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Structure and Function of Human Hemoglobin Covalently Labeled with Periodate-oxidized Adenosine Triphosphate*

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Periodate-oxidized adenosine triphosphate (*o*-ATP), a ribose ring-opened dialdehyde derivative of ATP, reacts specifically with human deoxyhemoglobin to give a single major covalently modified product after reduction with sodium borohydride. This product, designated di-ATP Hb, was isolated using ion-exchange chromatography and shown to have incorporated two molecules of *o*-ATP/tetramer. Peptide mapping and x-ray crystallography at 2.8-Å resolution indicate that a covalent adduct is formed between the ligand and residues Lys-82 EF6 of each β chain in the organic phosphate-binding site of the molecule. di-ATP Hb exhibits a significantly decreased oxygen affinity (P_{50} = 20.8 mm Hg *versus* 5.8 mm Hg control; 50 mM 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol, pH 7.4, 0.1 M Cl, 20 °C). The subunit cooperativity of di-ATP Hb is also reduced (n_{max} = 1.9 *versus* 2.7 control).

Adenosine triphosphate (ATP) exhibits a high affinity for the organic phosphate-binding site of human hemoglobin and affects oxygen affinity in a manner similar to 2,3-diphosphoglycerate (1). Periodate-oxidized ATP (*o*-ATP)[1] is a ribose ring-opened dialdehyde ATP analog (Fig. 1) which has been shown to act as an affinity label for several enzymes which use ATP as a substrate (2, 3). Because *o*-ATP is bifunctional, it has the capacity for introducing intermolecular cross-linkages through Schiff base formation with free amino groups. The organic phosphate-binding site of human hemoglobin is situated at the interface of the two β chains in the central cavity of the molecule and contains four primary amino groups, Lys-82 EF6 and Val 1 NA1 from β_1 and β_2. Because of its unique chemistry, *o*-ATP has been used to attempt structural and functional modification of hemoglobin in order to produce a cross-linked, low oxygen affinity derivative which might be suitable for use as a cell-free blood substitute (4–6). Such structural and functional modifications would address two of the major problems associated with infusion of native Hb solution as an acellular blood substitute, *i.e.* rapid renal clearance of dissociated αβ dimers and high oxygen affinity outside the milieu of the erythrocyte with its allosteric effectors. Other bifunctional reagents with high affinity for the organic phosphate-binding site have been shown to produce interdimerically (αβ–βα) cross-linked derivatives with significantly decreased oxygen affinity; for example, 2-nor-2-formylpyridoxyl-5′-phosphate (7), and 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) (8). However, it has been shown that cross-linked products derived from the reaction of human deoxyhemoglobin with *o*-ATP exhibit increased oxygen affinity, presumably due to differing structural constraints introduced by intersubunit cross-linking with this affinity ligand (6). Here we report on the structural and functional characterization of a non-cross-linked product of the reaction of human deoxyhemoglobin with *o*-ATP which has undergone a monofunctional substitution leading to lowered oxygen affinity and subunit cooperativity.

EXPERIMENTAL PROCEDURES

o-ATP Synthesis—Adenosine triphosphate and sodium periodate were obtained from Sigma. *o*-ATP was synthesized according to the procedure of Easterbrook-Smith *et al.* (2). Reaction yields were monitored by thin layer chromatography on polyethyleneimine-cellulose plates (E. Merck, Darmstadt, W. Germany) developed with 0.8 M NH_4HCO_3 using UV detection. The *o*-ATP was purified by chromatography on a Sephadex G-10 column equilibrated with deionized water at 4 °C. Aliquots were stored at −70 °C and protected from light.

