Structure and Oxygen Affinity of Crystalline des-His-146β Human Hemoglobin in the T State*

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To correlate directly structure with function, the oxygen affinity and the three-dimensional structure of crystals of the T quaternary state of des-His-146β human hemoglobin have been determined by polarized absorption microspectrophotometry and x-ray diffraction crystallography. In des-His-146β, the COOH-terminal histidine residues of the β-chains of hemoglobin A have been removed. Oxygen binding to crystalline des-His hemoglobin is non-cooperative and independent of pH. The oxygen affinity is 1.7-fold greater than that of the crystalline state of hemoglobin A. Removal of His-146β results in a small movement of the truncated COOH-terminal peptide and in a very small change in quaternary structure. Previously, similar studies on T state crystals of des-Arg-141α hemoglobin showed that removal of the COOH termini of the α-chains results in much larger effects on oxygen affinity and on quaternary structure. Kinetic studies in solution reveal that at pH 7.0, the rates of CO combination with deoxygenated des-His-146β in the absence and presence of inositol hexaphosphate are 2.5- and 1.3-fold, respectively, more rapid than for hemoglobin A. The values for des-Arg are 7.6- and 3.9-fold. The properties of the T state of hemoglobin both in the crystal and in solution are influenced to a greater degree by the interactions associated with Arg-141α than those associated with His-146β.

It was demonstrated by Mozzarelli et al. (1) that crystals of human deoxyhemoglobin, grown from solutions of polyethylene glycol (PEG), can bind oxygen reversibly. The T state hemoglobin molecules in these crystals have very low oxygen affinity that is comparable to the affinity measured for the binding of the first oxygen molecule to deoxyhemoglobin complexed with inositol hexaphosphate (IHP) in solution. Rivetti et al. (2) suggested that the low oxygen affinity of crystalline deoxyhemoglobin is due to crystal lattice stabilization of the T state structure so that the salt bridges associated with the Bohr effect are prevented from breaking upon ligand binding. This hypothesis is consistent with the absence of a Bohr effect in crystalline deoxyhemoglobin (2) and with the observation that the salt bridges associated with both the α-subunit and β-subunit COOH termini are intact in all of the partially liganded (3–7) and fully liganded (8, 9) T state x-ray crystal structures that have been determined to date.

Evidence in support of the idea that the salt bridges associated with the COOH-terminal Arg-141α are a key determinant of the low oxygen affinity of crystalline T state hemoglobin comes from our previous studies (10–12) on crystals of deoxyhemoglobin Rothschild (βW37R) and des-Arg deoxyhemoglobin. Rivetti et al. (10) demonstrated that in the absence of chloride ions a 10-fold increase in oxygen affinity for crystalline deoxyhemoglobin Rothschild (βW37R) is associated with a significant increase in the mobility of the α-subunit COOH termini in the deoxy crystal structure (11), but not with any detectable change in the stereochemistry of the deoxy heme groups. This suggested that the increased oxygen affinity of crystalline deoxyhemoglobin Rothschild is due at least in part to a weakening of the salt bridges associated with Arg-141α. More recent studies on crystalline des-Arg deoxyhemoglobin (12) confirm the hypothesis that the salt bridges at the α-subunit COOH termini are fundamentally associated with the low oxygen affinity of T state hemoglobin. Removal of Arg-141α eliminates the COOH-terminal salt bridges and results in a 15-fold increase in the oxygen affinity of the crystalline T state, a change in oxygen affinity that is similar in magnitude to that observed in solution for the first Adair constant (13). The increased oxygen affinity of crystalline des-Arg deoxyhemoglobin, like that of crystalline deoxyhemoglobin Rothschild, is associated with a large increase in the mobility of the α-subunit COOH termini.

Previously, Kilmartin and Hewitt (14) showed that the bulk properties of hemoglobin in solution are less sensitive to the removal of His-146β than to the removal of Arg-141α, and Perutz and Ten Eyck (15) reported a low resolution (3.5 Å) difference electron density map of des-His deoxyhemoglobin that confirmed the removal of His-146β and showed no evidence for any associated structural changes. In the present paper, we directly investigate the influence of the COOH-terminal salt bridges associated with the His-146β on the oxygen binding properties of the T state hemoglobin tetramer in the crystalline state and on the kinetics of carbon monoxide bind-

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Properties of T-State Crystals of Human Des-His Hb

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* These values are from Kavanaugh et al. (11).

