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α-Subunit oxidation in T-state crystals of a sebacyl cross-linked human hemoglobin with unusual autoxidation properties

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Abstract

In the crystal structure of human T-state hemoglobin with a sebacyl residue cross-linking the two β-subunit Lys^{82}'s (DecHb), the Fe atoms of the α-subunit hemes are found to be oxidized with a water molecule bound. The three-dimensional structure and heme geometries were compared to those of deoxyhemoglobin and other partially and fully oxidized hemoglobins [R. Liddington, Z. Derewenda, E. Dodson, R. Hubbard, G. Dodson, J. Mol. Biol. 228 (1992) 551]. The heme geometries of the α-subunits are consistent with those observed in oxidized structures. The proximal histidines of the α-subunits move toward the heme plane shifting the F-helix and FG-corner in a manner observed for partially oxidized human hemoglobin. This supports the hypothesis that these perturbations may precede the T- to R-state transition. Circular dichroism studies comparing DecHb and natural human hemoglobin in the deoxy and CO ligated forms confirm that the conformations of the deoxy forms are identical, but the ligated forms have slight differences in the solution structures. DecHb is found to be more resistant to autoxidation than natural hemoglobin. The time course of autoxidation of DecHb shows that it is virtually absent for the first 1500 min followed by a rapid increase. Thus, the discovery of the oxidation of the α-subunits in the deoxy-crystals is quite unexpected. The data confirm that ligation of the α-subunits precedes that of the β-subunits. This may suggest a low ligand affinity of the α-diligated form of hemoglobin. © 1998 Elsevier Science B.V.

Keywords: Cross-linked hemoglobin; Protein crystallography; T-state hemoglobin; Three-dimensional structure; Circular dichroism; Autoxidation

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1. Introduction

Autoxidation of oxyhemoglobin is a complex process not entirely understood resulting from the transfer of one electron from Fe(II) to the bound O_2 with the formation of Fe(III) and superoxide [1,2]. Autoxidation and oxygen affinity are correlated, and autoxidation rates are inversely proportional to the oxygen affinity of hemoglobins. In a study of the rate of azide induced autoxidation, this correlation has been reported to be present also in chemically cross-linked hemoglobins [3]. Autoxidation has been extensively investigated in myoglobin using heme pocket mutants [4]. An analysis of this phenomenon in hemoglobin is complicated by the following factors. In solution, tetrameric and dimeric forms of the hemoglobin molecule are at equilibrium. The rate of hemoglobin oxidation has been found to be concentration dependent, with the dimer having a rate of methemoglobin formation that is 20-fold faster than the tetramers [5].

The crystallographic structure determination of R-state and T-state hemoglobins that are fully or partially oxidized have provided details of the perturbation in the structure of hemoglobin resulting from the oxidation of the Fe atom, and in some cases, from the presence of a bound water molecule or other ligands. The crystallographic structure determination of the R-state horse methemoglobin provided the first detailed picture of the α- and β-subunit heme geometry [6–9]. The displacement of the Fe atom from the porphyrin plane was estimated to be 0.07 ± 0.06 Å in the α-subunit and 0.21 ± 0.06 Å in the β-subunits. These were considerably smaller displacements from the heme plane than that for the first reported for the Fe atom in metmyoglobin of 0.40 Å [9]. More recently, the out-of-plane distance for the Fe atom in recombinant aquometmyoglobin has been found to be ~ 0.1 Å for the wild-type protein and ~ 0.3 Å for apolar mutations of the distal histidine [10]. Similar geometry has been observed in the structure of R-state porcine aquomethemoglobin [11]. The Fe atom associated with the β-subunit lies ~ 0.15 Å farther out of the heme plane than that of the α-subunit. It was also observed that the distance between the Fe atom and the distal histidine is 0.6 Å larger in the β_1-subunit than in the β_2-subunit. These studies have been complemented by a number of low-resolution difference Fourier studies of R-state methemoglobins with a variety of bound ligands that show qualitatively a variability in the met structures (e.g., Ref. [12]). The variance is presumably a result of differences in ligand size, geometry, and electronics.

Several T-state methemoglobin structures complement the R-state structures mentioned above. One of the first studies examined T-state deoxyhemoglobin crystals treated with acrylamide and then oxidized with air to form methemoglobin [13]. The difference Fourier at 3.5 Å resolution showed that the Fe atoms move toward the heme planes. It was postulated that this movement is the beginning of coupled changes that eventually result in the T- to R-state transition. The movement of the Fe atom tilts the heme weakening hydrogen bonds that need to be broken to make the tertiary and quaternary conformational changes necessary to go from the T- to the R-state. The presence of the water molecule introduces an opening to both the α- and β-subunit distal heme pockets. The largest opening is observed for the β-subunit. This provides a rationale for why the α-hemes undergo autoxidation more readily than the β-hemes.

More recently, high-resolution, partially oxygenated and oxidized T-state structures of human hemoglobin (T(α-oxy)Hb and T(met)Hb, respectively) [2] have been reported [14–17]. The T-state crystals are grown in low salt using polyethylene glycol 1000 or 8000 as the precipitant. In the T-state methemoglobin structure, all four subunits were found to be partially oxidized [15,17]. The occupancies for the ligands were 0.86, 0.46, 0.51, and 0.51 for the α_1, α_2, β_1 and β_2, respectively. A comparison of the T(α-oxy) and T(met)Hb show insignificant movements of residues associated with the distal heme pocket, and little change in the conforma-

[2] Abbreviations: T(α-oxy)Hb, the structure of a partially oxidized T-state human hemoglobin reported in Refs. [13–16]; T(met)Hb, a partially reduced T-state human hemoglobin reported in Refs. [13–16]; DecHb, sebacyl $\beta_1 Lys^{82} - \beta_2 Lys^{82}$ cross-linked human deoxyhemoglobin; RSR-56, 2[4-[((3,5-dimethoxyanilino)carbonyl)-methyl]phenoxy]-2-methylpropionic acid; (α-met, β-deoxy)DecHb, the structure of partially oxidized, T-state sebacyl $\beta_1 Lys^{82} - \beta_2 Lys^{82}$ cross-linked human hemoglobin; DecBDS, activated sebacic acid.

tion of the α-heme is seen [15]. The proximal histidine moves closer to the heme plane and moves progressively closer to the axial position with increasing levels of ligation. The Fe atom also moves more toward the heme plane, but doming of the heme prevents it from moving completely into it.

In another study, high-salt crystals of deoxyhemoglobin, cocrystallized in the presence of an effector, 2[4-[((3,5-dimethoxyanilino)carbonyl)-methyl]phenoxy]-2-methylpropionic acid (RSR-56), were found diligated and led to the discovery that all four subunits of hemoglobin in these deoxy crystals with RSR-56 could be completely ligated [18]. The 3.0 Å difference Fourier study indicated that RSR-56 was bound in the central cavity of the tetramers. Ligands were bound only to the Fe atom of the α-hemes in the diligated case and to all four hemes in the completely ligated structure. The difference maps of the diligated structure have density features only around the region of the α-hemes and the allosteric effector. The fully ligated structure had difference density features near all of the hemes, the allosteric effector and near the α_1/β_2-interface. Again, the changes suggest those needed to make the T- to R-state allosteric transition.