Reaction of Hemoglobin with o-ATP—Hemoglobin A was isolated from freshly drawn human blood according to methods previously described (9). The hemoglobin was deoxygenated prior to reaction by flushing a solution in a round bottom flask on a rotary apparatus with nitrogen for 3 h at 0 °C. Hemoglobin concentration was 0.25 mM in 50 mM Tris, pH 7.8. Degassed *o*-ATP solution was introduced anaerobically to give a final concentration of 0.275 mM. Degassed sodium borohydride (10 mM) was added 30 min after addition of *o*-ATP. The reaction was stopped by chromatography on a G-25 Sephadex column equilibrated with 50 mM Tris, pH 7.8. Modified hemoglobin was purified by preparative ion-exchange chromatography on CM-Sephadex with a gradient consisting of 50 mM bis-Tris run from pH 6.7 to 7.2

HPLC of Hemoglobins and Peptides—Yields of modified hemoglobin were determined by HPLC on a cation exchange column (Polycat-A, Custom LC Inc., Houston, TX) (10) monitored at 419 nm using an IBM System 9000 for peak integration. Separation of globin chains was accomplished by reversed-phase HPLC on a Vydac C_4 column (The Separations Group, Hesperia, CA), monitoring at 220 nm (11). Tryptic peptide separation was performed on a Vydac C_{18} large-pore reversed-phase column following a modification of the method of Shelton *et al.* (12) using a gradient between 0.1% trifluoroacetic acid (A) and 0.1% trifluoroacetic acid in acetonitrile (B). The gradient ran from 0 to 13.6% B in 20 min, then to 34% B in 50 min, then to 100% B in 5 min, at a flow rate of 1 ml/min.

Proteolytic Digestion—Trypsin (Worthington) was added to acetamidated, heat-denatured Hb (1:50, w/w) and incubated for 8 h at 37 °C in 80 mM NH_4CO_3, pH 8.5 Hb (native or modified) was acetamidated at β-93 Cys in order to solubilize tryptic peptide βt-10

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[1] The abbreviations used are: *o*-ATP, periodate-oxidized ATP, DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; HPLC, high pressure liquid chromatography.

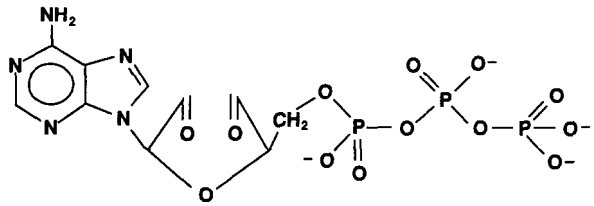

FIG. 1. **Postulated structure of periodate-oxidized adenosine triphosphate (Ref. 2).**

following a modification of the procedure of Goldstein *et al.* (13). Oxyhemoglobin (0.5 mM) was reacted with iodoacetamide (10 mM) for 2 h at 20 °C in 10 mM potassium phosphate, pH 7.0, and protected from light. The reaction was halted by chromatography on a column of Sephadex G-25.

Gel Electrophoresis—Separation of cross-linked globin from monomer was accomplished by electrophoresis in a 12% discontinuous polyacrylamide gel in the presence of sodium dodecyl sulfate and mercaptoethanol (14).

Functional Testing—Oxygen equilibrium curves were determined at a hemoglobin concentration of 15 μM in 50 mM bis-Tris buffer, pH 7.4, 0.1 M Cl^-, at 20 °C, using an automated recording apparatus (15). A Cary 219 spectrophotometer and a polarographic oxygen electrode (Beckman Instruments 39065) were employed. Data aquisition and reduction were performed with a PDP 11/VO3 computer (Digital Equipment Corp.) (16).

X-ray Crystallography—Di-ATP Hb was crystallized in the deoxy form according to Perutz (17). Friedel pairs of about 14,000 reflections extending to 2.8-Å resolution were collected from one crystal on a Hilger four-circle diffractometer. The data were corrected by the usual factors and scaled against the native data (21). The R-factor between Friedel pairs was 3.7% on intensity, that between derivative and native data was 6.5% on amplitude. An electron density difference map (di-ATP minus native deoxyhemoglobin) was calculated with phases from the native model (21). The map was contoured at ±0.06 $e/Å^3$, or approximately ±3 times the root mean square density over the whole cell (0.19 $e/Å^3$).