EXPERIMENTAL PROCEDURES

Preparation of Des-His-146 β HbA

Unless otherwise noted, all procedures were carried out in the presence of CO.

β chains were prepared from human HbA after treatment with an excess of PMB essentially as described by Bucci and Fronticelli (16). The PMB reaction mixture was gel-filtered against 0.015 M HCl-bis-Tris, pH 6.5, and applied to a CM52 column equilibrated with the same buffer. Under these conditions, the β-PMB chains passed through this column in a dilute form. PMB was removed from the β chains with dithiothreitol according the method of Parkhurst and Parkhurst (17). If necessary, β chains were further purified by HPLC using a glass TSK gel SP-5PW cation exchange column (Toso-Haas, 22 b 23 b). The final buffer contained CO-saturated 0.02 M HCl-bis-Tris, 10 mM sodium dithionite, and 3 mM potassium phosphate, pH 6.0, and the final buffer contained CO-saturated 0.02 M HCl-bis-Tris, 10 μM EDTA, pH 6.0. The des-His-146 β chains were formed by the digestion of βSH chains with carboxypeptidase B as described by Kilmartin et al. (18) with a few modifications. The βSH chains were gel-filtered against 0.04 M HCl-Tris, pH 8.2, and brought to a concentration of 10 mg/ml before the addition of carboxypeptidase B. After 3 h of digestion, followed by the addition of arginine HCl and dithiothreitol, excess carboxypeptidase B was removed. The reaction mixture was then frozen in liquid nitrogen. HPLC analysis of a small aliquot of this reaction mixture demonstrated the removal of about 25% His-146. Des-His-146 β chains were separated from undigested β chains on a CM52 column as already described (18). Purity of the preparation was determined by analytical HPLC using a glass TSK gel SP-5PW anion exchange column under conditions cited above and by polyacrylamide gel electrophoresis.

Preparation of des-His-146β HbA and removal of uncombined des-His-146β chains were accomplished as described previously (18). Purity of the des-His-146β HbA preparation was checked by polyacrylamide gel electrophoresis and analytical HPLC using a glass TSK gel DEAE-SPF anion exchange column under conditions described elsewhere (19). Before use for crystallization and kinetic studies, des-His-146β HbA was converted to the oxygen derivative (20).

Des-Arg-141α HbA was prepared as described previously (21).

X-ray Studies—Prior to crystallization the des-His hemoglobin was stripped of organic and inorganic ions by passing it over a Dintzis column (20) that was modified by the addition of a 1-mm layer of Dintzis resin to the top of the column. The stripped des-His oxyhemoglobin was frozen and stored in liquid nitrogen until used for crystallization. Crystals of des-His deoxyhemoglobin were grown at room temperature as described previously for deoxyhemoglobin A (11) from solutions consisting of 10 mg/ml hemoglobin, 10 mM potassium phosphate, pH 7.0, 100 mM potassium chloride, 3 mM sodium dithionite, and 10 to 10.5% PEG 6000. A single crystal (measuring 0.8 × 0.6 × 0.2 mm 3) that was isomorphous with crystals of deoxyhemoglobin A (Table I) was mounted in a quartz capillary for data collection. The des-His deoxyhemoglobin and all crystallization stock solutions were thoroughly deoxygenated, and all work was conducted in a nitrogen-filled glove box.

Diffraction data were collected on a Rigaku AFC6 diffractometer fitted with a San Diego Multixray Systems area detector. A total of 247,179 measurements were made of 35,576 unique reflections, representing 98% of all possible data out to a resolution of 2.12 Å. After the diffraction data were scaled and merged according to the procedure of Howard et al. (22), the discrepancy between symmetry related reflections (as judged by the R sym value) was 9.0% on intensity for all data out to 2.12 Å resolution.

