The Interaction of 2,3-Diphosphoglycerate with Various Human Hemoglobins

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ABSTRACT Oxygen equilibria were measured on a number of human hemoglobins, which had been "stripped" of organic phosphates and isolated by column chromatography. In the presence of 2×10^{-4} M 2,3-diphosphoglycerate (2,3-DPG), the P_{50} of hemoglobins A, A_2, S, and C increased about twofold, signifying a substantial and equal decrease in oxygen affinity. Furthermore, hemoglobins Chesapeake and $M_{Milwaukee-1}$ which have intrinsically high and low oxygen affinities, respectively, also showed a twofold increase in P_{50} in the presence of 2×10^{-4} M 2,3-DPG. In comparison to these, hemoglobins A_{1c} and F were less reactive with 2,3-DPG while hemoglobin F_1 showed virtually no reactivity. The N-terminal amino of each β-chain of hemoglobin A_{1c} is linked to a hexose. In hemoglobin F_1 the N-terminal amino of each γ-chain is acetylated. These results suggest that the N-terminal amino groups of the non-α-chains are involved in the binding of 2,3-DPG to hemoglobin.

INTRODUCTION

Hemoglobin function is dramatically altered in the presence of 2,3-diphosphoglycerate (2,3-DPG) and adenosine triphosphate (ATP), compounds found in high concentration in the red cell (1, 2). A growing body of evidence suggests that 2,3-DPG may serve as a fine control of intracellular hemoglobin function (3, 4). Under physiologic conditions 2,3-DPG appears to lower oxygen affinity by combining reversibly with 1 mole of deoxyhemoglobin (5). Further information about the nature of this interaction might accrue from a comparison of different human hemoglobins. Altered intracellular oxygen affinity may not necessarily be due to an intrinsic property of the hemoglobin molecule but rather to abnormal reactivity with organic phosphates. This appears to be true of hemoglobin F (6, 7). Furthermore, if a specific hemoglobin can be shown to interact abnormally with 2,3-DPG the locus of its structural alteration might provide information as to the site of 2,3-DPG binding. In this study we have compared the effect of 2,3-DPG on the oxygen equilibria of a variety of human hemoglobins.

METHODS

Blood samples were collected in citrate except for specimens containing hemoglobin Chesapeake[1] and hemoglobin $M_{Milwaukee-1}$[2] which were collected in acid-citrate-dextrose (ACD), packed in ice, and shipped by air freight. Experiments were completed within 8 days after the blood was drawn. Hemolysates were prepared as previously described (8) and were "stripped" of organic phosphates as follows. After overnight dialysis in the cold against a large volume of 0.1 M NaCl, containing 0.005 M bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (bis-Tris)[3] pH 7.20, specimens were passed through a chromatography column containing Sephadex G25[4] in the same buffer-salt (5). Protein-free extracts of hemolysates, so treated, contained no detectable phosphate, as determined by the method of Ames and Dubin (9).

In some experiments, isolated hemoglobins were prepared from "stripped" hemolysates by ion-exchange chromatography at 5°C. Hemoglobin samples were dialyzed against the initial eluting buffer and gassed with carbon monoxide before application to the column in order to minimize auto-oxidation. Hemoglobin A_2 was isolated from a normal adult hemolysate on DEAE-cellulose as described by Huisman and Dozy (10). In like manner, hemoglobins C and S were isolated from hemolysates of homozygous individuals. The

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[1] Kindly provided by Dr. Samuel Charache.

[2] Kindly provided by Dr. Anthony Pisciotta.

[3] General Biochemicals Div. (North American Mogul Products Co.), Chagrin Falls, Ohio.