RESULTS AND DISCUSSION

Separation of o-ATP-modified Derivatives—After reacting deoxyhemoglobin with *o*-ATP followed by reduction with sodium borohydride, the reaction mixture was chromatographed by cation-exchange HPLC (Fig. 2). The elution profile shows the appearance of one major reaction product eluting at 16.2 min. In addition, several minor peaks appear which elute in positions intermediate between HbA and the major reaction product. These minor peaks include products containing intersubunit cross-linkages which exhibit increased oxygen affinity (6). The major peak eluting at 16.2 min was preparatively purified by ion-exchange chromatography on CM-Sephadex with a pH gradient of 50 mM bis-Tris, pH 6.7–7.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of this modified hemoglobin revealed that it contained no intersubunit cross-linkages (data not shown). Because *o*-ATP has a large extinction coefficient at 259 nm, it was possible to obtain an estimate of the number of molecules of *o*-ATP associated with the modified hemoglobin by spectroscopy (Table I). The $\Delta\epsilon_{259}$ of 25.5 mM^{-1} corresponded to 1.7 molecules of *o*-ATP bound per tetramer, suggesting that the product was disubstituted. This product, designated di-ATP Hb, was produced consistently in a yield of 40–50% as determined by peak integration following cation-exchange HPLC. Although the molar ratio of *o*-ATP to hemoglobin tetramer was 1.1:1 in the reaction mixture, the maximum yield of di-ATP Hb observed was 50%. Increasing the reagent to Hb ratio to 2.2:1 did not result in any further increase in yield of di-ATP Hb. When the ratio of reagent to tetramer was further increased to 10:1, there was a marked increase in the extent of modification; this was apparently due to heterogenous nonspecific reactions as evidenced by numerous new

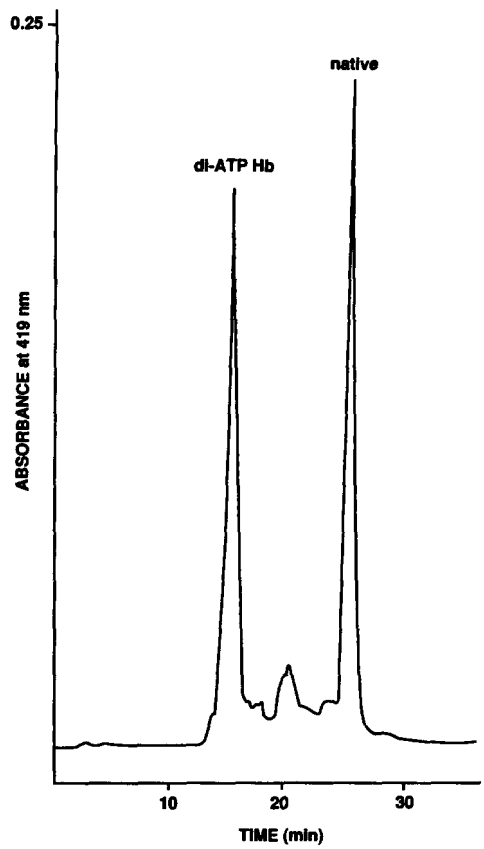

FIG. 2. **Cation-exchange HPLC of crude reaction mixture following reaction of deoxyhemoglobin with o-ATP.** Gradient and conditions are described under "Experimental Procedures."

TABLE I

Extinction coefficients at 259 nm (λ_{max} o-ATP) for native and o-ATP modified hemoglobins

Concentration of hemoglobins was determined for the cyanmet form on the basis of ϵ_{540} = 44 mM^{-1} cm^{-1} (19). For *o*-ATP, ϵ_{259} = 14.9 mM^{-1} cm^{-1} (2).