The initial des-His atomic model was derived from the 1.9-Å structure of deoxyhemoglobin A (11) with the COOH-terminal histidine residues of the β-subunits removed. The additional oxygen molecule necessary for completing the new α-carboxyl group of Tyr-145β was not included in this model. The standard crystallographic R value for the initial model was 20.6% for all diffraction data between 8.0 and 2.12 Å resolution with magnitudes greater than 2σ (34,083 reflections). Following 11 cycles of restrained least-squares refinement (using the program PROLSQ (23, 24), the R value dropped to 17.7%. After the new COOH termini were constructed by fitting oxygens into Fo – Fc, Fourier maps with the computer graphics program TOMP/FRODO (25, 26), 35 additional refinement cycles reduced the R value to 17.0%. The final deoxy-des-His atomic model had r.m.s. deviations from ideal bond lengths of 0.010 Å and r.m.s. deviations from ideal bond angles of 1.4°. Individual atomic temperature factors were refined throughout the des-His deoxyhemoglobin refinement.

Least-squares superposition methods (11) were used to partition the structural perturbations in des-His deoxyhemoglobin into components of tetramer movement within the crystal lattice, changes in tetramer quaternary structure, and deformations of tertiary structure. Superpositions of the atomic models of deoxyhemoglobin A and des-His deoxyhemoglobin were carried out using the method of Kabsch (27) as implemented in the program BMMFIT (28). When superimposing α-subunits, the first 5 residues and last 5 residues of each subunit were omitted from the superposition. When superimposing β-subunits, the first 5 residues and last 5 residues of each subunit were omitted from the superposition. The magnitudes of the structural perturbations were measured by calculating the vector magnitude of the main chain atomic displacements (11, 12). Specifically, the quantity as shown in Equation 1 is plotted versus residue number, where δj is the atomic displacement vector for the jth main chain atom of a residue.

Measurement of Oxygen Binding to Crystals of Hb

Crystals for microspectrophotometry were grown from PEG 8000 Mw solutions at room temperature as described previously (10). Once grown, crystals were washed sequentially with 20 and 36% (w/v) anaerobic PEG 8000 Mw solutions containing 10 mM potassium phosphate, 30 mM sodium dithionite, pH 7.2. Crystals were stored at 20 °C until used. The crystals of des-His Hb, resuspended at least seven times in a deoxygenated solution containing 54% (w/v) PEG 8000 Mw, 1 mM EDTA, 10 mM potassium phosphate, 6000 units/ml catalase, pH 7.2, were loaded in a Dvorak-Stotler flow cell (29) within a deoxygenated glove box. This procedure prevents crystal damage caused by exposure to air (see below).

Polarized absorption spectra of des-His-Hb crystals were recorded as a function of oxygen pressure at 15 °C with a Zeiss MPM03 microspectrophotometer, as described previously (1, 2). Fractional concentrations of deoxygenated, oxygenated, and oxidized hemes were determined by fitting the observed spectra to a linear combination of deoxy-Hb, oxy-Hb, and met-Hb reference spectra (2). Since both fully oxygenated and oxidized des-His Hb crystals are severely cracked, reference spectra from HbA crystals were used for data analysis. Of the 23 spectra collected between 4.7 and 59 torr, 15 were accepted on the basis of two criteria as follows: (i) fractional concentration of oxidized hemes less than 25% and (ii) the ratio of residuals of the calculated versus observed spectrum within the averaged r.m.s. + 3 S.D. To obtain stable fractional saturations, polarized absorption spectra were recorded over a period of several hours at constant oxygen pressure. This procedure limited data collection on a single crystal to no more than three data points.

Kinetic Measurements

Flash Photolysis—Carbon monoxide recombination following photodissociation was measured with a flash photolysis apparatus based upon the use of three photographic strobe lamps (10). The concentration of hemoglobin was reduced to 20 μM in heme equivalents, and the CO concentration was 40 μM. To ensure anaerobic conditions, residual oxygen was removed by addition of sodium dithionite to 1.5 mM.