The three-dimensional structure of a partially oxidized T-state sebacyl-cross-linked human deoxyhemoglobin, (α-met, β-deoxy)DecHb, has been determined [19,20]. Here we describe and compare the oxidized hemes of the (α-met, β-deoxy)DecHb α-subunits and the heme environments of a deoxyhemoglobin [21] and an oxidized T-state hemoglobin [17]. The quaternary and tertiary structures still have the T-state conformation, but measurable changes are found in the positions of the proximal histidine and the associated F-helix and FG-corner. These changes are consistent with those found for oxidized T-state hemoglobin [17] providing further support that these perturbations reflect the initial structural changes in going from the T- to R-state. Additionally, circular dichroism measurements show small differences between oxidized DecHb and oxidized HbA limited to the α_1/β_2-interface. At variance with autoxidation of normal hemoglobin and other hemoglobin systems, where the rate of autoxidation seems to be inversely proportional to oxygen affinity, DecHb has a lower affinity than normal hemoglobin and a much lower rate of autoxidation.

2. Materials and Methods [3]

2.1. Hemoglobin isolation, cross-linking and purification

The preparation and purification of cross-linked human hemoglobin have been reported and discussed previously [19]. Briefly, human hemoglobin was prepared and purified as described [22]. Activation of sebacic acid (DecBDS) was previously described [23]. The cross-linking was carried out by adding DecBDS at a 2:1 to 4:1 molar ratio to that of tetrameric hemoglobin. The DecBDS was added to a 6 g dl^{-1} solution of human deoxyhemoglobin in 0.05 M borate buffer at pH 9.0 and then stirred at 37°C for 90 min under nitrogen. Glycylglycine was added to a final concentration of 0.1 M to stop the reaction. The solution was then dialyzed against 0.1 M glycine for at least 4 h in the cold. The β–β cross-linked product was purified by repetitive chromatography using columns packed with Mitsubishi DEAE-MCI resin (Mitsubishi) and a Waters Delta Prep 4000 HPLC. After loading the column, the hemoglobin adduct was eluted using gradients formed by 0.015 M tris–acetate at pH 8.2 and 0.015 M tris–acetate buffer in 0.2 M sodium acetate at pH 7.7.

2.2. Crystallization, X-ray data collection and processing

Crystals of DecHb were obtained by adapting the procedure described by Perutz [24]. Single crystals measuring more than 1.0 mm in their largest dimension grew after several weeks from deoxyhemoglobin solutions under nitrogen containing a protein concentration of 1 g dl^{-1} in 2.2 M ammonium sulfate, 0.3 M ammonium phosphate, and 0.01 M

[3] Certain commercial equipment, instruments and materials are identified in this paper in order to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the material, instrument or equipment identified is the best available for the purpose.

ferric citrate at pH 6.5 at room temperature. X-ray diffraction data were collected from a single crystal with dimensions $0.8 \times 1.0 \times 1.0$ mm^3 using a Siemens electronic area detector with a Rigaku RU200B rotating anode as the X-ray source. Data processing was carried out using the XENGEN program system [25]. The space group is $P2_1$ with unit cell parameters of $a = 62.8$ Å, $b = 82.5$ Å, $c = 53.3$ Å, and $\beta = 99.2°$. The final 1.9 Å data set was composed of 42,167 independent reflections comprising 99.6% of those possible at this resolution. The *R*-merge from 108,860 observations was 0.104, and the average $I/\sigma(I)$ in the outermost shell was 1.9. Complete details of data collection and processing are described previously [19].

2.3. Crystallographic structure refinement

The starting coordinates [19] included four polypeptide chains (α_1, β_1, α_2 and β_2), four hemes, two sulfate anions and 393 water molecules. Details of the further refinement of this model have been reported [20]. Briefly, the refinement used a combination of simulated annealing using the X-PLOR program [26] and the least-squares, geometric-optimization procedure PROLSQ [27,28]. The original least-squares algorithm was modified by Furey et al. [29] for execution on a CRAY Y-MP computer. The basic strategy was to improve the phases, and therefore the model, by extended refinement interspersed with model building. After 20 additional rounds of refinement and coordinate adjustment, the overall geometry improved significantly, especially that of side chains [20]. At this stage, the model contained 130 more water molecules than the starting model and the electron density for the 10-carbon linker was almost complete. After the atoms of the sebacyl residue were added to the model, sixteen additional rounds of refinement and adjustment were carried out. At this point no significant peaks in the difference Fourier ($F_o - F_c$) map were observed. The lower resolution data excluded from the refinement were included in all map calculations. The program O [30] was used on an Indigo2 computer with GR3-XZ graphics system for the examination of $2F_o - F_c$, $F_o - F_c$ and omit maps, the adjustment of the model and the deletion and addition of solvent molecules. The final coordinates and structure factors have been deposited in the Brookhaven Protein Data Bank [31] and assigned the ID code of **1CLS** and **RICLSSF**.

2.4. Structure and heme geometry comparison

The comparison of the final (α-met, β-deoxy)DecHb structure with that of deoxyHbA, **2HHD** [21], and of T(met)Hb, **1HGB** [17], was carried out using the ALIGN program [32]. The structures were superposed and visualized using O [30]. The temperature factor values from corresponding atoms in the structures were compared after scaling. (α-met, β-deoxy)DecHb was used as the reference structure in the positional and temperature factor comparisons.

The comparison of the hemoglobin heme geometries was done in the following way. All of the pyrrole atoms and bridging carbons were used to calculate the average heme plane, to compensate for heme puckering and nonsymmetric deformation of the heme. The heme plane was generated by first progressively taking the sum of the unit vectors resulting from the cross product of a vector from each atom of the heme and a vector to every other atom of the heme as shown in Eq. (1).

$$\boldsymbol{N}_{\mathrm{Avg}} = \left(\frac{2}{N(N-1)(N-2)} \right) \times \sum_{i}^{N} \sum_{j \neq i}^{N} \sum_{k > j}^{N} \frac{(\boldsymbol{r}_j - \boldsymbol{r}_i) \times (\boldsymbol{r}_k - \boldsymbol{r}_i)}{\left| (\boldsymbol{r}_j - \boldsymbol{r}_i) \times (\boldsymbol{r}_k - \boldsymbol{r}_i) \right|} \quad (1)$$

Choosing an atom of the heme, $\boldsymbol{r}_i$, one can make two vectors, $\boldsymbol{r}_j - \boldsymbol{r}_i$ and $\boldsymbol{r}_k - \boldsymbol{r}_i$, to two other heme atoms, $\boldsymbol{r}_j$ and $\boldsymbol{r}_k$. The cross product of these two vectors results in a vector that is orthogonal, or normal, to the two. Dividing the normal vector by its magnitude results in a unit normal vector. The cross products of the vectors of all the heme atoms to each other were converted into unit vectors, summed, and divided by the number of all the normal unit vectors to yield an average normal vector of the heme plane, $\boldsymbol{N}_{\mathrm{Avg}}$. The average x, y, and z coordinates of the heme atoms, provides a point on the average plane. The plane can then be defined by the equation, $Ax + By + Cz = D$, where A, B, and C are the constants of the average normal vector in the $\hat{i}$, $\hat{j}$, and $\hat{k}$ directions, respectively. Inserting the average x, y, and z coordinates into the equation and calculating the result, yields the

constant D, and defines a unique plane. The off-normal angle of the ligand was calculated by taking the dot product of the vector from the iron to the first ligand atom, $\boldsymbol{r}_{\mathrm{li}} - \boldsymbol{r}_{\mathrm{Fe}}$ and the normal vector of the average heme plane, $\boldsymbol{N}_{\mathrm{Avg}}$.

