Hemoglobin Rahere, A Human Hemoglobin Variant with Amino Acid Substitution at the 2,3-Diphosphoglycerate Binding Site

Functional Consequences of the Alteration and Effects of Bezafibrate on the Oxygen Bindings

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Abstract

We encountered an abnormal hemoglobin (Rahere), with a threonine residue replacing the $\beta 82$ (EF6) lysine residue at the binding site of 2,3-diphosphoglycerate, which was responsible for overt erythrocytosis in two individuals of a Japanese family. Hemoglobin Rahere shows a lower oxygen affinity on the binding of 2,3-diphosphoglycerate or chloride ions than hemoglobin A. Although a decrease in the positive charge density at the binding sites of 2,3-diphosphoglycerate in hemoglobin Rahere apparently shifts the allosteric equilibrium toward the low affinity state, it greatly diminishes the cofactor effects by anions. The oxygen affinity of the patient's erythrocytes is substantially lowered by the presence of bezafibrate, which combines with sites different from those of 2,3-diphosphoglycerate in either hemoglobin Rahere or hemoglobin A.

Introduction

Since the discovery of the role of 2,3-diphosphoglycerate (DPG)[1] in regulating oxygen transport (1, 2) led to the identification of the binding sites of anionic cofactors in the hemoglobin molecule (3, 4), an understanding of linkage between oxygen binding and cofactor binding has been sought through studies on hemoglobin variants with alterations at the 2,3-DPG binding site (5–9). Recently Perutz and Poyart (10) reported that low molecular weight compounds, such as 2-(4-chlorophenoxy)-2-methylpropionic acid (clofibric acid) and 2-[4-(2-*p*-chlorobenzamidethyl)-phenoxy]-2-methylpropionic acid (bezafibrate), have the remarkable ability to modify hemoglobin function in erythrocytes, a finding that opened the way to studies of the molecular control of hemoglobin function and its clinical application. The present paper describes the identification, in patients with erythrocytosis, of hemoglobin Rahere, in which a threonine residue substitutes for the $\beta 82$ lysine residue at the binding site of 2,3-DPG (5). This study was undertaken to determine how this substitution affects the binding of natural anionic cofactors with hemoglobins,

and how bezafibrate interferes with alteration of the allosteric effects.

Case report. A 37-yr-old Japanese man was admitted to Kyushu University Hospital, Fukuoka City, Japan for evaluation of glycosuria and erythrocytosis. 20 yr previously, he was noted to have erythrocytosis, but had no further examinations performed at the time. The patient appeared to be plethoric, without other physical abnormalities. Hemoglobin was 20.5 g/dl; erythrocytes, 5.88 million/mm³; hematocrit, 61%; mean corpuscular hemoglobin, 34.8 pg; mean corpuscular hemoglobin concentration, 33.6%; mean corpuscular volume, 103.7 μm³; reticulocytes, 0.5%; leukocytes, 6,600/mm³; and platelets, 12.3×10^4/mm³. Sternal marrow examination showed normoblastic hyperplasia, with a myeloid-to-erythroid ratio of 0.90 to 1. The total erythrocyte mass was 47.7 ml/kg of body weight, exceeding the normal limit of 36.0 ml/kg of body weight. Oxygen and carbon dioxide tensions in the arterial blood were 87.1 mmHg and 38.3 mmHg, and in the venous blood were 47.7 mmHg and 42.6 mmHg, respectively. Blood glucose levels were 120 mg/dl at fast, and 147 mg/dl 120 min after ingestion of 75 g glucose. The glycosylated hemoglobin fraction (A_1c) was determined to be 51.8% of the total hemoglobin. The unusually high hemoglobin A_1c concentration prompted us to search for an abnormal hemoglobin as a possible cause of erythrocytosis in this individual.

Family study discovered that an apparently healthy, 18-yr-old son of the patient had the erythrocytosis and hemoglobin variant seen in the father. Hematological data were: hemoglobin, 18.4 g/dl; erythrocytes, 5.71 million/mm³; hematocrit, 51%; and leukocytes, 6,300/mm³.

Methods

Packed erythrocytes were washed with 0.9% NaCl and subsequently lysed in water and CCl_4. The methods for electrophoresis of hemoglobins either on a thin-layer starch gel plate (11) or on a cellulose acetate sheet (12), determination of relative concentration of hemoglobins by ion-exchange high performance liquid chromatography (HPLC) (13), purification of hemoglobins by chromatography on a DEAE-cellulose column (14), preparation of globins, and separation and aminoethylation of α-chains and β-chains (15), are as described previously. Erythrocyte concentrations of 2,3-DPG were determined by the method of Ericson and Verdier (16) using a Boehringer kit (Boehringer Mannheim Diagnostics, Houston, TX).