[4] Pharmacia Fine Chemicals Inc., Piscataway, N. J.

hemoglobin S hemolysate contained 11% hemoglobin F, confirmed by alkali denaturation (11). Hemoglobins A and Chesapeake were separated on carboxymethyl cellulose as described by Charache, Weatherall, and Clegg (12). Hemoglobin $M_{Milwaukee-1}$ was prepared on Bio-Rex 70 as previously described (13). Hemoglobin F was prepared from hemolysates of cord blood. Hemoglobins F and A were separated on Bio-Rex 70 by the method of Allen, Schroeder, and Balog with developer No. 2 (14). Hemoglobins F_I and F_{II} were isolated with developer No. 4 (14). Both were equally resistant to alkali denaturation (11). In like manner, hemoglobin A_{Ic} was isolated from a normal adult hemolysate on Bio-Rex 70, with developer No. 4 (14). In all the chromatograms done on Bio-Rex 70 the eluting buffer contained 0.01 M cyanide. This apparently had no deleterious effect in hastening auto-oxidation as shown by the following experiment. Oxyhemoglobin A was incubated at 37°C in either developer No. 2 or in a buffer identical in pH and ionic strength but lacking cyanide. During 4 hr the amount of ferrihemoglobin cyanide formed in the first flask never exceeded the amount of ferrihemoglobin in the second.

After chromatography, hemoglobin-rich fractions were pooled and concentrated with a Diaflow apparatus[5] in the cold. The hemoglobin was then dialyzed overnight against two changes of 0.1 M NaCl. Immediately before the experiment, carbon monoxide was removed by rotating the hemoglobin solution in a tonometer immersed in an ice bath and exposed to strong incandescent light. Full conversion to oxyhemoglobin was checked spectrophotometrically before oxygen equilibria were run. Replacement of CO by O_2 was considered complete when $OD_{576}/OD_{560} \geq 1.78$.

2,3-Diphosphoglycerate was obtained as the pentacyclohexylammonium salt.[6] This large cation was removed as follows (15). 20 ml Dowex 50 W-X8, 200-400 mesh, ionic form H^+[7] was placed in a 100 ml graduated cylinder and washed six times with warm water. 225 mg 2,3-DPG salt dissolved in 12.5 ml H_2O was added, shaken for 30 sec, allowed to settle, and made up to 100 ml with water. The supernate was then passed through a fine sintered glass funnel. The concentration of 2,3-disphosphoglyceric acid in the filtrate was checked by titration with standard sodium hydroxide. At pH 10.0 all five titratable hydrogen ions were removed. The concentration of 2,3-DPG was also checked by a total phosphate determination (9). Agreement between the titration and the gravimetric and phosphate determinations was within 5%. The DPG standard solution was then neutralized to pH 7.0. Aliquots of the same solution were used in all experiments.

Oxygen equilibria were done on solutions consisting of "stripped" hemoglobin (4 to 6×10^{-5} mole/liter, tetramer) and known concentrations of 2,3-DPG in 0.1 M NaCl and 0.05 M bis-Tris buffer, pH 7.20. This buffer was selected since, unlike phosphate, it contains no negatively charged ions that might interfere with the interaction of hemoglobin and 2,3-DPG and it has an appropriate pK of 6.5 (15). 4.0 ml of solution was placed in a 250 ml tonometer attached to a 2 mm cuvette. Oxygen equilibria were done by the method described by Allen, Guthe, and Wyman (16) and by Riggs (17). Generally two tonometers were run simultaneously, one containing no 2,3-DPG and the other containing 2×10^{-4} M 2,3-DPG. Specimens were deoxygenated by successive evacuations and nitrogen flushes in a closed system. After deoxygenation was considered complete as judged by $OD_{555}/OD_{540} \geq 1.23$, the tonometer was flushed and evacuated once more. The tonometers were rotated in a water bath at 10.0 ±0.1°C for 10 min after the introduction of known volumes of air saturated with water vapor. Absorption spectra from 700 to 500 mμ were recorded by a Cary 14 R spectrophotometer, the cell compartment of which was also cooled to 10°C. Calculations were made as outlined by Briehl (18). The data shown in the following tables and figures were plotted logarithmically according to the Hill equation:

$$\left(\frac{Y}{1-Y}\right) = \left(\frac{P_{O_2}}{P_{50}}\right)^n$$

where Y is fractional saturation of hemoglobin with oxygen and P_{O_2} is the partial pressure of oxygen for each separate equilibration. P_{50}, an inverse measure of oxygen affinity, is the P_{O_2} at which hemoglobin is half saturated, and n represents a measure of heme-heme interaction.