2.5. Autoxidation

Autoxidation experiments were carried out in a Hewlett-Packard 8452A diode array spectrophotometer. The initial protein concentration was 0.1 mg

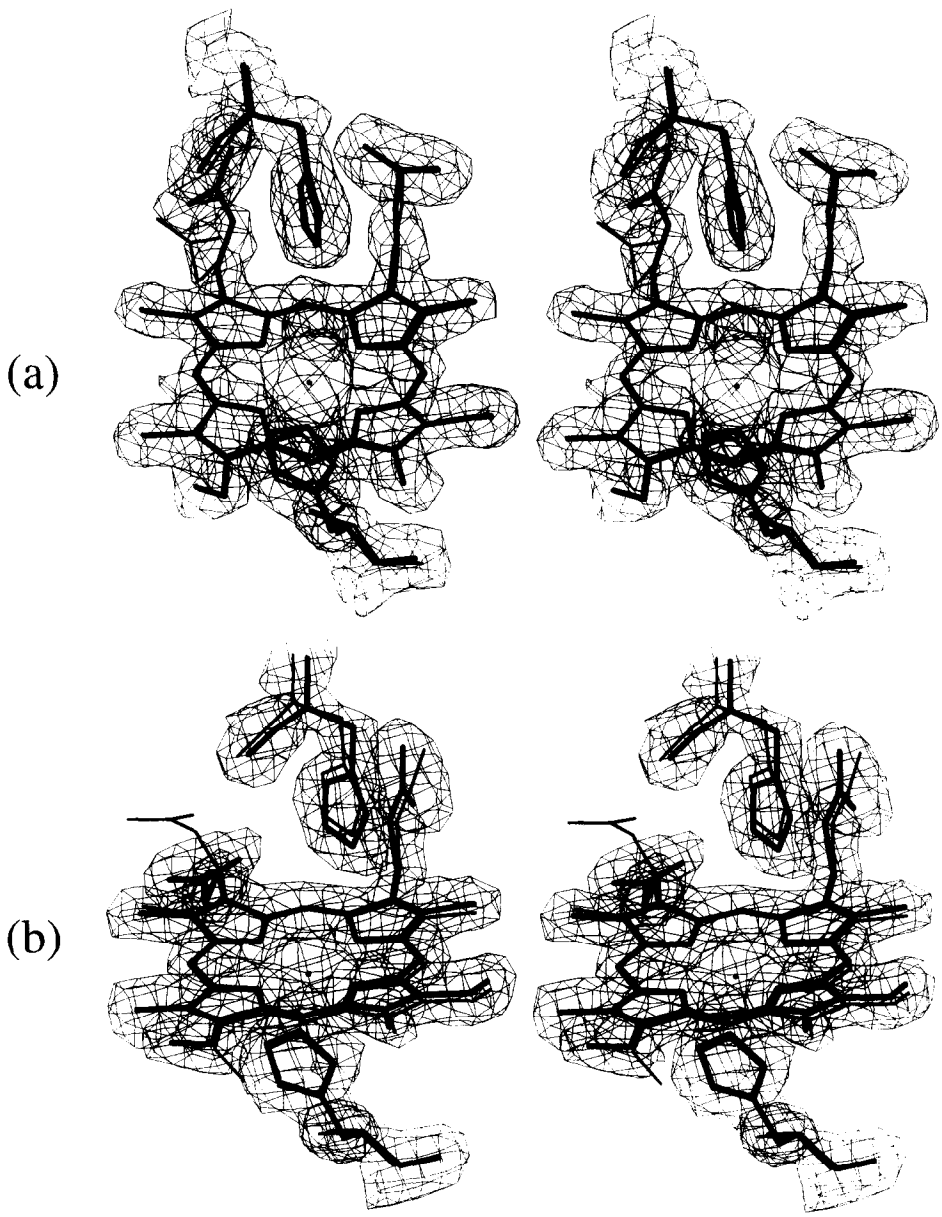

Fig. 1. Stereoviews of the electron density for the heme regions of (a) the α_1-subunit, and (b) the β_1-subunit. The $2F_o - F_c$ electron density maps are contoured at 1.0 σ. Thick lines connect atoms of (a) the α_1-subunit and (b) the β_1-subunit, while thin lines connect atoms of (a) the α_2-subunit and (b) the β_2-subunits superimposed on those of the the α_1- and β_1-subunits, respectively.

Table 1
A comparison of heme pocket geometries of the T-state deoxyhemoglobin (**2HHD** [20]), (α-met, β-deoxy)DecHb (**1CLS**, this work), and fully oxidized hemoglobin (**1HGB** [16])

ID	Subunit	B_{Fe} (Å^2)	B_O^a (Å^2)	Occ_O^a	N^b–Fe (Å)	N^b–O^a (Å)	O^a–Fe (Å)	O off-normal angle (°)	Fe distal to plane (Å)	Fe–N^c (Å)	Proximal His distal to plane (Å)	Proximal His off-normal angle (°)
2HHD	deoxy-α_1	9.5			4.6				−0.6	2.3	−2.9	9.0
	deoxy-α_2	10.8			4.3				−0.5	2.3	−2.8	12.5
	deoxy-β_1	10.3			4.0				−0.4	2.4	−2.8	9.7
	deoxy-β_2	8.9			4.2				−0.3	2.4	−2.6	9.6
1CLS	met-α_1	15.7	14.5	1.0	4.4	2.7	2.1	4.4	−0.3	1.9	−2.2	2.6
	met-α_2	16.7	17.8	1.0	4.3	2.6	2.1	2.9	−0.3	2.2	−2.5	11.4
	deoxy-β_1	13.6			4.1				−0.5	2.2	−2.6	6.7
	deoxy-β_2	15.4			4.4				−0.4	2.1	−2.4	9.4
1HGB	met-α_1	21(17)	20(17)	1(.86)	4.3	2.5	2.3	4.9	−0.2	2.2	−2.3	7.4
	met-α_2	22(21)	20(21)	1(.46)	4.3	2.5	2.3	4.5	−0.3	2.4	−2.6	12.6
	met-β_1	16(17)	20(17)	1(.51)	4.0	2.5	2.1	3.4	−0.2	2.2	−2.4	1.2
	met-β_2	27(25)	20(25)	1(.51)	3.9	2.4	2.1	5.2	−0.2	2.1	−2.3	4.2

[a]Oxygen atom of met H_2O.
[b]$N^{\in 2}$ atom of distal His.
[c]$N^{\in 2}$ atom of proximal His.
[d]Values in parentheses are taken from Ref. [16] and those out of parentheses from the Protein Data Bank entry **1HGB** [16].