Tryptic peptides were made from aminoethylated globins in ammonium bicarbonate buffer at pH 8.0 and 37°C for 4 h. For analyses of the amino acid composition, peptides were separated by HPLC on a TSK LS-410S ODS column (5 μm, 0.4 × 30 cm; Toyo Soda Manufacturing Co., Ltd., Tokyo), or on a TSK IEX-510 SP SIL cation-exchange column (5 μm, 0.4 × 30 cm), using phosphate-acetonitrile gradient elutions (17, 18). All fractions were lyophilized and analyzed for amino acid composition after hydrolysis in 6 N HCl at 110°C for 20 h (19).

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Received for publication 8 March 1985.

1. *Abbreviations used in this paper:* DPG, diphosphoglycerate; HPLC, high performance liquid chromatography; P_{50}, blood oxygen tension at 50% oxygen saturation; R, high affinity; T, low affinity.

Oxygen equilibria were determined automatically in 0.11 M phosphate buffer for erythrocytes, and in 0.05 M bis-Tris (Tris) or Hepes (Sigma Chemical Co., St. Louis, MO) buffer for hemoglobin solutions, as described by Imai et al. (20). Hemoglobins were stripped of anions by gel filtration on a Sephadex G25 column (Pharmacia Fine Chemicals, Piscataway, NJ) (21). 2,3-Diphosphoglyceric acid (Sigma Chemical Co.) was prepared by conversion of the salt to the free acid with Dowex 50-X8 (Bio-Rad Laboratories, Richmond, CA) and neutralization of the acid with NaOH (21). The effects of 2-[4-(2-p-chlorobenzamidethyl)phenoxy]-2-methylpropionic acid (bezafibrate: Boehringer Mannheim Diagnostics) on the oxygen equilibria of erythrocytes and hemoglobin solutions were studied as described elsewhere (10). Hemoglobin concentrations were determined spectrophotometrically, using the extinction coefficients of hemoglobin A (9). Methemoglobin concentrations in solutions of hemoglobin isolated by chromatography remained <1%, as determined before equilibria measurements by the method described previously (9).

Results

Electrophoresis of hemolysates detected a hemoglobin variant, subsequently identified as hemoglobin Rahere (5), that migrated anodally to hemoglobin A at pH 8.6. Electrophoretic mobility of the variant on a cellulose acetate sheet was +3.07 at pH 8.6, measured by the method of Schneider and Barwick (12). Hemoglobins A_2, A, Rahere (or A_1), and the glycosylated derivative of hemoglobin Rahere composed 2.83%, 44.9%, 47.7%, and 4.5% of the total hemoglobins, respectively. Concentration of 2,3-DPG was 3.48 μmol/ml erythrocytes in the patient (mean of duplicate determinations), being slightly below the normal range of 4.34±0.58 (SD) μmol/ml erythrocytes.

Amino acid substitution. Separation of trypsin peptides of the aminoethylated β-chains from the variant is shown in Fig. 1. It was apparent that normal βT9 and βT10 peptides were absent in the peptide chromatograms, while three new peptides, βT8/9/10, βT9/10, and βT9/10/11, were found, each corre-

Table I. Amino Acid Composition of the βT9/10 Peptide from Hemoglobin Rahere*

Amino acid	βT8/9/10	βT9/10	βT9/10/11
Aspartic acid	4.04 (4)	4.01 (4)	5.63 (6)
Threonine‡	2.90 (2)	2.70 (2)	2.95 (2)
Serine	1.91 (2)	1.73 (2)	2.03 (2)
Glutamic acid	1.19 (1)	1.12 (1)	2.13 (2)
Glycine	3.18 (3)	3.47 (3)	3.38 (3)
Alanine	2.96 (3)	2.91 (3)	3.13 (3)
Valine	1.11 (1)	1.06 (1)	1.95 (2)
Leucine	5.99 (6)	6.29 (6)	7.13 (7)
Phenylalanine	1.98 (2)	2.03 (2)	2.89 (3)
Lysine‡,§	2.69 (4)	1.60 (3)	2.08 (3)
Histidine	2.02 (2)	2.08 (2)	2.94 (3)
Arginine			0.87 (1)
Proline			0.87 (1)
Residue number	66–95	67–95	67–104

* Numbers in parentheses refer to the theoretical values expected from the normal β-chain sequence.
‡ Values that differ significantly from those expected are underlined.
§ Values for lysine include contributions from the S-aminoethyl derivative of cysteine.