RESULTS

The effect of 2,3-DPG on the oxygen equilibria of "stripped" hemolysates is shown in Table I. The affinity of A hemolysates, "stripped" by Sephadex G-25, was very close to that found by Benesch, Benesch, and Yu (5) when the two sets of data were corrected to the same pH and temperature. In duplicate experiments, the P_{50} of hemoglobins A, S, and C increased approximately twofold in the presence of 2×10^{-4} M 2,3-DPG, indicating a substantial and equal decrease in oxygen affinity for these three hemoglobins. Benesch, Benesch, and Yu have used the ratio P_{50} DPG/P_{50} "stripped" as an indirect measure of the binding of 2,3-DPG to deoxyhemoglobin (15).

The 2, 3-DPG reactivity of hemoglobins isolated by column chromatography is shown in Table II. "Stripped" isolated hemoglobins A, S, and C had a slightly higher oxygen affinity than their respective "stripped" hemolysates. This may have been due to mild denaturation engendered by the column chromatography. However, no significant auto-oxidation occurred since no difference in the absorption spectra of the hemolysates and isolated hemoglobins could be detected. Again, in the pres-

TABLE I

Effect of 2,3-DPG on Oxygen Equilibria of Hemolysates

Hemoglobin	"Stripped" Hb		2×10^{-4} M DPG		P_{50}DPG/P_{50}St
	P_{50}	n	P_{50}	n	
AA	1.46	3.2	3.09	2.7	2.03
	1.51	2.7	2.93	2.8	
SS	1.99	2.7	3.24	2.9	1.76
	1.60	2.8	3.06	2.7	
CC	1.59	2.7	3.59	2.9	2.17
	1.56	3.0	3.24	3.0	

[5] Amicon Corp., Lexington, Mass.
[6] Calbiochem, Los Angeles, Calif.
[7] Bio-Rad Labs, Richmond, Calif.

TABLE II

Effect of 2,3-DPG on Oxygen Equilibria of Isolated Hemoglobins

Hemoglobin	Preparation	"Stripped" Hb		2×10^{-4} M DPG		P_{50} DPG/P_{50} St
		P_{50}	n	P_{50}	n	
A	DEAE	1.27	2.6	1.83	2.7	1.73
		1.03	2.5	2.14	2.5	
A_2	DEAE	1.05	2.8	2.29	2.7	2.02
		1.07	2.5	2.00	2.5	
S	DEAE	1.36	2.6	2.17	2.7	1.60
C	DEAE	1.05	2.9	2.38	2.8	2.26
A	CMC	1.30	3.0	2.57	2.9	1.97
Chesapeake	CMC	0.28	1.4	0.48	1.6	1.72
A	Bio-Rex 70	1.41	2.9	2.85	2.7	2.02
M_{mil}	Bio-Rex 70	6.44	1.1	14.0	1.3	2.35
		5.79	1.0	14.8	1.2	
A_{II}	Bio-Rex 70	1.28	2.4	2.68	2.4	2.10
		1.30	2.4	2.76	2.5	
A_{IC}	Bio-Rex 70	1.04	2.4	1.28	2.4	1.23
		1.09	2.4	1.35	2.4	
F_{II}	Bio-Rex 70	1.90	2.6	2.22	2.7	1.20
		1.86	2.7	2.28	3.0	
F_I	Bio-Rex 70	2.37	2.6	2.44	2.3	1.02
		2.29	2.3	2.31	2.4	