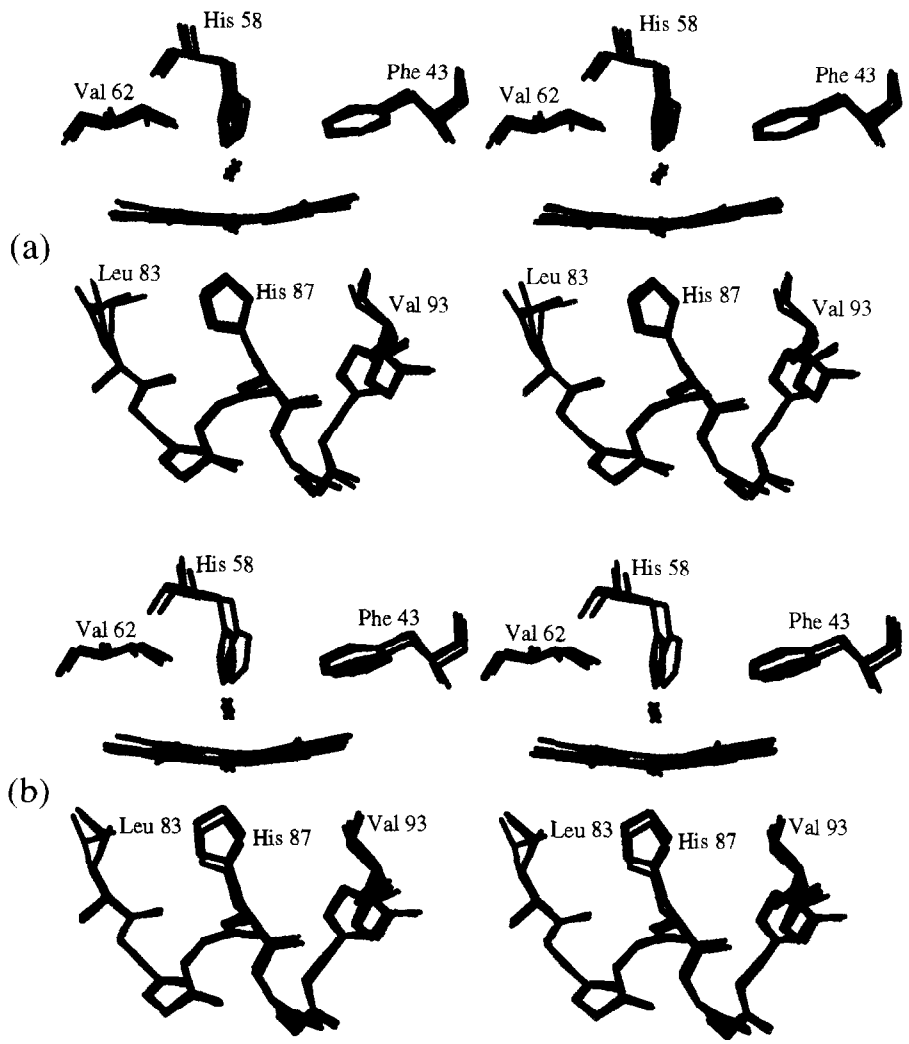

Fig. 2. Stereoviews of the superposition of the (α-met, β-deoxy)DecHb (blue), deoxyHbA (**2HHD**) [20] (red), and T(met)Hb (**1HGB**) [16] (green) (a) α_1-heme pockets and (b) the α_2-heme pockets. The orientation of the atoms of each structure is based on the superposition of the heme groups.

ml^{-1}, in 0.1 M phosphate buffer with 1 mM EDTA at pH 7.0. Beef liver catalase (Boehringer Mannheim) was added to the sample in a molar ratio of 0.003 M $heme^{-1}$ in order to prevent peroxide induced oxidation, and all experiments were carried out at 37°C. Superoxide dismutase (SOD) was not found to alter the rate of autoxidation for HbA in the presence of catalase plus EDTA, and therefore was not used in these experiments.[4] Absorption spectra were recorded every 20 to 30 min for 72 h. The hemoglobin samples were continuously equilibrated with a stream of 30% wet O_2 flowing through a rubber septum over the surface of the liquid to maintain saturation during the analysis. Moreover, moderate stirring of the sample was carried out during the measurement to insure a uniform distribution of oxygen throughout the cuvette.

The absorption spectra were decomposed linearly into a series of predetermined standard spectra of oxyhemoglobin, deoxyhemoglobin, and aquomethemoglobin (at pH 7.0). A standard baseline was used

[4] Karavitis, Fronticelli, Bucci, unpublished work.

to correct for the turbidity appearing at later times. The wavelength dependence of the baseline was estimated from the difference of the absorption spectra of solutions and after clearing. The decomposition also allowed correction of the optical density of the spectra for the loss of protein due to turbidity. The fraction of methemoglobin was plotted as a function of time and then fit to a first-order kinetic equation,

$$\mathrm{Hb(III)} = A_i(1 - e^{-k_i t}) + B_i \qquad (2)$$

where Hb(III) is the fraction of methemoglobin, A_i is the maximum fractional amount of the ith component, k_i is the corresponding first-order rate constant, t is the time in minutes, and B_i its initial fractional amount.

2.6. Circular dichroism measurements

An AVIV 60DS spectropolarimeter was used to carry out circular dichroism measurements. The hemoglobin was in 50 mM phosphate buffer at pH 7.0. The protein concentration was 0.5 mg ml^{-1}. Deoxygenation was obtained by adding sodium dithionite to oxygenated derivatives. Carbon monoxy derivatives were obtained by equilibrating the oxygenated solutions with carbon monoxide at atmospheric pressure.

3. Results

3.1. Three-dimensional structure of (α-met, β-deoxy)DecHb

The final model of the T-state (α-met, β-deoxy)DecHb resulting from the least-squares refinement includes the α_1-, β_1-, α_2-, and β_2-subunits with their associated hemes, the sebacyl residue that covalently cross-links the two β-subunits, 2 sulfate anions and 478 water molecules. Each of the Fe atoms of the α_1- and α_2-subunit hemes have a water molecule as a ligand in the distal heme pocket. The associated ligand and the heme geometry show that both Fe atoms are oxidized. The final structure has a crystallographic R factor of 0.168 for all data with $I \geq \sigma(I)$ between 6.0 and 1.9 Å with RMS deviations from ideal distance of 0.018 Å.