Hemoglobin	Abs_{540}	Abs_{259}	ϵ_{259} (mM^{-1})	$\Delta\epsilon_{259}$ (mM^{-1})
Native (CN-Met)	0.264 (6.00 μM)	0.618	103.0	0
di-ATP (CN-Met)	0.269 (6.11 μM)	0.785	128.5	25.5

peaks appearing during cation-exchange HPLC (data not shown). One possible mechanism to account for the observed 50% limit in yield of di-ATP Hb involves the dissociation and reassociation of hybrid species which can take place following oxygenation during ion-exchange chromatography. In order for affinity labeling to take place, specific binding must occur followed by covalent bond formation. It has been shown that only one molecule of the organic phosphate 2,3-diphosphoglycerate binds to the $\beta_1\beta_2$ cleft of deoxyhemoglobin (18). Even more steric hindrance would be expected in the case of *o*-ATP for a second molecule binding to the cleft after a first molecule has bound. Thus, an initial specific reaction of each deoxyhemoglobin tetramer with one molecule of *o*-ATP, followed by subunit dissociation and reassociation upon oxygenation and ion-exchange chromatography would give rise to the following equilibrium.

$$2\ \alpha_2\beta\beta^{ATP} \leftrightarrow 2\ \alpha\beta + 2\ \alpha\beta^{ATP} \leftrightarrow \alpha_2\beta_2 + \alpha_2\beta_2^{ATP}$$

Such a mechanism would lead to a yield of di-ATP Hb with an upper limit of 50%. In order to confirm this mechanism, however, further studies using anaerobic ion-exchange chro-

matography in order to trap hybrid molecules in the deoxy state will be required.

Tryptic Peptide Mapping—In order to determine the sites of modification by *o*-ATP, ion-exchange purified HbA and di-ATP Hb were iodoacetamidated, heat denatured, and subjected to trypsin digestion. After trypsin treatment for 8 h at 37 °C, the tryptic hydrolysates were chromatographed by reversed-phase HPLC. The α-globin tryptic peptides derived from di-ATP Hb were identical to those of native α-globin. In contrast, the tryptic map of β-globin tryptic peptides from di-ATP Hb revealed the absence of the adjacent tryptic peptides βt-9 and βt-10 (Fig. 3). In native β-globin, tryptic peptides βt-9 and βt-10 are split as a result of enzymatic hydrolysis at Lys-82 EF6, a residue found in the $\beta_1\beta_2$ cleft which plays an important role in binding organic phosphates through ionic interactions (18). The absence of βt-9 and βt-10 suggests that reductive alkylation of the ε-amino group of Lys-82 EF6 by *o*-ATP had occurred, inhibiting tryptic cleavage at this site and resulting in the formation of a larger, presumably insoluble peptide. As would be expected, the tryptic fragment βt-10, 11, which in native Hb hydrolysates is normally present in variable yield due to incomplete cleavage, was also missing from the di-ATP Hb hydrolysate. No other changes in the peptide pattern were observed. These results support the conclusion from spectroscopic studies indicating that the protein is disubstituted, as both β chains have been modified and the protein is not cross-linked.

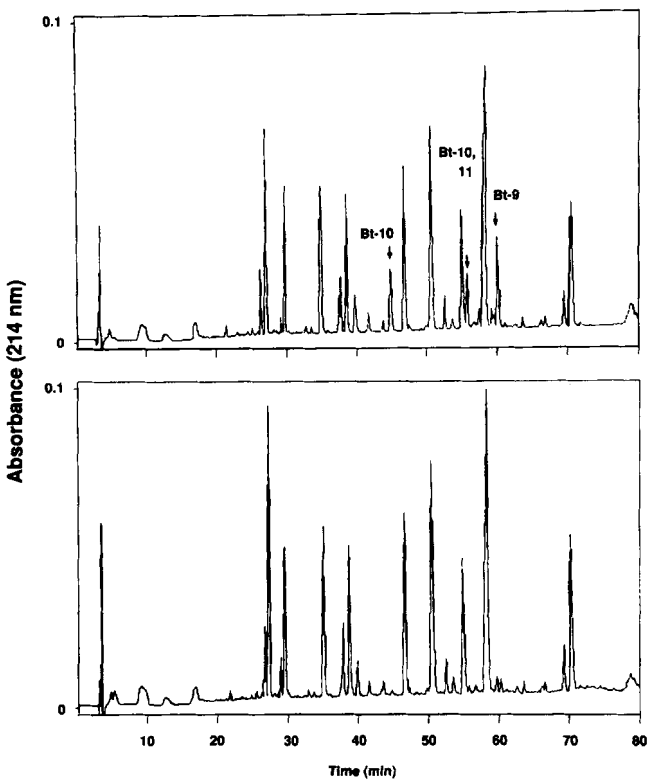

FIG. 3. **C_{18} HPLC tryptic peptide maps of iodoacetamidated HbA (*top*) and di-ATP Hb (*bottom*).** *Arrows* indicate positions of tryptic peptides βt-9, βt-10, and βt-10,11 which are missing from the di-ATP Hb hydrolysate.