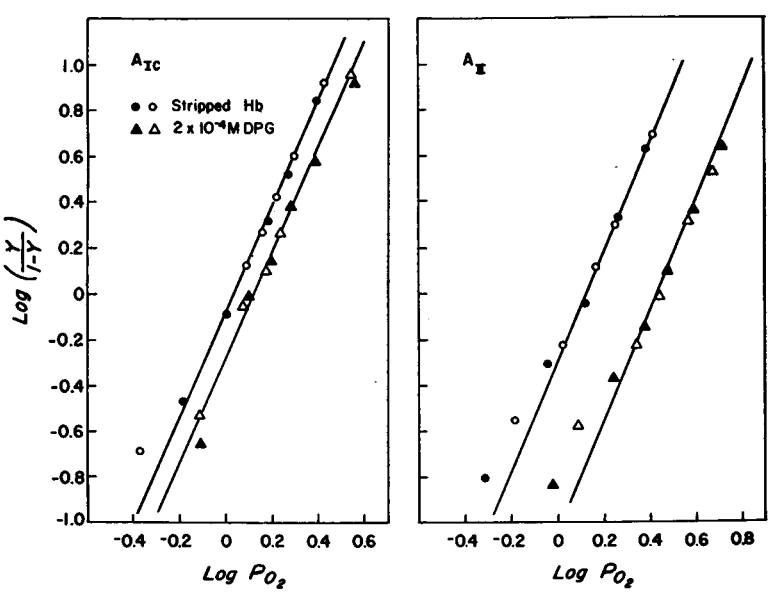

FIGURE 1 Oxygen equilibria of hemoglobins A_{IC} and A_{II} in 0.1 M NaCl, 0.05 M bis-Tris buffer, pH 7.20, 10°C. Open and solid symbols represent duplicate experiments.

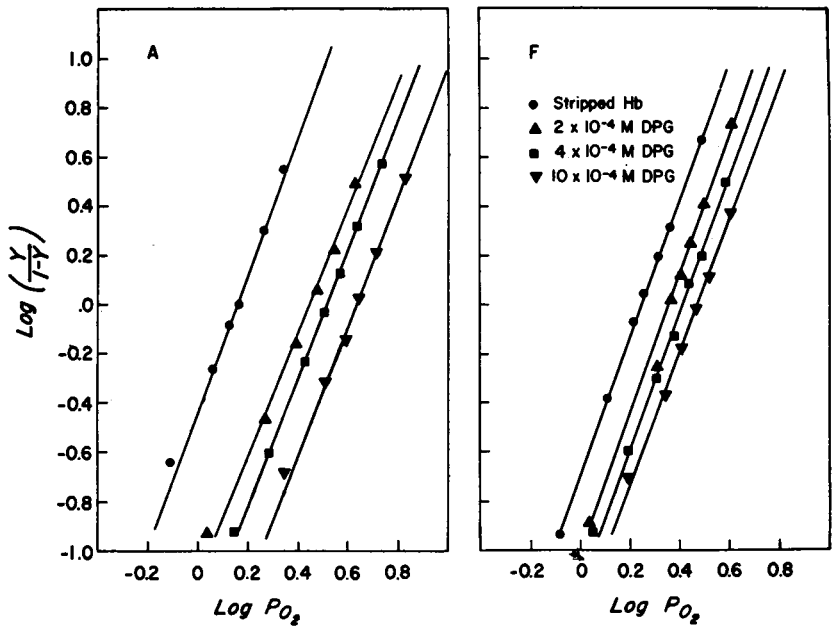

FIGURE 2 Oxygen equilibria of hemoglobins A and F in 0.1 M NaCl, pH 7.20, 10°C with increasing concentrations of 2,3-DPG (see figure key).

ence of 2×10^{-4} M 2,3-DPG, the P_{50}'s of isolated hemoglobins A, S, C, and also A_2 were about twice that of the "stripped" hemoglobins.

Hemoglobins Chesapeake and Milwaukee-1 have been shown to have high and low oxygen affinities respectively (12, 13, 19). The P_{50} values shown in Table II for these "stripped" hemoglobins agree fairly well with previously reported values (13, 19). In the presence of 2×10^{-4} M 2,3-DPG, the P_{50}'s were each approximately doubled, indicating that these hemoglobins had normal reactivity with 2,3-DPG.