Only a brief description of the (α-met, β-deoxy)DecHb structure will be presented here since the overall three-dimensional structure is described elsewhere [20]. The 10-carbon sebacyl residue found in the β-cleft covalently links the two βLys^{82} side chains. The sebacyl residue assumes a zigzag conformation with *cis* pseudo-peptide bonds formed by the N^{ζ} atoms of βLys^{82} residues and the sebacyl carbonyl oxygen atoms. The atoms of the cross-link have an occupancy factor of 1.0 with an average temperature factor for all atoms of 34 Å^2. An RMS deviation of 0.27 for all C^{α}'s of the tetramer is observed when (α-met, β-deoxy)DecHb is compared deoxyHbA refined using a similar protocol, **2HHD** [21]. Thus, no significant perturbations in the tertiary or quaternary structure are introduced by the presence of the sebacyl residue. However, the sebacyl residue does displace seven water molecules in the β-cleft and the conformations of the β_1Lys^{82} and β_2Lys^{82} are altered as a consequence of the cross-linking. The carbonyl oxygen that is part of the amide bond formed with the N^{ζ} of β_2Lys^{82} forms a hydrogen bond with side chain of β_2Asn^{139} that is in turn hydrogen bonded to the side chain of β_2Arg^{104}.

3.2. Oxidized α-subunit heme environment

Although the crystals of (α-met, β-deoxy)DecHb were grown under the high-salt conditions that favor the formation of deoxy T-state crystals, a water molecule was bound to the Fe atom in each of the α-subunit distal heme-pockets. In contrast, no ligand was associated with the Fe atoms of the β-subunits. The $2F_o - F_c$ electron density maps for the distal and proximal histidines and the hemes of the α_1- and β_1-subunits are shown in Fig. 1a and b. The electron density is clearly visible above the Fe atom in the α-subunit and absent in the β-subunit. The water molecules in the α_1- and α_2-subunits have full occupancy and the temperature factors of 14.5 and 17.8 Å^2, respectively. These values are similar to those found for the respective Fe atoms, 15.7 and 16.7 Å^2. The geometry of the structure associated with the ligand in both the α_1- and α_2-subunits is similar (see Table 1). The distances between the O atom of the water molecules and the Fe atoms for

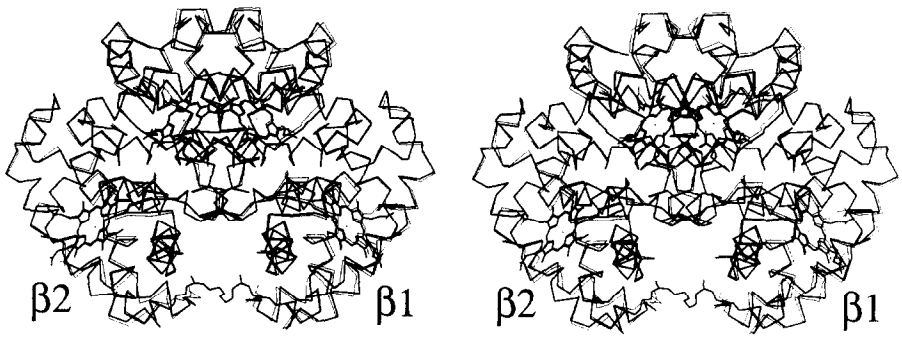

Fig. 3. Stereoview of the superposition of the C_α trace of (α-met, β-deoxy)DecHb superimposed on that of T(met)Hb (**1HGB**) [16]. In addition to the C_α trace for each molecule, the hemes are included. (α-met, β-deoxy)DecHb is represented by thick lines, while the T(met)Hb is represented by thin lines.

both the α_1- and α_2-subunits of (α-met, β-deoxy)DecHb are 2.1 Å. The water molecules are also involved in hydrogen bonds with the distal histidines. The distances between the O atom of the water molecules and the $N^{\epsilon 2}$ of $\alpha_1 His^{58}$ and $\alpha_1 His^{58}$ are 2.7 and 2.6 Å, respectively.

3.3. Comparison of (α-met, β-deoxy)DecHb and deoxyHbA

An overall comparison of the structure of (α-met, β-deoxy)DecHb with that of deoxyHbA, **2HHD** [33], has been reported earlier [20]. The RMS deviation for all 574 C^α atoms between the deoxyHbA and (α-met, β-deoxy)DecHb structures is 0.27 Å. The presence of the sebacyl cross-link shown bridging the two β-subunits induces no obvious tertiary or quaternary conformational changes in the T-state tetramer. Conformational differences in side chains and a reorganization of the solvent near the cross-link are observed. A comparison of the temperature factors of (α-met, β-deoxy)DecHb and deoxyHbA shows good agreement even for the protein atoms associated with the cross-link. Thus, the temperature factors in this region show no stabilizing or destabilizing effects of the presence of the cross-link.

In contrast to the overall structural similarities, the comparison of the α-subunit heme environments shows significant differences because of the oxidation of the Fe atom and the presence of the water molecule in (α-met, β-deoxy)DecHb. The differences in geometry are summarized in Table 1 and

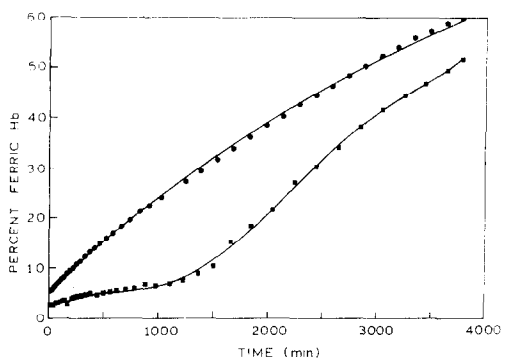

Fig. 4. The time course of autoxidation of natural HbA (●) and DecHb (■) in 0.1 M phosphate buffer pH 7.0 at 37°C. The interpolating lines represent the fit obtained using a single rate, $k = 2.20 \pm 0.04 \times 10^{-4}$ min^{-1}, for natural HbA and two rate constants, $k_1 = 4.34 \pm 0.1 \times 10^{-5}$ min^{-1} and $k_2 = 2.70 \pm 0.04 \times 10^{-4}$ min^{-1}.

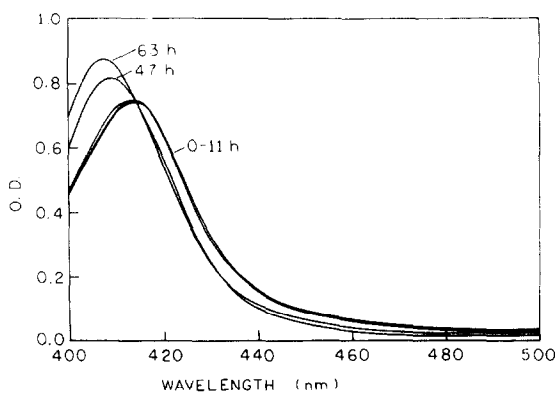

Fig. 5. Absorption spectra of DecHb taken at different times during the autoxidation.

illustrated in Fig. 2. In the α_1- and α_2-subunits the iron atoms move closer to the heme plane by 0.3 and 0.2 Å, respectively. The proximal histidines also move closer to the Fe atoms (by 0.4 and 0.1 Å, respectively), and therefore, closer to the heme plane (by 0.7 and 0.3 Å). In turn, the residues of the F-helix and FG-corner move toward the heme. The shifts in position of these residues are larger in the α_1-subunit than in the α_2-subunit. On the other hand, the heme environments of the two β-subunits are quite similar to those in deoxyHbA (see Table 1).

