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Noncooperativity of the $\alpha\beta$ Dimer in the Reaction of Hemoglobin with Oxygen

(human/dissociation/equilibrium/sulfhydryl/absorption/x-ray analysis)

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ABSTRACT **The theory that the $\alpha\beta$ dimer is the functional unit of cooperativity in hemoglobin has been tested by determination of the oxygen equilibrium curve of stable deoxy dimers, obtained by the addition of 0.9 M $MgCl_2$ to human des-Arg 141α-hemoglobin. Cooperativity was absent in this medium, but was regained on transfer of the hemoglobin to a dilute phosphate buffer, where tetramers reformed. X-ray analysis of crystals of oxy- and deoxy-des-Arg hemoglobins showed that the removal of Arg 141α would leave the structure of $\alpha\beta$ dimers unchanged. Nonreactivity of the sulfhydryl groups at 112β proved that the subunits in deoxy dimers form the same contact as in oxy dimers, namely $\alpha_1\beta_1$, and that no significant dissociation into free subunits occurs in 0.9 M $MgCl_2$. The absorption spectrum of the deoxy dimers corresponded to the sum of the spectra of the free deoxy α and β subunits, and was different from that of the deoxy tetramer, showing the constraining salt bridges formed by the C-terminal residues in the tetramer to be necessary for the spectral changes normally observed on association of the deoxy subunits.**

Hemoglobin is a tetramer made up of two kinds of subunits, known as α and β, which are identical in pairs. Its four iron atoms react cooperatively with oxygen, and the degree of cooperativity is usually expressed by the coefficient n in Hill's equation (1) which could, in a tetramer, theoreticall attain a value of four, but is never observed to be more than three.

When studying the effect of neutral salts on the molecular weight and oxygen equilibrium of human hemoglobin, Rossi Fanelli, Antonini, and Caputo found that high salt concentrations, such as 2 M NaCl, led to dissociation of the hemoglobin tetramer, the degree of apparent dissociation being about equal for oxy- and deoxyhemoglobin (2). Under these circumstances, one would have expected Hill's constant to decrease but this was not observed; on the contrary, there appeared to be a small increase of n and a decrease of the oxygen affinity (3). Later work established that the tetramer dissociates predominantly into dimers, rather than monomers (3), that the dimers are made up of one α and one β subunit (4–6), and that they have the structure $\alpha_1\beta_1$ (7). This implies, paradoxically, that the $\alpha_1\beta_1$ dimer has a Hill's constant of three. As a tentative interpretation of the paradox Antonini suggested that the $\alpha\beta$ dimer is the functional unit of hemoglobin and possesses strong cooperativity. Since a dimer cannot have Hill's constant greater than two, he attributed the excess to "additional effects over and above intramolecular interactions within the $\alpha\beta$ dimer" (8).

Abbreviation: BES = *N*,*N*-Bis-(2-hydroxyethyl)-2-aminoethanesulphonic acid.

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It was clear from the outset that the paradox might be resolved if deoxyhemoglobin failed to dissociate in strong salt, but not until recently were the sedimentation velocities and equilibria of very dilute deoxyhemoglobin solutions in concentrated salt solutions carefully checked (9, 10). After introducing proper precautions to prevent oxidation or oxygenation of hemoglobin during the ultracentrifuge run, Kellett could observe no dissociation of the deoxy form in any of the salt solutions formerly believed to produce dimers. The sensitivity of his methods would have allowed such dissociation to be detected if the dissociation constant of deoxyhemoglobin were greater than 10^{-4} that of oxyhemoglobin. On the basis of Kellett's results, the high value of n in concentrated salt solutions is no longer paradoxical, because the cooperative effects could take place in the tetrameric deoxy form, and dissociation into dimers could follow oxygenation. On the other hand, his results do not exclude the presence of cooperative effects within the $\alpha\beta$ dimer.

Evidence against cooperativity of the dimer has been advanced on structural and kinetic grounds. Oxy- and deoxyhemoglobin have different quaternary structures; in the transition between them the contacts $\alpha_1\alpha_2$, $\alpha_2\beta_1$, and $\alpha_1\beta_2$ play a vital part. In the $\alpha_1\beta_1$ dimer, these interfaces would be open so that no interactions could take place between them (11–13). Oxy- and deoxyhemoglobin also differ kinetically, oxygen reacting much faster with the form that has the quaternary oxy structure. In kinetic studies, short-lived deoxy dimers obtained by three different methods reacted rapidly with ligands, and failed to show the usual transition between slowly and rapidly reacting forms (14–16).