X-ray Crystallography—The electron density difference map of deoxy di-ATP Hb minus HbA obtained at 2.8-Å resolution is shown in Figs. 4 and 5. The most prominent features in the map are the symmetric positive double peaks in the central cavity between the two Lys-82 EF6 (Fig. 4). The *o*-ATP molecules can be fitted into these peaks with the Lys-82 EF6 ε-amino adduct as either a bifunctional morpholino derivative (Fig. 5, *a* and *b*) or as a monofunctional secondary amine involving a single aldehyde from C3′ of *o*-ATP (Fig. 5, *c* and *d*). At 2.8-Å resolution it is not possible to distinguish between these two possibilities, although the morpholino configuration appears to give a slightly better fit. In any case it is clear from the map that the adenine rings take up a parallel "stacked" configuration approximately 3.5 Å apart. The map also shows a prominent peak centered near the site of Asn-139 H17. Since the atoms of this side chain have high temperature factors in the native structure, this peak probably represents a reduction of the Asn mobility due to its being trapped between the ligand and the H helix. The negative peaks between the positive peaks discussed above probably result from the exclusion of water. Small negative peaks on the cavity side of helix H, near position H12, and even smaller positive ones away from the cavity near H10 and H15 suggest a slight opening of the cavity by *o*-ATP. Positive and negative peaks surrounding the side chain of His-143 H21 indicate that this group moves toward the ATP and interacts either with the phosphate or with the 3′ hydroxyl in the morpholino configuration. The exact position of the phosphate is not well determined in the map. It may interact

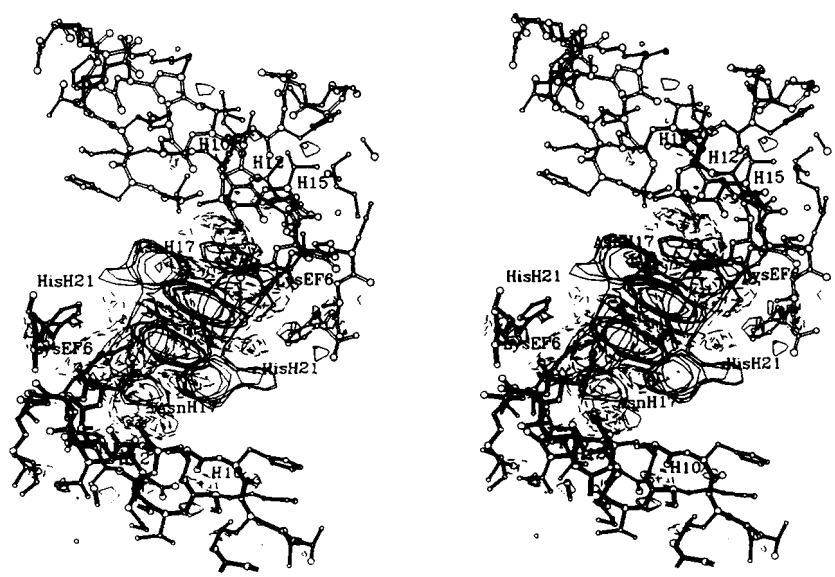

FIG. 4. **The difference map deoxy-di-ATP Hb minus HbA showing the region of the *o*-ATP-binding site.** Solid contours, +0.06 $e/Å^3$; dashed contours, −0.06 $e/Å^3$. The atomic model is of native deoxy-HbA. There were no significant features in the difference map outside of the region illustrated.