Hemoglobin A_{1c}, a minor component comprising about 6% of the normal adult human hemolysate, was compared with the main component, designated as hemoglobin A_{II}, and isolated from the same column. The two hemoglobins differ only in that the N-terminal amino groups of the β-chains of hemoglobin A_{1c} are each bound covalently to a hexose by a Schiff's base linkage (20, 21). Oxygen equilibria of these two hemoglobins with and without 2,3-DPG are shown in duplicate in Fig. 1 and Table II. While hemoglobin A_{II} showed a 102% (twofold) increase in P_{50} in the presence of 2×10^{-4} M 2,3-DPG, only a 23% rise was observed with hemoglobin A_{1c}.

As shown in Fig. 2, hemoglobins A and F differed in their reactivity with 2,3-DPG. At each of three concentrations of 2,3-DPG (2, 4, and 10×10^{-4} mole/liter), the increase in P_{50} (as compared to "stripped" hemoglobin) of hemoglobin F was only one-third that of hemoglobin A. Similar results have been reported by Bauer, Ludwig, and Ludwig (6) and Tyuma and Shimizu (7). Hemoglobin F in cord blood contains one minor component, F_1 (comprising about 15%), and the major component, designated as hemoglobin F_{II} (14). The two differ only in that in hemoglobin F_1, acetyl groups are linked covalently to both N-terminal amino groups of the γ-chains (22, 23). The oxygen equilibria of hemoglobin F_1 and F_{II} (isolated from the same hemolysate) with increasing concentrations of 2,3-DPG is shown in Fig. 3. As expected, hemoglobin F_{II} showed the same oxygen affinities at 0, 2, 4, and 10×10^{-4} M 2,3-DPG as hemoglobin F (Fig. 2). In contrast, the oxygen affinity of hemoglobin F_1 was unaffected by all three concentrations of 2,3-DPG tested. Identical results were obtained on a repeat experiment (Table II).

In the oxygen equilibria reported here, there was no significant difference in Hill's coefficient n of "stripped" hemoglobins compared with the same hemoglobins containing 2,3-DPG. This was true even with hemoglobins Chesapeake and Milwaukee-1 which had low n values. In accord with these results, Benesch, Benesch, and Yu had previously reported that n values were unaffected by the addition of more than equimolar 2,3-DPG to "stripped" hemoglobin (5).

DISCUSSION

The oxygen affinity of intracorpuscular hemoglobin can be affected by a variety of factors:

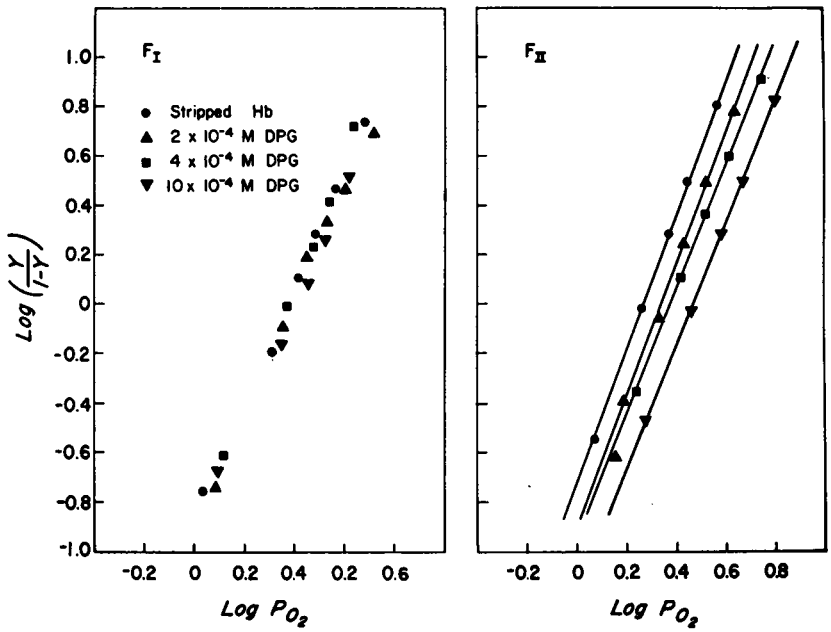

FIGURE 3 Oxygen equilibria of hemoglobins F_I and F_{II} in 0.1 M NaCl, pH 7.20, 10°C with increasing concentrations of 2,3-DPG (see figure key).