However, because of the short-lived nature of the dimer it can be argued that these results are open to alternative interpretations. We have, therefore, tried to prepare deoxy dimers in a stable yet structurally unaltered form, and to determine their oxygen equilibrium curve.

The great resistance of deoxyhemoglobin to dissociation into dimers must be due to additional bonds between the subunits that exist in deoxy- but not in oxyhemoglobin. The structures of the two forms show that these additional bonds consist of salt bridges between polar groups of opposite charge (12, 13). Dominant among them appear to be the bridges between the guanidinium group of the C-terminal arginine of one α subunit and the carboxyl group of aspartate H9 (126) of its neighbor. It seemed to us that once these bridges were removed, high salt concentrations might well cause the remaining salt bridges between the subunits to break, and the deoxy tetramer might dissociate into stable dimers. However, such an experiment would give a valid

answer only if one could rely on the removal being without effect on the structure of the α and β subunits.

The position of the C-terminal arginines in oxyhemoglobin is indicated in Fig. 1. In the tetramer they lie buried in the internal cavity between the two α subunits. In the $\alpha_1\beta_1$ dimer the arginine lies at the surface of the α subunit and has no neighboring groups of complementary charge with which it could link up; it would be held merely by the peptide bond linking it to tyrosine 140 and would be otherwise free to rotate, so that its loss or the exposure of the α-carboxyl group of tyrosine 140 would be unlikely to affect the structure of the dimer.

We have removed the C-terminal arginines of the α chains by digestion with carboxypeptidase B, crystallized the resulting des-Arg hemoglobin in both the oxy and deoxy forms, and determined their three-dimensional structures at 3.5-Å resolution. The results show that the removal of the arginines causes small disturbances in the quaternary structure of the tetramer because, due partly to the removal of the arginine spacers between the two α chains and partly to the formation of new salt bridges, the two α subunits are drawn more closely together. However, this effect is evidently due to interaction between the two subunits and could not influence the structure of the $\alpha_1\beta_1$ dimer. We have compared the sedimentation constants of des-Arg and native hemoglobin. In 0.05 M phosphate buffer (pH 7.0) neither the CO nor the deoxy forms of either hemoglobin appeared to dissociate. In 0.9 M $MgCl_2$ and 0.1 M BES (pH 7.2), normal CO-hemoglobin dissociated but normal deoxyhemoglobin remained tetrameric; on the other hand, both CO- and deoxy-des-Arg-hemoglobin dissociated equally, which showed that we had succeeded in preparing stable deoxy dimers. We determined the oxygen equilibrium curves of des-Arg hemoglobin. In 0.2 M phosphate buffer (pH 7.0), repeated measurements gave a Hill's constant of $n = 2.0$, showing that the des-Arg tetramer is cooperative. In 0.9 M $MgCl_2$ (pH 7.2), on the other hand, Hill's constant dropped to 1.0, showing that in this medium the dimer is noncooperative.

Human hemoglobin contains three pairs of sulfhydryl groups, of which one lies exposed at the surface (93β), while the other two (104α and 112β) are hidden in the $\alpha_1\beta_1$ contact and remain unreactive even in strong salt solution where oxyhemoglobin dissociates into dimers. This observation led Rosemeyer and Huehns to conclude that the oxyhemoglobin dimer has the structure of $\alpha_1\beta_1$ (7). Does the deoxy dimer have the same structure? We have tried to answer this question by testing the reactivities of its three pairs of sulfhydryl groups with ^{14}C-labeled iodoacetamide. Those at 104α always remained unreactive, even in free deoxy α chains. Those at 93β were always reactive. The sulfhydryl groups at 112β were reactive in free deoxy β chains, but remained unreactive in both normal and des-Arg deoxyhemoglobin in 0.9 M $MgCl_2$, showing that the $\alpha_1\beta_1$ contact remained closed on dissociation into dimers and that significant dissociation into free chains was not taking place.