3.4. Comparison of (α-met, β-deoxy)DecHb with T(met)Hb

The differences in the overall structure of the cross-linked (α-met, β-deoxy)DecHb and T(met)Hb [17] are similar to those found between (α-met, β-deoxy)DecHb and deoxyHbA. The RMS deviation is 0.27 Å for the 574 C^{α} atom pairs of the (α-met, β-deoxy)DecHb and T(met)Hb structures. The superposition of (α-met, β-deoxy)DecHb and T(met)Hb is shown in Fig. 3. Since both T-state structures have the α-subunits oxidized, the geometry of the heme environments is nearly the same (see Fig. 2 and Table 1). The perturbations in both the T-state structures near the α-subunit hemes relative to that of deoxyHbA are almost identical. In both cases, the α_1-subunit has the largest change in position of the F-helix and FG-corner with smaller changes observed for the α_2-subunit. However, because the β-subunits of T(met)Hb are oxidized, the heme environments are significantly different from those of (α-met, β-deoxy)DecHb (see Table 1).

3.5. Autoxidation of DecHb

The time courses of autoxidation of HbA and DecHb are shown in Fig. 4. For HbA the experimental data points can be described by Eq. (2) with a single exponential with a rate of 2.2 ($\pm$0.04) $\times$ 10^{-4} min^{-1}. In the cross-linked derivative the phenomenon was more complex. Autoxidation is practically absent during the first 1500 min. After this time, a rapid increase in methemoglobin formation is observed. The slow and fast phases can be described by Eq. (2) with rates of 4.34 ($\pm$0.10) $\times$ 10^{-5} min^{-1} and 2.70 ($\pm$0.04) $\times$ 10^{-4} min^{-1} for the slow and

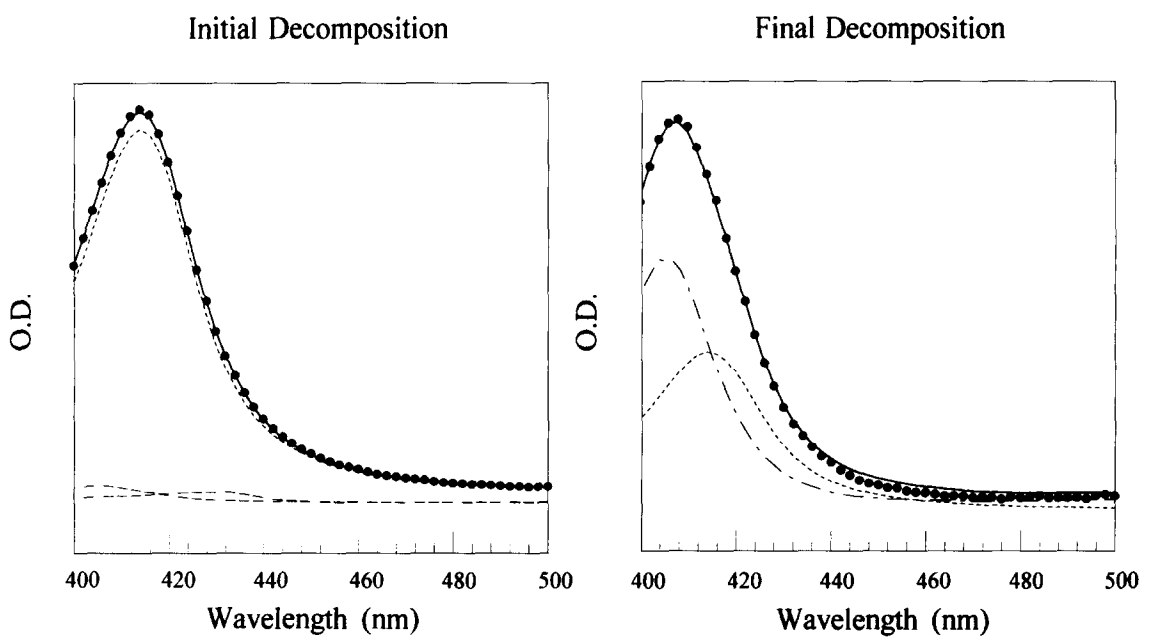

Fig. 6. Decomposition of the absorption spectra of DecHb at zero time (left) and after 63 h (right) during the autoxidation. At zero time, the various species were 95% oxy-, 3% ferric-, and 2% deoxyhemoglobin. After 63 h they were 48% oxy- and 52% ferricDecHb with no trace of the deoxy form.

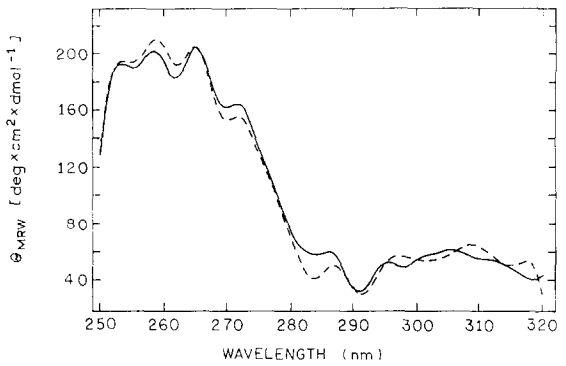

Fig. 7. The CD spectra of ferric natural HbA (dashed line) and DecHb (continuous line) in 0.05 M phosphate buffer at pH 7.0. The protein concentration was near 1 mg ml^{-1}.

fast rates, respectively. Absorption spectra of DecHb measured at different times between 0 and 63 h are shown in Fig. 5. The isosbestic points are perfectly conserved throughout the experiment indicating the presence of only two components in the mixture. In fact, at no time does the decomposition show the presence of more than two components, namely oxy- and ferric-DecHb, as shown in the decomposition of the spectrum taken after 63 h, Fig. 6.

3.6. Circular dichroism measurements

The CD spectra of carbon monoxy and deoxy-DecHb were practically superimposable to those of normal HbA in the far and near UV and in the Soret regions. Small, but distinct, differences between the CD spectra of the ferric forms of DecHb and HbA are observed in the near UV region, only. In particular, the spectrum of metDecHb did not show a trough at 280 nm, the region where the signal of βTrp^{37} is present. This is shown in Fig. 7.