Stopped Flow—The kinetics of combination of deoxyhemoglobin Hb with CO were measured in an Olis stopped-flow apparatus based on the Gibson-Durrum design (30) as described previously (19). Anaerobic conditions were obtained using sodium dithionite. The hemoglobin-con
concentration was 2 μM in heme, and the CO concentration was 40 μM after mixing.

Both stopped flow studies and flash photolysis measurements were carried out at pH 6, 7, and 8. The buffers were prepared by titrating an amount of HCl equal to 0.1 M chloride with solid bis-Tris base (for pH 6 and 7) and solid Tris base (for pH 8). Inositol hexaphosphate, IHP (Sigma), was obtained in the sodium form. The pH of the stock solution (0.2M) was adjusted to pH 5.6 with the protonated form of Amberlite IR-120 resin. IHP was added to give a final concentration of 0.1 mM. Reactions were followed at 420 and 435 nm, and the temperature of the sample maintained at 20 °C. Data collection and processing was by an Olis model 4000 data acquisition and instrument control system as already described (31). Rigorous error analysis for the fitted rate constants is difficult. Because of the large numbers of data points obtained in each kinetic experiment, the estimated errors of the fits are very small. However, reproducibility between and among experiments indicates a more reasonable estimate of error to be ±10% for the rate constants and ±15% for the fractional contributions of the kinetic components of the reaction.

RESULTS

X-ray Studies—The electron density images in Fig. 1 show that the COOH-terminal β-subunit residues can be positioned accurately in both deoxyhemoglobin A and des-His deoxyhemoglobin. Moreover, the total absence of any electron density for His-146β in the deoxy des-His electron density map (Fig. 1B) confirms the complete removal of the COOH-terminal histidines in the purified des-His hemoglobin.

Partitioning of Structural Perturbations—Superposition analyses can be used to partition the structural differences between hemoglobin A and a modified hemoglobin into components of rigid-body rotation of the entire tetramer, changes in quaternary structure, and changes in tertiary structure (11). The δ-profiles in Fig. 2A were calculated without superimposing the deoxyhemoglobin A and des-His deoxyhemoglobin atomic models. Therefore, these δ-profiles include rigid-body motion of the deoxy des-His tetramer within the crystal lattice. In the absence of rigid-body motion of the entire tetramer, the non-crystallographic 2-fold symmetry of the hemoglobin tetramer requires that δ-profiles calculated for the symmetry related β1- and β2-subunits have similar shapes and magnitudes. The same is true for the α1- and α2-subunits. There are, however, significant differences between the β1-subunit (thin lines) and β2-subunit (thick lines) δ-profiles in Fig. 2A, where the r.m.s. value of δβ1-δβ2 is 0.12 Å. Likewise, an r.m.s. value of 0.14 Å for δα1-δα2 reflects significant differences in the shapes and magnitudes for the α1 and α2 δ-profiles calculated prior to

**Fig. 1. Stereo diagrams showing the electron density at the β chain COOH termini of deoxyhemoglobin A (A) and des-His deoxyhemoglobin (B).** Shown are the spatial relationships between His-146β (top of figure) and residues Asp-94β (far left of figure), Cys-93β (center of figure), and Tyr-145β (right side, background of figure). The (F_{observed} – F_{calculated}) electron density images were calculated with residues 88β through 98β and residues 140β through the COOH terminus omitted from the atomic models. The contouring is at 2.5 times the r.m.s. density of the map.
any superposition of the atomic models (data not shown). If these differences in $\delta$-profiles are largely the result of rigid-body motion, they should be reduced by superimposing the des-His deoxyhemoglobin and deoxyhemoglobin A tetramers. The $\delta$-profiles in Fig. 2B show this to be the case. That is, the shapes of the $\beta_1$ and $\beta_2$ $\delta$-profiles calculated after superposition of the tetramers are much more similar (the r.m.s. value of $\delta_{\beta_1} - \delta_{\beta_2}$ is reduced to 0.05 Å). The same is true of the $\alpha_1$ and $\alpha_2$ $\delta$-profiles (the r.m.s. value of $\delta_{\alpha_1} - \delta_{\alpha_2}$ is reduced to 0.05 Å). The rigid-body motion of the des-His deoxyhemoglobin tetramer amounts to a rotation of approximately 0.2° and a translation of 0.07 Å about an axis that passes through the outer edge of the $\alpha_2$-subunit.