The association of free deoxy α and β chains to form deoxy tetramers is accompanied by a spectral change (17). According to the cooperative theory advanced by Perutz, this should be an expression of the constraints imposed upon the hemes by the C-terminal salt bridges. Since these are absent in dimers, we find their absorption spectrum to be the same as the sum of the spectra of the free chains and different from that of the deoxy tetramer. Similar observations have been made on short-lived dimers (15, 16).

METHODS

Preparation of Des-Arg Hemoglobin. Arginine 141α was removed from human (or horse) CO-hemoglobin by digestion with chromatographically purified carboxypeptidase B (400 mg hemoglobin to 1 mg of enzyme) for 3 hr in freshly prepared 0.2 M sodium barbitone buffer (pH 8.2) at 25°C. An aliquot removed for analysis of amino acids showed that digestion had gone to completion. The solution was then passed through a Sephadex G-25 column equilibrated with 0.01 M sodium phosphate buffer (pH 6.9), followed by passage through a column of DE-52 cellulose equilibrated with the same buffer. Carboxypeptidase B was retained by this column, while the hemoglobin passed through.

Before functional studies or crystallization, the carbon monoxide was removed (18); medical grade oxygen was used. Oxygen equilibrium curves (18) were determined with both horse and human des-(Arg 141α) hemoglobins in 0.2 M phosphate buffer (pH 7.0); identical results were obtained. The methemoglobin content at the end of the experiment was 10%.

Des-Arg hemoglobin in 0.9 M $MgCl_2$ autoxidized much faster, so that we were forced to adopt a special technique. A 2–3% (w/v) solution of oxyhemoglobin in 0.1 M BES buffer (pH 7.2) was placed in the cuvette, while a solution of 1 M $MgCl_2$ in 0.1 M BES buffer (pH 7.2) was placed in the body of the tonometer. The hemoglobin was deoxygenated without allowing it to make contact with the $MgCl_2$. The two solutions were then mixed and the oxygen equilibrium curve was determined as before, but in order to minimize oxidation to methemoglobin (which reached a value of 14%) only two points were taken from each sample. A curve was then built up from several samples. As a test for possible irreversible changes, $MgCl_2$ was removed from one set of samples, after determination of their oxygen dissociation curve, by passage through a Sephadex G-25 column equilibrated with 0.2 M phosphate buffer in 1 mM EDTA at pH 7.0, and a second oxygen equilibrium curve was determined in this buffer.

Sedimentation Velocities. Des-Arg deoxyhemoglobin was too unstable in 0.9 M $MgCl_2$ to make an ultracentrifuge run in the absence of reducing agent. Accordingly 2 mg/ml of $Na_2S_2O_4$ were added to 0.9 M $MgCl_2$–0.1 M BES (pH 7.2) containing 2 mg/ml of deoxyhemoglobin in a nitrogen-filled glove box. Still in the glove box, the solution was syringed into one sector of a 12-mm aluminum–Epon double-sector centrifuge cell, which had been deoxygenated under reduced pressure in a desiccator for at least 1 day, and the cell was sealed. The spectrum of the hemoglobin in the cell was checked for oxygenation or oxidation on a Unicam SP 8000 spectrophotometer. The cell was then run in a Spinco model E ultracentrifuge at 20°C and 56,000 rpm in an AnD rotor. Sedimentation diagrams were recorded with Schlieren optics at intervals of 16 min on Ilford R10 plates, with 40 sec of exposure and an Ilford 203 filter. The plates were measured on a Nikon microcomparator.

After the centrifuge run, the spectrum of the normal deoxyhemoglobin in the region 460–640 nm corresponded to that normally obtained on deoxygenation of hemoglobin in the absence of dithionite and $MgCl_2$. In particular, the ratio of the absorbances at 480 and 555 nm was unchanged, showing

that no side reactions had been caused by the dithionite (19). In contrast, the spectrum of deoxy des-Arg hemoglobin corresponded to the sum of that observed for the isolated α and β chains in the deoxy form, that is, the shoulder at 590 nm was absent. After removal of the $MgCl_2$ on a Sephadex column in the nitrogen box, the normal spectrum of des-Arg deoxyhemoglobin was obtained, including the shoulder at 590 nm and a slightly diminished value of the ratio between the absorbances at 460 and 555 nm, as compared to their ratio in hemoglobin A.