4. Discussion

4.1. Structure of a partially oxidized T-state

The (α-met, β-deoxy)DecHb structure represents that of the first refined, high-resolution, high-salt, partially oxidized T-state human hemoglobin. Earlier difference Fourier studies of oxidized high-salt crystals required cross-linking with acrylamide [13] or the presence of an effector, RSR-56 [18], to stabilize the partially oxidized hemoglobin in the T-state. The presence of the sebacyl cross-link in diligated (α-met, β-deoxy)DecHb appears to serve the same purpose. The cross-linking with acrylamide, the RSR-56 effector, or the presence of the sebacyl cross-link appear to be required to stabilize the T-state conformation upon ligation. The α-subunits are preferentially ligated in the T-state structures.

The comparison of partially oxidized and ligated low-salt crystal forms of T-state human hemoglobin [14–17] with high-salt (α-met, β-deoxy)DecHb illustrates that not only are the T-state structures virtually identical, but so are the changes observed in the heme-pocket regions of the α-subunits. The movement of the F helices and FG corners with respect to the hemes in the α-subunits are similar despite the differences in crystallization conditions and crystal packing contacts. In fact, in both the high-salt and low-salt structures larger changes are observed in one of the α-subunits relative to the other. These observations are supportive of the hypothesis that the changes observed are those that precede the T- to R-state allosteric transition.

4.2. Autoxidation of DecHb

It is a common observation that autoxidation of oxyhemoglobin is favored by low oxygen affinity of the system. Natural mutant hemoglobins with lower oxygen affinity exhibit an increased rate of autoxidation [34]. Also, low-oxygen-affinity cross-linked hemoglobins, like those linked between the two α-subunit Lys^{99} residues [35], have higher autoxidation rates, while cross-linked hemoglobins with higher oxygen affinity show reduced oxidation rates [3].

Without an inducer, autoxidation of normal human hemoglobin was shown to be concentration dependent, with hemoglobin dimers having about a 20-fold higher rate of autoxidation than the tetramers [5]. Thus, at the low protein concentrations used in our measurements (0.1 mg ml^{-1}), dimers contributed significantly to the observed rate of autoxidation that appeared monoexponential.

In DecHb the intermolecular cross-link between the βLys^{82} residues prevents the dimerization of the molecule, and little methemoglobin was formed dur-

ing the first 23 h. This was unexpected in view of the low oxygen affinity of the sample. It is consistent however, with the stabilization of the tetrameric structure produced by the cross-link. After 23 h the process becomes autocatalytic, the autoxidation rate increases about 20-fold and approaches the rate measured for normal hemoglobin, presumably for the dimeric form.

The autocatalytic autoxidation behavior of DecHb has an additional important implication. It indicates that the ferrous and ferric subunits have different conformations. When unlike subunits are forced together by the cross-link, the system acquires an instability resolved only by complete oxidation of the tetramer. DecHb cannot form dimers. However, there is a general parallelism between these data and those of Zhang et al. [5] on normal hemoglobin. In that study, the difference in the autoxidation rate of the dimers was approximately 20 times greater than that of the tetramers. The higher rate of the dimer autoxidation was proposed to show modification of the heme environment due to the loss of the α_1/β_2-contacts [5]. In DecHb the conformational change due to heme oxidation alters the interfaces of the mixed tetramers making their hemes less resistant to oxidation. Autocatalysis is not detectable in the autoxidation of HbA probably because the process is diminished by the kinetics of the dimers.

Finding a clear correlation between the crystallographic data of partial oxidation of DecHb and its autocatalytic behavior in solution is difficult. It is possible that the hybrid α-met/β-deoxy tetramers represent the molecules in solution at the end of 23 h of incubation, before autocatalysis starts. What is not clear is why the α-subunits did not remain oxygenated as in the various reports of other T-state ligated hemoglobins [14–17]. We may speculate that the different liganded structure proposed for DecHb [19] affects the α-subunits decreasing their oxygen affinity, thereby favoring oxidation over oxygenation.

It is notable that in all of the hybrid ferrous–ferric and partial ferric system so far investigated by X-ray diffraction studies, the α-subunits are either preferentially or the only ones to be in ligated conditions. This strongly suggests that the doubly α-ligated form of hemoglobin has a peculiar stability. Edelstein [36] has shown that the introduction of a stable intermediate of ligation in the system would decrease its ligand binding cooperativity. This, however, is not true if the new intermediate has ligand affinity lower than that of the preceding ligation step. In DecHb an initial negative binding cooperativity leads to a low affinity at the second step of oxygenation. A burst of positive cooperativity follows when the increasing ligand concentration forces the further ligation of the tetramer [19]. Therefore, if this system is exposed to low partial pressures of oxygen it will relax into a partial ligation selectively involving only the initial binding sites, which make the molecule less sensitive to oxygen. The data suggest that these initial sites are the hemes of the α-subunits.

While the presence of an intermediate at low oxygen affinity is evident in DecHb, it is not clear whether this is a general phenomenon in hemoglobin. A very low value to the overall Adair's constant β_3 has been assigned to the third step of oxygenation [19,37], which indicates the presence of a low oxygen affinity intermediate. Also, all of the crystallographic evidence regarding ferrous–ferric hybrid crystals, as mentioned, strongly supports the view that this is a general phenomenon in human hemoglobin. It is possible that ligand binding cooperativity in hemoglobin is produced by the very low oxygen affinity of the β-subunits, after the initial ligation of the α-hemes.

At least in the crystal lattices, this intermediate is essentially a modified T-like structure. It should be stressed that these intermediates of total oxygenation probably sit in a broad and shallow minimum of conformational energy. Thus, crystal forces and the presence of PEG, acrylamide, cross-links, and α-subunit effectors may have helped the selectivity of the crystal formation in favor of the more stable T-state conformation.

4.3. Circular dichroism measurements

The comparison of the atomic coordinates of (α-met, β-deoxy)DecHb and deoxyHbA shows that the structures of the two proteins are essentially identical. However, the functional properties of the cross-linked hemoglobin have been modified by the presence of the covalent bridge between the β-subunits [19]. The oxygen affinity is lowered as indicated by the $p50$ that goes from 15 to 26 Torr. The Bohr

effect is also decreased. Analysis of the functional data is consistent with the crystallographic data in that they show that the deoxy structure has identical oxygen affinity in the natural and in the cross-linked systems. The data also support the hypothesis that the change in oxygen affinity is due to a modification of the ligated structure of the system.

As reported by Bucci et al. [19], these functional differences are due to differences in the ligated state of the proteins. No clear difference is found in the CD spectra of the deoxy- and CO-forms of DecHb and normal hemoglobin. Circular dichroism of hemoglobin in the Soret region is sensitive to modification of the hemoglobin structure. In fact, the signal is due primarily to the relative position of the heme to that of phenylalanine and tyrosine residues near the heme pocket, which are positioned in different helical segments of the three-dimensional structure. In the far UV the signal of human hemoglobin is largely dominated by that of the helical structure. These signals are not modified in ferrous DecHb, suggesting that the conformational change produced by the cross-link does not affect the secondary and tertiary structure of hemoglobin, at least in its fully ligated and fully unligated forms. Unfortunately, the CD behavior of the intermediates of ligation cannot be measured. Distinct small differences are found in the near UV region between the ferric forms of DecHb and HbA. This may reflect different states of polymerization of the two systems, since DecHb does not form dimers. The presence of the cross-link appears to only effect the α_1/β_2-interface, without modifications of the heme pocket (Soret region) and of the secondary structure of the protein (far UV region).