After the rigid-body motion has been removed, the remaining structural differences between deoxyhemoglobin A and des-His deoxyhemoglobin (Fig. 2B) are the result of rearrangements in quaternary structure and/or changes in tertiary structure. The tertiary component of the structural changes can be isolated from the quaternary component by superimposing corresponding subunits of des-His deoxyhemoglobin and deoxyhemoglobin A. When this is done (Fig. 2C), the only significant structural changes are located at the $\beta$-subunit COOH termini, where the changes are approximately 7.5–10 times the r.m.s. $\delta_{\beta_1} - \delta_{\beta_2}$ value of 0.05 Å. Elsewhere in the $\beta$-subunits, the $\delta$ values are only about 2 to 3 times the r.m.s. $\delta_{\beta_1} - \delta_{\beta_2}$ value. The same is true for $\alpha$-subunits (data not shown). The only exception is the NH$_2$ terminus of the $\beta_1$-subunit, where the $\delta$ value is ~8 times the r.m.s. $\delta_{\beta_1} - \delta_{\beta_2}$ value. This difference, however, most likely reflects the inherent mobility of the $\beta$-subunit NH$_2$ terminus (see below), rather than a significant structural change that results from the removal of the COOH-terminal histidine.

The small differences between the shapes of the $\delta$-profiles in Fig. 2C and the corresponding $\delta$-profiles in Fig. 2B are indicative of a slight quaternary structure change in the des-His deoxyhemoglobin tetramer. The existence of a small quaternary rearrangement in des-His deoxyhemoglobin is more clearly demonstrated by the $\delta$-plot in Fig. 2D, where the $\delta$-profile for the $\beta_1$-subunit was calculated after the $\alpha_2$-subunits were superimposed, and the $\delta$-profile for the $\beta_2$-subunit was calculated after the $\alpha_1$-subunits were superimposed. This type of $\delta$-plot, a quaternary $\delta$-plot, is very sensitive to any change in the position of the $\alpha_1$-$\beta_1$ dimer relative to the orientation of the $\alpha_2$-$\beta_2$ dimer. If there is no change in the relative orientation of the dimers, the $\delta$-profiles in a quaternary $\delta$-plot would be flat and featureless, as is the case when the $\beta$V1M model is compared with the deoxyhemoglobin A model (dashed line). The $\beta$V1M mutation does not result in any quaternary rearrangements, but it does cause a change in the tertiary structure of the $\beta$-subunit NH$_2$ termini (32). In contrast to the $\beta$V1M $\delta$-profile, the $\delta$-profiles for the des-His deoxyhemoglobin have clear maxima for residues located on the outside of the tetramer and minima for residues located within the dimer-dimer interface, confirming the presence of a slight quaternary structure change in des-His deoxyhemoglobin. Rigid-body calculations (33) show that this quaternary structure change amounts to a rotation of one dimer relative to the other by just over 0.4°.

The structural perturbations detected in the des-His deoxyhemoglobin $\delta$-plots are illustrated in Fig. 3. The only significant changes involve the main chain atoms of residues Lys-144$\beta$ and Tyr-145$\beta$, as well as a slight repositioning of the Tyr-145$\beta$ side chain. The remainder of the des-His deoxyhemoglobin atomic model, including the hemes and proximal histidines, is indistinguishable from that of deoxyhemoglobin A. In particular, despite the loss of the inter- and intrasubunit salt bridges associated with His-146$\beta$, no significant increases in mobility or changes in conformation are detected for the side chains of either Lys-40$\alpha$ or Asp-94$\beta$. No increase in the mobility of the $\beta$-subunit COOH termi is observed in crystals of des-His deoxyhemoglobin (Fig. 4).