To test whether the des-Arg hemoglobin still exhibited normal cooperativity in phosphate buffer free of $MgCl_2$ after centrifugation, its oxygen equilibrium curve was determined. After the sedimentation velocity was measured, the cells were opened in the nitrogen-filled glove box and the hemoglobin solution was transferred to a conical flask, which was then sealed. On removal from the glove box, the flask was filled with CO and the sample was passed through a Sephadex G-25 column equilibrated with 0.2 M phosphate buffer (pH 6.98)–1 mM EDTA. The CO was removed and the equilibrium curve was determined as above.

Reactivity of Sulfhydryl Groups. The reactivity of the sulfhydryl groups was measured under the same conditions as those occurring during the sedimentation velocity runs. 535 μmol of [^{14}C]iodoacetamide (0.3 μCi/μmol) was added under nitrogen to 40 mg of normal deoxyhemoglobin, des-Arg deoxyhemoglobin, and deoxy β chains in 2.0 ml of 0.9 M $MgCl_2$–0.1 M BES (pH 7.2) containing 8 mg of dithionite. The rate of reaction was variable, since iodoacetamide appeared to react with the oxidation products of dithionite. The β chains incorporated radioactivity fastest, followed by des-Arg and normal hemoglobin. Deoxy α chains did not appear to incorporate any radioactivity under these conditions. When at least 0.5 mol of iodoacetamide had been incorporated per $\alpha\beta$ dimer, the samples were filtered through Sephadex G-25 and globin was prepared and digested with trypsin (20). The soluble peptides at pH 6.5 were separated by electrophoresis at pH 6.5, chromatography in butanol acetic acid–water–pyridine X:Y:Z:Q, 15:3:12:10 and electrophoresis at pH 3.5 and pH 2.1. The insoluble peptides were digested with chymotrypsin, followed by pepsin, and separated similarly.

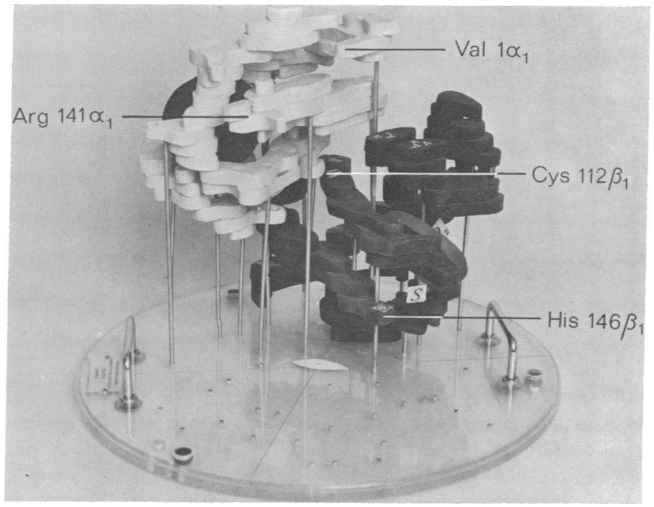

FIG. 1. Structure of the $\alpha_1\beta_1$ dimer, showing that the termini of both chains are exposed and far removed from contact with neighboring chains.

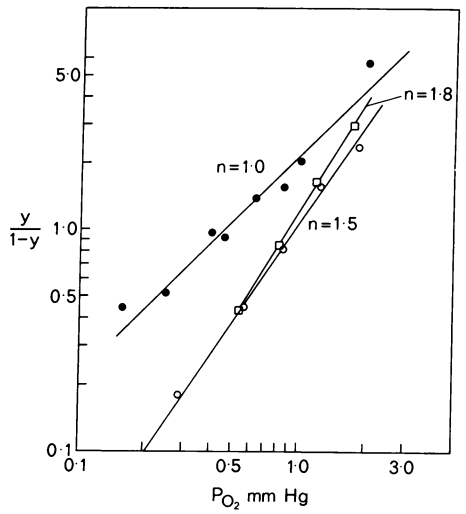

FIG. 2. *Oxygen equilibrium curves of human des-Arg hemoglobin. Full circles:* 0.9 M $MgCl_2$–0.1 M BES (pH 7.2). *Open circles*: same sample after determination of the equilibrium curve in $MgCl_2$. $MgCl_2$ was removed by passing the sample through a Sephadex G-25 column equilibrated with 0.2 M phosphate buffer (pH 6.9). At this stage, the methemoglobin content had risen to about 17%, which would reduce the observed value of *n*. *Squares:* sample after determination of the sedimentation velocity in $MgCl_2$ and transfer to 0.2 M phosphate buffer as described above.