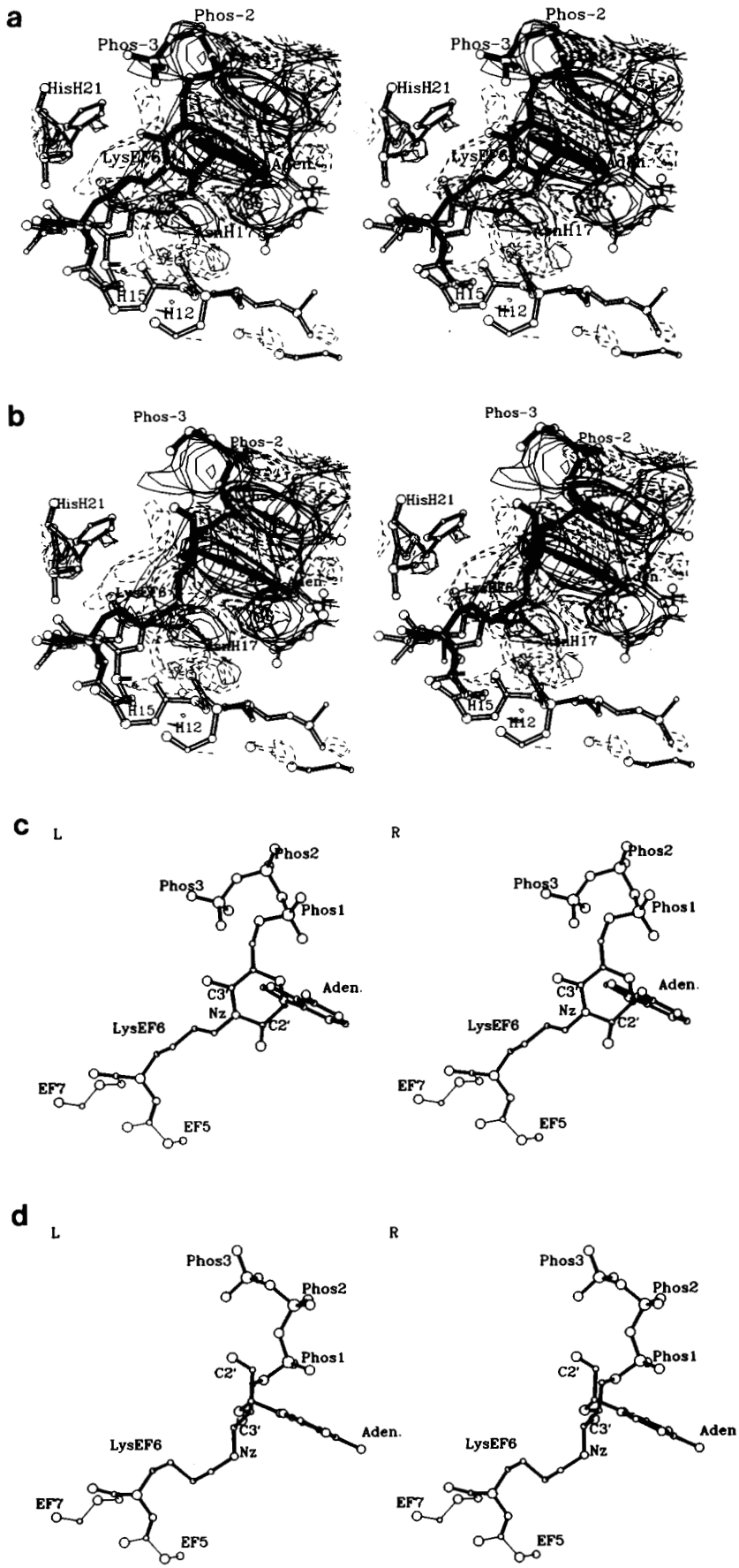

FIG. 5. **Detailed view of *o*-ATP bound to Lys-82 EF6 either bifunctionally (*a* and *b*) or monofunctionally at C3′ only (*c* and *d*).** Parts *a* and *c* show the fit to the difference map; solid contours: +0.06 $e/Å^3$; dashed contours: −0.06 $e/Å^3$; intermediate bonds: native model with the side chain of Asn-139 H17 shifted according to the difference map; lightest bonds: symmetry-related *o*-ATP. Parts *b* and *d* show the binding in more detail, with bonded atoms labeled. The viewpoint is the same in all four parts of the figure.