Oxygen Binding to T State Des-His Hb Crystals—Des-His Hb crystals are well shaped rectangular plates. Exposure of crystals to oxygen pressures in excess of 65 torr, at 15 °C, causes first the formation of diagonal bands and then irregular cracks. Polarized absorption spectra were recorded during an oxygen pressure jump between 0 and 76 torr. Fractional concentrations of oxygenated hemes vary with time following complex kinetics that ultimately lead to a fully oxygenated Hb (Fig. 5A). When the oxygenation is carried out in two steps, 0–50 torr and 50–76 torr, a stable fractional saturation is obtained after the first step, and a fully oxygenated Hb is obtained in the second step (Fig. 5B) with the concomitant cracking of the crystal.

Reversible oxygen binding to intact des-His Hb crystals was determined between 0 and 59 torr by collecting single crystal polarized absorption spectra along the $a$ and $c$ crystal axes. Fractional saturations with oxygen and fraction of oxidized hemes were determined by fitting the observed spectra to the reference spectra (see “Experimental Procedures”). The dependence of fractional saturation with oxygen on oxygen pressure, as shown in Fig. 6, was analyzed by fitting to Equation 2.

$$Y = \frac{p_o (\text{torr})^n}{p (\text{torr})^n + p_{50}^n}$$  

(Eq. 2)

where $Y$ is the fractional saturation with oxygen; $p$ is the oxygen pressure; $p_{50}$ is the oxygen pressure at half-saturation with oxygen; and $n$ is the Hill coefficient. Along the $a$ crystal
axis, the $p_{50}$ and the Hill coefficient $n$ are 81.2 ± 9.7 and 0.98 ± 0.10, respectively, and along the $c$ crystal axis are 76 ± 9.2 and 1.01 ± 0.11. These $p_{50}$ values are 1.7 times lower than those determined for HbA crystals, 136 torr along the $a$ crystal axis and 133 torr along the $c$ crystal axis (Table II).

The effect of pH on the oxygen affinity of des-His Hb crystals was investigated by measuring single crystal polarized absorption spectra between pH 6 and 8.5, at constant oxygen pressure. The fractional saturation is almost independent of pH.

**Kinetics of CO Binding in Solution**—Data for the combination of CO with deoxygated des-His Hb are presented in Table III. Results obtained with both HbA and des-Arg-141a HbA are included for comparison. The combination of CO with deoxygen-HbA, following rapid mixing, is typically characterized by a reaction rate that is limited by the rate of binding of the first oxygen molecule to the low affinity T state of the Hb tetramer. The overall reaction is frequently autocatalytic, a kinetic reflection of cooperative ligand binding. Comparing the CO combination rates for normal human HbA and des-His Hb one observes a far greater difference in the absence of IHP than when this allosteric effector is present. HbA reacts autocatalytically with CO with an overall rate constant which is lowered by somewhat less than a factor of 2 by the addition of IHP at all pH values examined. In contrast, the rate constant for the reaction of des-His Hb with CO decreases 2.7-fold at pH 6, 3.5-fold at pH 7, and 2-fold at pH 8 in response to IHP. As a result, in the absence of IHP at pH 7 des-His Hb reacts with CO 2.5-fold more rapidly that HbA, but when IHP is present the loss of His-146 increases the CO combination rate constant by only 30%.

Data for the recombination of CO with HbA and des-His Hb following flash photolysis are presented in Table IV. The initial state of the hemoglobin molecule in these measurements is ligand-saturated, with the result that the protein exists in a dynamic equilibrium between its tetrameric state and the $ab$ dimer. The latter has a high ligand affinity and binds ligand rapidly and without cooperativity. The rapid process observed in the recombination kinetics is the binding of CO to the $ab$ dimers. Since the rate of transition of the tetrameric R state to the T state, following photolysis of the ligands, is more rapid than the rate of CO binding at the CO concentration used in these studies, the slow process in the recombination kinetics is the reaction of CO with the T state tetramers. The relative amounts of fast and slow kinetic phases offer a measure of the degree of dissociation of the CO-saturated Hb tetramer under the experimental conditions. With HbA we observe that at pH 7, in the absence of IHP, 59% of the heme groups are found in $ab$ dimers. Addition of IHP reduces the percentage of dimers to 50%.
FIG. 5. Effects of oxygenation on the affinity of des-His Hb crystals. Polarized absorption spectra were consecutively recorded on a crystal exposed to an oxygen jump from 0 to 76 torr in a single step (a) and in two steps, 0 to 50 torr and 50 to 76 torr (b). Fractional saturation with oxygen (Δ, ▲) and fraction of oxidized hemes ( ○, ▼) along the a (open symbols) and c (closed symbols) crystal axes were determined as described under “Experimental Procedures.”