Crystallization and X-ray Analysis. Human and horse des-Arg oxy- and deoxyhemoglobins were crystallized (21). Unit cells and space groups were determined from precession photographs. Intensities within a limiting sphere of 3.5-Å^{-1} radius were recorded on a Crystalign four-circle diffractometer. Difference Fourier syntheses were calculated using $|F_{\text{des-Arg}}| - |F_{\text{Hb A}}|$ as coefficients and $\alpha_{\text{Hb A}}$ as phases. A Fourier synthesis of the human des-Arg derivatives was calculated using $|F_{\text{Hb A}}| + 2\,[|F_{\text{des-Arg}}| - |F_{\text{Hb A}}|]$ as coefficients and $\alpha_{\text{Hb A}}$ as phases (22). For human deoxyhemoglobin, we used the phases determined by Muirhead and Greer (12) and for horse methemoglobin those determined by Perutz *et al.* (23).

RESULTS

Fig. 2 shows a comparison between three oxygen equilibrium curves, one of des-Arg hemoglobin in 0.9 M $MgCl_2$ (n = 1.0), another taken afterwards from the same sample in 0.2 M phosphate buffer without $MgCl_2$ (n = 1.5), and a third obtained from a sample removed from the ultracentrifuge cell after centrifugation in $MgCl_2$ (n = 1.8). Allowing for the extra methemoglobin present in the second sample, we see the loss of cooperativity in $MgCl_2$ solutions to be reversible.

Fig. 3 shows the Schlieren diagram of two cells, one containing normal human deoxyhemoglobin and the other des-Arg deoxyhemoglobin, during the same ultracentrifuge run. Both solutions contain 0.9 M $MgCl_2$–0.1 M BES buffer (pH 7.17). The sedimentation diagram shows that des-Arg deoxyhemoglobin sediments more slowly than the normal form. The sedimentation constants are listed in Table 1. In the absence of $MgCl_2$, normal and des-Arg deoxy have the same sedimentation constants, corresponding predominantly to the tetrameric forms. Normal deoxy has a sedimentation constant corresponding to a high degree of tetrameric aggre-

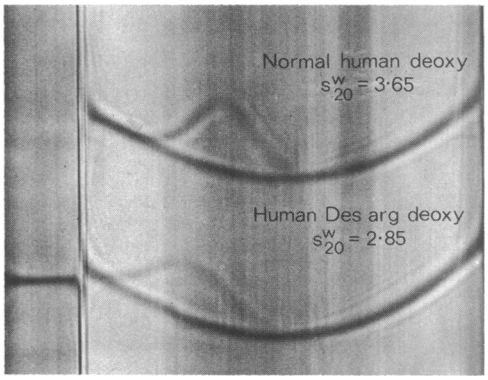

FIG. 3. Sedimentation velocity diagrams of normal and des-Arg deoxyhemoglobin. Buffer 0.9 M $MgCl_2$–0.1 M BES (pH 7.17); 13 mM $Na_2S_2O_4$; 75 μM hemoglobin. Speed, 56,000 rpm.

gation in the presence or absence of $MgCl_2$; des-Arg deoxyhemoglobin in $MgCl_2$ has about the same sedimentation constant as normal CO-hemoglobin in $MgCl_2$, which is known to be completely dissociated into dimers in that medium.

Samples of deoxy normal hemoglobin, deoxy des-Arg hemoglobin and deoxy β chains were reacted with radioactive iodoacetamide. After digestion with trypsin, 85–90% of the counts in normal and des-Arg hemoglobin were present in a peptide containing residues 83–95, showing that cysteine 93β had reacted. In β chains, 60% of the counts were present in this peptide; the rest were insoluble and had to be digested with pepsin to give a peptide containing residues 112–114, which showed reaction at cysteine 112β. When the insoluble tryptic peptides from normal and des-Arg hemoglobins were digested with pepsin, two weakly radioactive peptides were observed at pH 6.5 that had a completely different mobility from those occurring in the peptic digest of the insoluble tryptic peptides from β chains, which showed that no reaction