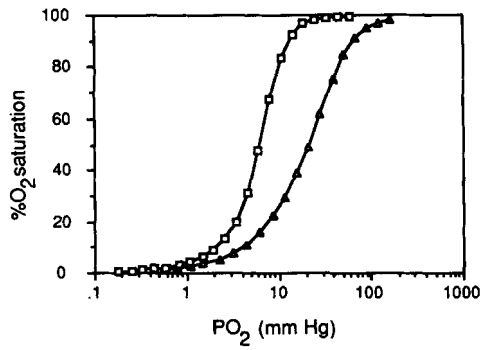

FIG. 6. **Oxygen equilibrium curves of HbA (*squares*) and di-ATP Hb (*triangles*).** Recordings were performed in 50 mM bis-Tris, pH 7.4, 0.1 M Cl^-, 20 °C. Hb concentration was 15 μM tetramer.

also with His-2 NA2 or the α-amino terminus of the β chain, but the map shows no evidence of movement or localization of these groups. (The periodic progression of positive and negative peaks across the central cavity might suggest an artifact due to gross mis-measurement of a single reflection. In order to test this possibility, a difference map was calculated omitting all 75 reflections with ΔF greater than the root mean square of ΔF. The difference maps calculated with and without these large ΔF values were virtually identical, ruling out the possibility of such an artifact.)

Functional Testing of o-ATP-modified Hb—Fig. 6 shows a comparison of the oxygen equilibrium curves for HbA and di-ATP Hb. Under the conditions tested (50 mM bis-Tris, pH 7.4, 0.1 M Cl^-, 20 °C), the P_{50} of di-ATP Hb is significantly increased relative to HbA (20.8 mm Hg *versus* 5.8 mm Hg). In addition, the subunit cooperativity of di-ATP Hb is reduced (n_{max} = 1.9; control n_{max} = 2.7).

Conclusions—It has been demonstrated that *o*-ATP serves as an efficient affinity label for the organic phosphate-binding site of deoxyhemoglobin via adduct formation with β Lys-82 EF6. It is noteworthy that while both affinity ligands *o*-ATP and pyridoxal phosphate label the organic phosphate-binding site, the amino acid residue involved in *o*-ATP adduct formation is different from that of pyridoxal phosphate, which has been shown to involve an adduct with β Val-1 NA1 (20). Because of the lower p*K* and hence greater nucleophilicity of the β-amino termini compared with the ε-amino group of Lys-82 EF6, they are more susceptible to alkylation by carbonyl attacking groups. The modification of β Lys-82 EF6 by *o*-ATP and lack of evidence of any reaction products involving the amino termini imply a stereochemical binding conformation in the central cavity which would specifically favor reaction with the ε-amino group.

It has been previously demonstrated that the formation of multiple cross-linked, singly-modified, high-affinity reaction products from the reaction of *o*-ATP with deoxyhemoglobin can be favored by the use of the milder reducing agent cyanoborohydride in place of borohydride (6). In contrast, reduction with borohydride leads to selective formation of the product described in this work, di-ATP Hb, which exhibits a significantly lowered oxygen affinity and subunit cooperativity. The structure of the molecule determined by x-ray crystallography suggests a possible mechanism for these functional changes. Both molecules of *o*-ATP are protruding into the $\beta_1\beta_2$ cleft with the planes of the adenine rings 3.5 Å apart and parallel to each other. The subunit motion in the EF regions of the central cavity induced by oxygenation would tend to push the adenine rings closer together, resulting in steric repulsion inhibiting the T to R conformational transition. The expected functional effect of this would be a reduction in oxygen affinity due to the shift of the allosteric equilibrium towards a T-like state. Such a mechanism would also be consistent with the observed reduction in subunit cooperativity in di-ATP Hb.

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