FIG. 6. Oxygen binding curve to des-His Hb crystals. Fractional saturations with oxygen were determined from spectra recorded along the a (open symbols) and the c crystal axes (closed symbols) either by increasing (Δ, ▲) or decreasing oxygen pressures ( ○, ▼). Data were analyzed by a nonlinear fitting to Equation 2. Inset, Hill plot calculated from the data reported in the figure.

IV are consistent with this pattern. However, with des-His-146β dissociation into dimers in the absence of organic phosphates exhibits a reversed pH dependence, decreasing with increasing pH. This may be a consequence of the marked decrease in the alkaline Bohr effect which results from this chemical modification.

DISCUSSION

Removal of the His-146β results in changes in the bulk solution properties of human hemoglobin. In particular, at pH 7 in 0.2 M phosphate buffers, this enzymatic modification de-
increases the $p_{50}$ of hemoglobin by between 3- and 4-fold and reduces the Hill constant from 3.1 to 2.6 (14), whereas the alkaline Bohr effect is halved (36). Because of the reduced Bohr effect, the consequences of the chemical modification become progressively smaller with increasing pH, and at pH 9 des-His hemoglobin has only a 60% higher oxygen affinity than HbA (14). Kilmartin et al. (37) further demonstrated that, at pH 7 in bis-Tris buffer in the absence of organic phosphates such as IHP, removal of His-146β increases the affinity of the deoxygenated protein for the first oxygen molecule, $K_1$, by 4- to 5-fold. Correlating changes in the bulk properties of hemoglobin with structural changes can be difficult because in solution hemoglobin can exist in a wide range of conformational states. By studying oxygen binding to crystalline deoxyhemoglobin and determining its three-dimensional structure, we are able to compare directly the structure and oxygen affinity of hemoglobin A and des-His hemoglobin in a single quaternary structure, the crystalline T state. This approach of investigating protein function in the same crystalline state used for structure determination has been fruitful in understanding structure-function relationships in hemoglobin and in several other proteins (38 and references therein, 39 and 40).

We find a 70% increase in oxygen affinity of crystalline des-His hemoglobin relative to that of crystalline HbA. No significant Bohr effect is detected. Although the inability to measure the equilibrium of oxygen binding to des-His crystals above 50% saturation increases the errors associated with the computation of $p_{50}$ and $n$, the findings reported here remain sound. Measuring the first 50% of the saturation curve, by necessity, detects the oxygen binding sites with the highest affinity, excluding the possibility that the effect of removing His-146β on oxygen affinity could be greater than the 70% increase reported. On the other hand, errors in the measurements of $p_{50}$ preclude the detection of differences in the apparent oxygen affinities measured along the a and c axes of the crystal of the magnitude reported by Rivetti et al. (2) for crystals of HbA. Those authors calculated the difference in the projections of α and β hemes along the a and c axes of the deoxygenterated and liganded T state of HbA. From this and the difference in the apparent oxygen affinities measured along the two crystal axes, they estimated the magnitude of the inequality of the oxygen affinities of the α and β chains (see also Mozzarelli et al. (34)). At present the information required for a similar analysis of des-His crystals is lacking and, given the instability of des-His crystals to ligand saturation, it is unlikely to be available any time soon.

Analysis of the refined des-His deoxyhemoglobin atomic model reveals that loss of His-146β results in only minor changes in the T state structure consistent with the small magnitude of the increase in the T state oxygen affinity. With the exception of a very small change in the position of the β-subunit COOH termini and very slight change in quaternary structure, the des-His deoxyhemoglobin structure is essentially identical to that of deoxyhemoglobin A. One somewhat surprising observation is that the loss of the salt bridges associated with His-146β does not result in greater mobility for either the truncated β-subunit COOH termini or the side chains of residues Lys-40a or Asp-94β.