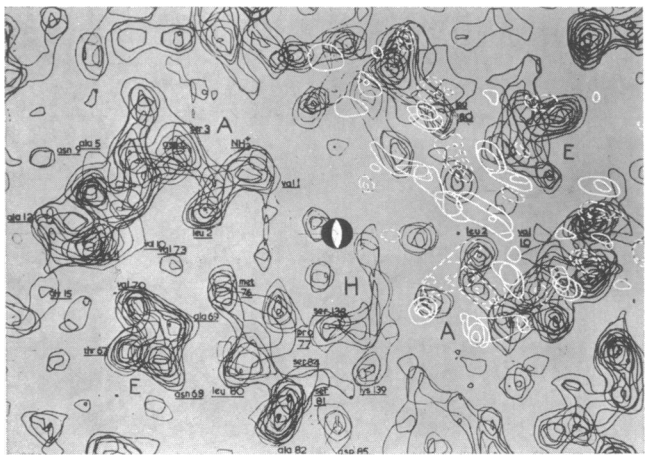

FIG. 4. Electron-density map of normal human deoxyhemoglobin superimposed on the difference map of des-Arg minus normal deoxyhemoglobin, both at 3.5-Å resolution. *Black lines:* contours of the normal form; *full white lines:* electron density added to; *broken white lines:* electron density removed from the normal form. The sign in the center is the dyad axis, so the sections are normal to the dyad, which runs vertically through the center. For clarity, difference contours are shown on one side of the dyad only. The sections show parts of helices *A*, *E*, and *EF* corner, with systems of positive and negative peaks indicating a tilting of the α chain towards the central cavity.

TABLE 1. *Sedimentation velocities of human des-Arg and normal hemoglobins at pH 7.2, 20°C and tetramer 30-μM hemoglobin*

Buffer	Sample	$s_{20}{}^w$
0.9 M $MgCl_2$–0.1 M BES–13 mM $Na_2S_2O_4$	Des-Arg deoxy	2.6
0.9 M $MgCl_2$–0.1 M BES–13 mM $Na_2S_2O_4$	Normal deoxy	3.7
0.05 M phosphate–13 mM $Na_2S_2O_4$	Des-Arg deoxy	4.1
0.05 M phosphate–13 mM $Na_2S_2O_4$	Normal deoxy	4.1
0.9 M $MgCl_2$–0.1 M BES	Des-Arg CO	3.0
0.9 M $MgCl_2$–0.1 M BES	Normal CO	2.8
0.9 M $MgCl_2$–0.1 M BES–13 mM $Na_2S_2O_4$	Des-Arg deoxy	2.85*
0.9 M $MgCl_2$–0.1 M BES–13 mM $Na_2S_2O_4$	Normal deoxy	3.65*

* For this experiment, the hemoglobin concentration was 75 μM.

had occurred at cysteine 112β in normal and des-Arg deoxyhemoglobin.

Both the oxy and deoxy forms of human des-Arg hemoglobin crystallized with the same unit-cell dimensions and space group as the corresponding forms of normal hemoglobin. In normal deoxyhemoglobin, arginine 141α forms the salt bridges shown below (where Gua^+ is guanidinium):

$$\text{Arg } 141\alpha_1 \begin{cases} \text{COO}^- \cdots\cdots {}^+\text{NH}_3 - \text{Val } 1\alpha_2 \\ \text{Gua}^+ \cdots\cdots {}^-\text{OOC} - \text{Asp } 126\alpha_2 \end{cases}$$

In oxyhemoglobin, the C- and N-terminal residues are normally free. The difference Fourier syntheses show that the newly liberated α carboxyl group of tyrosine 140α_1 tends to form salt bridges with the α-amino group of valine 1α_2 in deoxy, and with the ϵ-amino group of lysine 127α_2 in oxyhemoglobin. The removal of the arginines leaves the structure of the molecule undisturbed except for a slight distortion of

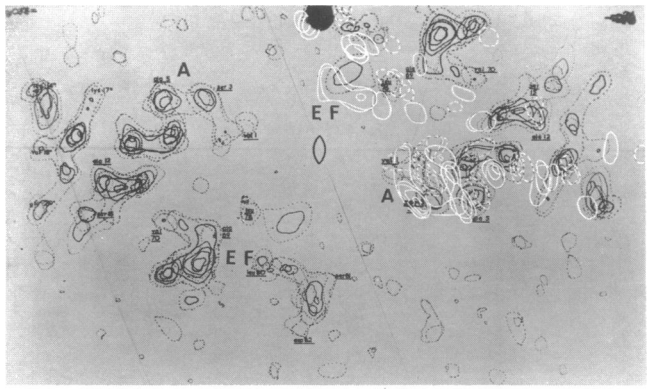

FIG. 5. Electron-density map of normal horse methemoglobin (which is isomorphous with oxy) at 2.8 Å superimposed on the difference map of des-Arg minus normal at 3.5-Å resolution. Contours as in Fig. 4, except that the lowest contour of the electron density map is indicated by a *broken black line.* Helix *A* and *EF* region with positive and negative peaks are indicative of a movement of the α chain towards the internal cavity.

