding by HbCPA in Bis-Tris buffer confirm and...

*This conclusion is based on studies of the subunit dissociation properties of carboxypeptidase-digested hemoglobin, with and without IP₆ (in preparation).
extend previous observations on HbCPA in dilute inorganic phosphate. Thus, it has been shown that HbCPA behaves in kinetic experiments as a homogeneous material characterized by a much larger combination velocity constant for both O₂ and CO than that observed for the unmodified protein. This fact accounts, to a large extent, for the observed increase in ligand affinity of HbCPA. The central observation of this work concerns the effect of IP₆ on the functional properties of HbCPA. HbCPA was regarded as representative of the high reactivity forms of hemoglobin. Its functional behavior was associated with an absence of ligand-linked changes in quaternary structure, as indicated by crystallographic observations (ref. 2 and M. F. Perutz and L. Mazzarella, personal communication). After IP₆ addition, HbCPA no longer behaves as a highly reactive, noncooperative and kinetically homogeneous molecule, but acquires properties that are, in some respects, similar to those of unmodified hemoglobin.

The effect of IP₆ on HbCPA is, in many ways, reminiscent of results obtained with artificial CNImetHb intermediates. In particular, it may be recalled that under many conditions the intermediates show both fast and slow phases in combination velocity measurements (15, 16). Cassoly et al. (16) have shown that addition of IP₆ (or P₂Glr) to the intermediates increases the percentage of slowly reacting material. Thus, for both HbCPA and the artificial CNImetHb intermediates, addition of IP₆ results in decreased reactivity. This finding is consistent with the idea that IP₆ may be capable of stabilizing molecular conformations characteristic of the “low-affinity forms” of hemoglobin.

The x-ray work of Perutz and coworkers (17) has suggested a structural picture of the role played by the C-terminal residues of the β chains in determining the functional properties of hemoglobin. In particular, there is evidence for the involvement of the C-terminal histidine (β 146) in the Bohr effect (18) and of the penultimate tyrosine (β 145) in the homotropic interaction shown by hemoglobin (17). The absence of hemeheme interaction of HbCPA in Bis-Tris may be correlated with the absence of the two penultimate tyrosines of the β chains (17). By stabilizing to a significant extent the low-affinity conformation, otherwise essentially absent in HbCPA, IP₆ thus provides the basis for the observed increase in n. To be sure, since evidence of the site or stoichiometry of IP₆ binding is lacking, no clear structural interpretation of this effect can be given. Nevertheless, apart from possible analogies with binding of organic phosphates to normal hemoglobin [which yield a stoichiometry of one P₂Glr or IP₆ molecule per Hb tetramer (19)], experiments in progress give some pertinent information. Thus, we observe that digestion of HbCPA by carboxypeptidase B yields a molecule (des lys tyr his β, des arg α) whose functional properties are unaffected by addition of IP₆ (results to be published). From this result it would be natural to conclude that some of the residues removed by the additional digestion may be involved either in the binding of IP₆ or in its linkage with oxygen.

The large Bohr effect exhibited by HbCPA in the presence of IP₆ might appear difficult to reconcile with previous evidence that β 146 His contributes about half of the total Bohr protons to the normal Bohr effect (17, 18). Experiments on normal hemoglobin, however, suggest that IP₆ may act indirectly to enhance the Bohr effect of HbCPA, just as it does in the case of unmodified hemoglobin (Fig. 2). In either case the origin of the additional Bohr protons is uncertain. They might come either from the groups normally involved in the Bohr effect, from other residues that become oxygen-linked after IP₆ is bound, or from IP₆ itself. The evidence presently available indicates that in normal Hb the additional Bohr protons arise from both the α and the β chains of normal hemoglobin. As shown in Fig. 3, the value of n for normal hemoglobin with added IP₆ is not pH dependent. On the other hand, under the same conditions, the value of n is significantly dependent upon pH in the case of HbCPA. Possibly this finding is related to the fact that in HbCPA only one set of Bohr groups, namely those of the α chains, are left to be affected by IP₆. This might explain the asymmetry of the effect of IP₆ on the α and β chains and, thereby, the dependence of n on pH.

The details of the effect of protein concentration on the relative amounts of fast- and slowly-reacting forms, as seen both in rapid mixing and flash photolysis experiments, will be discussed in a later paper, in connection with additional studies of molecular weight now in progress. But, without going into this matter here, it is clear that the addition of IP₆ to HbCPA results in a partial restoration of normal functional properties. Greater insight into the nature of the Bohr effect and the mechanisms giving rise to cooperative effects

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**Table 2. Overall oxygen dissociation constant by rapid mixing of oxy HbCPA (±IP₆) with sodium dithionite**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>pH</th>
<th>k (sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M phosphate</td>
<td>7</td>
<td>10.5</td>
</tr>
<tr>
<td>(7.5 mM) IP₆</td>
<td>7</td>
<td>11.5</td>
</tr>
<tr>
<td>2% Borate</td>
<td>9.2</td>
<td>8.0</td>
</tr>
<tr>
<td>(7.5 mM) IP₆</td>
<td>9.2</td>
<td>9.3</td>
</tr>
</tbody>
</table>

HbCPA at 5 μM (after mixing), observation wavelength 437.5 nm, temperature = 20°.
may result from structural studies complementary to this functional analysis.

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