As already noted, the kinetics of the reaction of deoxy-Hb with CO in solution reflect the functional properties of the deoxygenterated T state. It has been pointed out (2, 34, 38) that the oxygen affinity of the crystalline T state of HbA is very similar to the affinity of the first oxygen molecule, $K_1$, for deoxy-HbA in solution in the presence of IHP. Therefore, in solution IHP alters the properties of the T state of HbA, essentially by shifting the conformational equilibrium of the T state toward structures with low ligand affinity. Crystallization of the T state in the presence of PEG does much the same thing, i.e., lattice forces stabilize T state conformations with low oxygen affinity. Along with lowering the oxygen affinity of the T state, the addition of IHP markedly reduces the effect of removing His-146β on the functional properties of the T state in solution as measured by CO combination rates. At pH 7, in the absence of IHP, the rate constant for the combination of CO with deoxy des-His hemoglobin is 2.5-fold greater than that for oxy-HbA. This kinetic difference is consistent with the 4- to 5-fold increase in $K_1$ reported for des-His hemoglobin in the absence of IHP in bis-Tris buffer at pH 7 and 25 °C (37). In the presence of IHP the rate constant for the combination of CO with des-His hemoglobin is only 30% greater than that for HbA, consistent with the 70% increase in the oxygen affinity of crystalline des-His hemoglobin reported here. The smaller difference between the rate constants for des-His hemoglobin and HbA in the presence of IHP is the result of IHP having a greater effect on the kinetics of CO binding to des-His hemoglobin, a 3.5-fold reduction as compared with only 1.8-fold for HbA.

The effects of removing His-146β and Arg-141α on the properties of T state crystals are quite different. Crystals of des-His have a 1.7-fold higher oxygen affinity than those of HbA, but they are sufficiently destabilized by the chemical modification to be irreversibly altered when oxygen binds at greater than 50% saturation. Crystals of des-Arg bind oxygen with 15-fold greater oxygen affinity than crystals of HbA, but these crystals are sufficiently stable to permit reversible saturation with oxygen over the entire saturation range. Moreover, crystals of des-Arg exhibit a significant acid Bohr effect in oxygen binding, whereas such an effect exists in des-His crystals, it is very much smaller. These changes in functional properties appear to be correlated with the magnitudes of the structural changes resulting from these chemical modifications. In fact, the magnitude of the structural changes in des-His deoxyhemoglobin is about a third as large as the corresponding changes previously observed for des-Arg deoxyhemoglobin (12). In addition, the increase in B factors of the COOH termini of the α chains of des-Arg but not of des-His crystals implies a greater local flexibility of des-Arg hemoglobin. The greater flexibility of the COOH termini of the α chains may facilitate a conformational response to ligand binding, thereby increasing oxygen affinity. This greater flexibility of des-Arg also may be related to the acid Bohr effect which is observed in its crystals but not in crystals of HbA (2), des-His hemoglobin, and hemoglobin Rothschild (10).

The kinetics of CO binding to the deoxygenterated derivatives of des-His and des-Arg hemoglobins demonstrate that the difference in the effects of removing these two COOH-terminal residues on the functional properties of the T state of hemoglobin is significant. As seen in Table III, in the absence of organic phosphates at pH 7, des-Arg reacts 7.6-fold more rapidly with CO than does HbA, as compared with the 2.5-fold increase in rate for des-His hemoglobin. Addition of IHP reduces the CO combination rate constants of des-Arg and des-His hemoglobin by the same factor, 3.5-fold. Since this organic phosphate reduces the rate of CO binding to HbA only 1.8-fold, in its presence CO binds 3.9-fold more rapidly to des-Arg hemoglobin than to HbA while, as already discussed, binding only 30% faster to des-His hemoglobin. Together these studies show that the functional properties and the structure of the hemoglobin T state are much more sensitive to modification of the α chain COOH termini than the β chain COOH termini.
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Structure and Oxygen Affinity of Crystalline des-His-146β Human Hemoglobin in the T State

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