The sections shown in Figs. 4 and 5 are above the C-terminal arginines and do not contain the negative peaks due to their removal. The pertinent sections are shown in ref. 24.

the tetramer that is most pronounced in electron density sections near the top of the model pictured in Fig. 1. These contain systems of positive and negative peaks that indicate a closing of the gap between the two α subunits by about 1 Å in deoxy- and 2 Å in oxyhemoglobin (Figs. 4 and 5), due probably in part to the absence of the arginine spacer between the subunits and in part to attractive force exerted by the newly formed salt bridges. These forces could not operate in the $\alpha\beta$ dimer, so that its structure should be unaffected by the removal of the arginines. Further sections of these difference maps are being published elsewhere (24).

DISCUSSION

Our results show that des-Arg deoxyhemoglobin in 0.9 M $MgCl_2$ dissociates into dimers, and that its oxygen equilibrium curve is noncooperative. In 0.2 M phosphate buffer (pH 6.9), des-Arg deoxyhemoglobin is tetrameric and binds oxygen cooperatively, with a Hill's constant of 2.0, which suggests that the lack of cooperativity is due solely to the removal of the C-terminal arginines. Normal deoxyhemoglobin in 0.9 M $MgCl_2$ is substantially tetrameric, and its oxygen equilibrium curve is cooperative ($n = 1.8$), so that the absence of cooperativity of des-Arg hemoglobin is not due solely to the effect of the $MgCl_2$. Moreover, a sample of des-Arg hemoglobin that showed a noncooperative equilibrium curve in $MgCl_2$ regained its cooperativity on removal of the $MgCl_2$, which showed the effect of $MgCl_2$ to be reversible. It seems reasonable to conclude, therefore, that the absence of cooperativity is a property of the $\alpha\beta$ dimer.

No reaction of cysteine 112β in deoxy des-Arg dimers with iodoacetamide was detected under conditions where such a reaction occurs in β chains. This shows that no monomers are present, and that the dimer formed is the same as the oxyhemoglobin dimer: $\alpha_1\beta_1$. It is conceivable that the dimer "closes up", in both oxy- and deoxyhemoglobin and that the new contact formed leaves cysteine 112β unreactive. Our results do not exclude this possibility, although it is unlikely on stereochemical grounds.

Arginine 141 lies on the surface of the α subunits and is free to take up several alternative positions in oxyhemoglobin. In deoxyhemoglobin, it forms salt bridges with polar groups of the opposite α chain. Consequently, we expected that its removal would affect neither the tertiary structure of the α subunits nor the quaternary structure of the oxy tetramer, but that it would loosen the quaternary structure of the deoxy tetramer. In fact, the uncovering of the α carboxyl group of tyrosine 140 affects the quaternary structures of both oxy and deoxy forms, as its attraction for amino groups in the opposite α chain draws the two α subunits closer together. However, this effect should not influence the structure of the $\alpha\beta$ dimer, where the α carboxyl group of tyrosine 140 would be freely exposed on the surface and far removed from any amino groups of the β subunit.

Our conclusions concerning the noncooperativity of the $\alpha\beta$ dimer are supported by a simultaneous and independent study of G. C. Kellett (25), who finds that normal human deoxyhemoglobin in 2 M NaI dissociates into dimers and binds ethyl isocyanide noncooperatively. The noncooperativity of the deoxy dimers, their high oxygen affinity, and the identity of their absorption spectrum with the sum of the spectra of the free chains, rather than with the spectrum of the deoxy tetramer, are all consistent with, even though they do not prove, the cooperative mechanism advanced by Perutz, in which the salt bridges formed by the terminal residues in the deoxy tetramer impose constraints upon the subunits that lower their oxygen affinity.

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