(*a*) It increases with rising pH (i.e. Bohr effect) or falling temperature.

(*b*) In a number of clinical and physiologic conditions, affinity has been found to vary inversely with intracellular levels of organic phosphates, particularly 2,3-DPG (3, 4, 24–27).

(*c*) Oxygen equilibrium may be altered by failure of intracellular hemoglobin to interact normally with organic phosphates. Hemoglobin F is much less affected by given concentrations of 2,3-DPG than hemoglobin A (6, 7). The data shown in Fig. 1 confirm this observation. This explains why cord blood has a high oxygen affinity despite a normal concentration of 2,3-DPG. Similar findings have been reported for the blood of an individual homozygous for hereditary persistence of fetal hemoglobin (28).

(*d*) Oxygen affinity may be affected by the presence within the red cell of a hemoglobin variant which itself is functionally abnormal. In this study, for example, hemoglobin Chesapeake "stripped" of organic phosphates had the expected high oxygen affinity, but a normal reactivity with 2,3-DPG. Likewise hemoglobin Milwaukee-1 showed a low affinity but again a normal interaction with 2,3-DPG (Table II).

Hemoglobins S and C react normally with 2,3-DPG (Tables I and II). Oxygen equilibria of homozygous hemoglobin C red cells have been reported to be normal (29). The oxygen affinity of homozygous hemoglobin S blood is reduced in proportion to the severity of the individual's degree of anemia (30). This "shift to the right" is most likely due to increased levels of 2,3-DPG in response to the hypoxia of anemia (31).

Benesch, Benesch, and Yu have reported that under physiologic conditions of pH and ionic strength, 1 mole of 2,3-DPG binds 1 mole of deoxyhemoglobin tetramer (5). They went on to show that while isolated α-chains do not bind, both the oxy and the deoxy forms of β_4 (hemoglobin H) react with 2,3-DPG (32). Thus it is reasonable to conclude that 2,3-DPG binds somewhere to the β-chains of deoxyhemoglobin, probably at a locus near or in the diad axis of symmetry (32). Benesch and associates have found that pyridoxal phosphate can be bound covalently to deoxyhemoglobin A via a Schiff's base (33). This modified hemoglobin has an irreversibly lowered oxygen affinity. This linkage is probably to an amino group on the β-chains, either at the N-terminus, or at one of the 11 lysines of the β-chain.

Our results indicate that N-terminal amino group of the non-α-chain is involved in the binding of the 2,3-DPG to hemoglobin. Hemoglobin A_{Ic} and F_1 in which the N-terminal amines of the non-α-chains are bound to a hexose and an acetyl group respectively, showed a markedly diminished reactivity to 2,3-DPG. This could be due either to an attenuation of the amine's positive

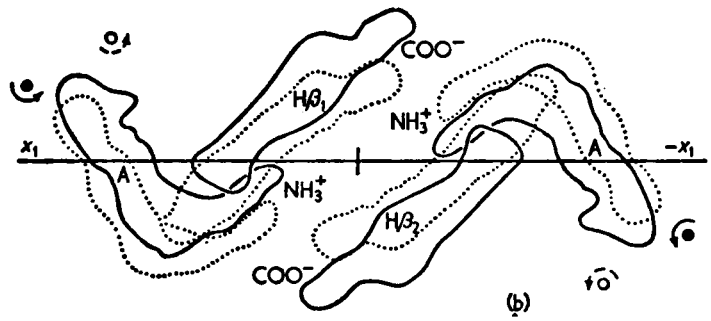

FIGURE 4 Composite sections through the A and H helices of the β-chains of hemoglobin A. Full lines: deoxyhemoglobin; broken lines: oxyhemoglobin. Figure reproduced from paper of Muirhead, Cox, Mazzarella, and Perutz (41).

charge or to steric hindrance, blocking the attachment of 2,3-DPG.