As mentioned above, the autocatalytic behavior of the kinetics of autoxidation of oxyDecHb strongly suggests the presence of a conformational change of the protein upon its transition from the ferrous-ligated form to the ferric form. In the near UV range, the CD spectra of the ferric derivative does not show the trough at 280 nm present in normal ferric hemoglobin. In the normal protein, the signal is due to the dichroism of $\beta\,\mathrm{Trp}^{37}$ at the α_1/β_2-interface. The appearance of the signal, when normal hemoglobin is oxidized, supports the hypothesis of Zhang et al. [5] that the higher autoxidation rate of the dimers is due to modifications of the α_1/β_2-interface. It is possible that the presence of the cross-link alters the environment around this residue.

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References

[1] J.J. Weiss, Nature 202 (1964) 83.
[2] W.J. Wallace, R.A. Houtchens, J.C. Maxwell, W.S. Caughy, J. Biol. Chem. 257 (1982) 4966.
[3] V.W. MacDonald, K.D. Vandergriff, R.M. Winslow, D. Currell, C. Fronticelli, J.C. Hsia, J.C. Bakker, Biomater. Artific. Cells Immobilizat. Biotechnol. 19 (1993) 425.
[4] R.E. Brantley, S.J. Smerdon, A.J. Wilkinson, E.W. Singleton, J.S. Olson, J. Biol. Chem. 268 (1993) 6995.
[5] L. Zhang, A. Levy, J.M. Rifkind, J. Biol. Chem. 266 (1991) 24698.
[6] M.F. Perutz, M.G. Rossmann, A.F. Cullis, H. Muirhead, G. Will, A.C.T. North, Nature 185 (1960) 185.
[7] M.F. Perutz, H. Muirhead, J.M. Cox, L.C.G. Goaman, F.S. Mathews, E.L. McGandy, L.E. Webb, Nature 219 (1968) 29.
[8] M.F. Perutz, H. Muirhead, J.M. Cox, L.C.G. Goaman, Nature 219 (1968) 131.
[9] R.C. Ladner, E.J. Heidner, M.F. Perutz, J. Mol. Biol. 114 (1977) 385.
[10] M.L. Quillin, R.M. Ardvini, J.S. Olson, G.N. Phillips Jr., J. Mol. Biol. 234 (1993) 140.
[11] D.S. Katz, S.P. White, W. Huang, R. Kumar, D.W. Christianson, J. Mol. Biol. 224 (1994) 541.
[12] K. Moffat, J.F. Deatherage, D.W. Seybert, Science 206 (1979) 1035.
[13] L. Anderson, J. Mol. Biol. 79 (1973) 495.
[14] A. Brzosowski, Z. Derewenda, E. Dodson, G. Dodson, M. Grabowski, R. Liddington, T. Skarżyński, D. Vallely, Nature 307 (1984) 74.
[15] R. Liddington, Z. Derewenda, G. Dodson, D. Harris, Nature 331 (1988) 725.
[16] D.A. Waller, R.C. Liddington, Acta Crystallogr., Sect. B 46 (1990) 409.
[17] R. Liddington, Z. Derewenda, E. Dodson, R. Hubbard, G. Dodson, J. Mol. Biol. 228 (1992) 551.
[18] D.J. Abraham, R.A. Peascoe, R.S. Randad, J. Panikker, J. Mol. Biol. 227 (1992) 480.
[19] E. Bucci, A. Razynska, H. Kwansa, Z. Gryczynski, J.H. Collins, C. Fronticelli, R. Unger, M. Braxenhardt, J. Moult, X. Ji, G.L. Gilliland, Biochemistry 35 (1996) 3418–3425.
[20] X. Ji, M. Braxenthaler, J. Moult, C. Fronticelli, E. Bucci, G.L. Gilliland, Proteins Struct. Funct. Genet. (1997), submitted.

[21] C. Fronticelli, I. Pechik, W.S. Brinigar, L. Kowalczyk, G.L. Gilliland, J. Biol. Chem. 269 (1994) 23965.
[22] E. Bucci, H. Malak, C. Fronticelli, I. Gryczynski, G. Laczko, J.R. Lakowicz, Biophys. Chem. 32 (1988) 187.
[23] A. Razynska, J. Rak, C. Fronticelli, E. Bucci, J. Chem. Soc., Perkin Trans. 2 (1991) 1531.
[24] M.F. Perutz, J. Cryst. Growth 2 (1968) 54.
[25] A.J. Howard, G.L. Gilliland, B.C. Finzel, T.L. Poulos, D.H. Ohlendorf, F.R. Salemme, J. Appl. Crystallogr. 20 (1987) 383.
[26] A.T. Brünger, X-PLOR, Version 3.1 Manual, Yale Univ. Press, New Haven, 1992, p. 187.
[27] W. Hendrickson, J. Konnert, in: R. Diamond, S. Ramaseshan, K. Venkatesan (Eds.), Computing in Crystallography, Indian Academy of Sciences, 1980, p. 1301.
[28] W. Hendrickson, Methods Enzymol. 115 (1985) 252.
[29] W. Furey, B.C. Wang, M. Sax, J. Appl. Crystallogr. 15 (1982) 160.
[30] T.A. Jones, J.-Y. Zou, S.W. Cowan, M. Kjeldgaard, Acta Crystallogr., Sect. A 47 (1991) 110.
[31] F.C. Bernstein, T.F. Koetzle, G.J.B. Williams, E.F. Meyer Jr., M.D. Brice, J.R. Rogers, O. Kennard, T. Shimanouchi, M. Tasumi, J. Mol. Biol. 112 (1977) 535.
[32] Y. Satow, G. Cohen, E.A. Padland, D.R. Davies, J. Mol. Biol. 190 (1986) 593.
[33] I. Pechik, X. Ji, K. Fidelis, M. Karavitis, J. Moult, W.S. Brinigar, C. Fronticelli, G.L. Gilliland, Biochemistry 35 (1996) 1935.
[34] R.E. Dickerson, I. Geis, Hemoglobin: Structure, Function, Evolution and Pathology, The Benjamin/Cummings Publishing Company Inc., Menlo Park, California, 1983.
[35] R. Chatterjee, E.V. Welty, R.Y. Walder, S.L. Pruitt, P.H. Rogers, A. Arnone, J.A. Walder, J. Biol. Chem. 261 (1986) 9929.
[36] S. Edelstein, J. Mol. Biol. 257 (1996) 737.
[37] S.J. Gill, C.H. Robert, M. Coletta, E. DiCera, M. Brunori, Biophys. J. 50 (1986) 747.