It is possible that the decreased interaction of hemoglobins A$_{Ic}$, F$_{I}$, and F$_{II}$ with 2,3-DPG could be due to equal binding of 2,3-DPG to the oxy and deoxy forms rather than decreased binding to the deoxy forms. However, this explanation would require that the change in conformation between the oxy and deoxy forms be significantly less for these hemoglobins in comparison to hemoglobin A. This seems unlikely since these hemoglobins have nearly normal heme-heme interaction and oxygen affinity. Secondly, it is difficult to see how the binding of a strong anion such as 2,3-DPG could be *increased* in oxyhemoglobins A$_{Ic}$ and F$_{I}$ which are more negatively charged than hemoglobins A and F$_{II}$. Finally, direct binding studies have shown that deoxyhemoglobin F binds 2,3-DPG more readily than oxyhemoglobin F but less than deoxyhemoglobin A (34).

Following the suggestion of Perutz, De Verdier, and Garby have proposed the β-H21 histidine residue as a likely binding site (34, 35). They have pointed out that the marked difference in binding between pH 7 and 8 (35, 15) suggests the imidazole of a histidine residue. Furthermore, in hemoglobin F the γ-chains have a nonionized serine instead of histidine at H21. Finally, hemoglobin Hiroshima, a variant associated with high oxygen affinity, has aspartic acid substituted in this β-H21 site (36). Unfortunately, the oxygen equilibrium of hemoglobin Hiroshima has only been studied in 0.1 M phosphate (37, 38) so that one does not know whether it has an intrinsically high affinity or, like hemoglobin F, an impaired interaction with organic (and inorganic) phosphates.

On the other hand, the H24 (C-terminal) histidines are unlikely to be involved in 2,3-DPG binding to deoxyhemoglobin. Perutz, Muirhead, Mazzarella, Crowther, Greer, and Kilmartin have recently shown that in deoxyhemoglobin, the imidazoles of these residues are each linked to aspartates FG1 of the same β-chain (39). This conformation-dependent interaction contributes considerably to the Bohr effect. Benesch et al. have found that the Bohr effect is unchanged after the addition of an excess of 2,3-DPG to "stripped" hemoglobin (15). Finally, Chanutin and Curnish showed that hemoglobin treated with carboxypeptidase A, so as to remove the β-C-terminal tyr-his, still has normal 2,3-DPG reactivity (40).

On the basis of the model of human deoxyhemoglobin worked out by Muirhead, Cox, Mazzarella, and Perutz from X-ray crystallographic data (41), a specific binding site may be proposed. At the entrance to the central cavity the A and H helices of the β-chain are symmetrically arranged in close proximity (Fig. 4). Upon deoxygenation the entrance to the internal cavity widens because of rotation (and a lesser extent translation) of the β-chains. Greer and Perutz have recently tried to fit a model of 2,3-DPG to an atomic model of human deoxyhemoglobin built on the basis of a Fourier synthesis at 3.5 A resolution.[8] They were able to place one molecule of 2,3-DPG in the internal cavity in such a way that the phosphates were within hydrogen bonding distance of the two N-terminal amino groups of the β-chains, the imidazoles of the β-H21 histidines and the ε-amino groups of the β-EF6 lysines. In contrast, the internal cavity of *oxyhemoglobin* was too narrow to allow binding at this site. Furthermore, they found that upon oxygenation, the N-terminal amino groups of the β-chains moved farther apart, making it impossible for both of them to bind to the same molecule of 2,3-DPG. Such a difference between the binding of 2,3-DPG to deoxy- and oxyhemoglobin would account for the effect of 2,3-DPG on oxygen affinity.

Our oxygen equilibrium data fit well with this proposed binding site. Hemoglobin A, which has full reactivity with 2,3-DPG has both β-H21 His and free β-N-

[8] Perutz, M. F. Personal communication.

terminal amino groups. Hemoglobin A_{Ic}, which has β-H21 His but lacks free β–N-terminal amino groups shows an intermediate 2,3-DPG effect. Hemoglobin F_{II}, which has free γ–N-terminal amino groups, but lacks γ-H21 His also has intermediate reactivity. Finally hemoglobin F_I, lacking both the γ-H21 His and free γ–N-terminal amino groups shows virtually no 2,3-DPG effect at all.

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