sponding to the residues 66–95, 67–95, and 67–104, respectively. The amino acid compositions of the three new peptides are shown in Table I, showing that 1 mol of lysine normally found in the βT9 peptide is replaced by 1 mol of threonine, so that no cleavage by trypsin occurs at the lysine residue. The composition of the other peptides from the β-chains was normal. The amino acid composition of the trypsin peptides derived from the α-chains was identical to that of the normal α-chains. We conclude
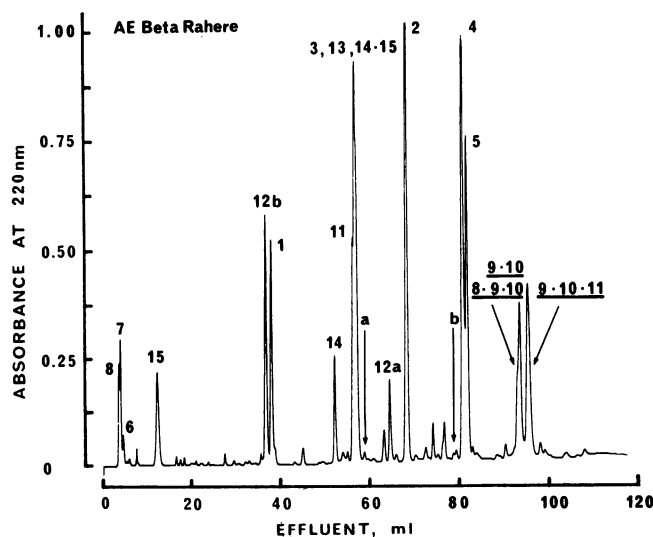

Figure 1. Separation of tryptic peptides from β-chains of hemoglobin Rahere on a reversed-phase HPLC column (17). Tryptic peptides are numbered in the sequence in which they occur in the polypeptide chains. Letters (a) and (b) show the positions for normal βT10 and βT9 peptides, respectively. Underlined numbers indicate the altered peptides.
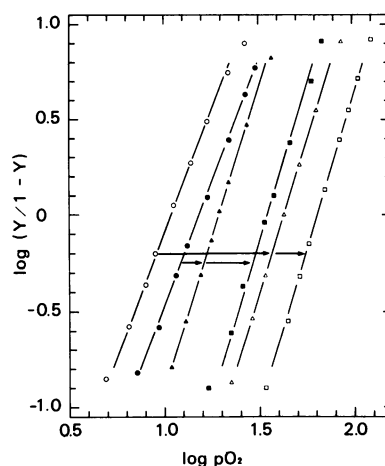

Figure 2. Effects of 2,3-DPG and bezafibrate on Hill plots of oxygen binding by 62 μM (heme) solutions of hemoglobins A (open symbols) and Rahere (closed symbols) at 37°C in 0.05 M Hepes buffer, pH 7.4. Triangles and squares denote data obtained with saturating concentration (100-fold over tetramer) of 2,3-DPG and 5 mM bezafibrate, respectively. Hemoglobins are, from left to right: (o) A, stripped; (●) Rahere, stripped; (▲) Rahere with 2,3-DPG; (■) Rahere with 2,3-DPG and bezafibrate; (△) A with 2,3-DPG; and (□) A with 2,3-DPG and bezafibrate.

Table II. Effects of Chloride, 2,3-DPG, and Bezafibrate on the Oxygen Affinities and Bohr Effects of Hemoglobin A and Hemoglobin Rahere

Cofactors	Log P_{50} at pH 7.4		$-\text{dlog } P_{50}/\text{dpH}$ at pH 7.4	
	A	Rahere	A	Rahere
	mmHg	*mmHg*		
None	1.04	1.19	0.36	0.24
0.1 M NaCl	1.29	1.30	0.43	0.28
1.55 mM 2,3-DPG	1.54	1.29		
5 mM BFR	1.47	1.49	0.63	0.44
0.1 M NaCl, 1.55 mM 2,3-DPG	1.55	1.30	0.55	0.34
0.1 M NaCl, 5 mM BFR	1.61	1.61		
1.55 mM 2,3-DPG, 5 mM BFR	1.82	1.55		

* Oxygen partial pressure at 50% saturation point, measured at 37°C in 0.05 M Hepes buffer and hemoglobin concentration of 62 μM (heme). BFR, bezafibrate.

from these results that the substitution must be a threonine residue for a lysine residue at position 82 of the β-chains. This variant, therefore, is identical with hemoglobin Rahere that has been found in an English family by Lorkin et al. (5).

Oxygen equilibria. Fig. 2 shows that stripped hemoglobin Rahere has lower oxygen affinities than do solutions of hemoglobin A in 0.05 M Hepes buffer at pH 7.4. In addition, the variant is much less affected by 2,3-DPG cofactor. The Bohr effects are compared in Table II, showing that the slopes of the curves, as expressed by $-\Delta \log P_{50}/\Delta pH$ (where P_{50} is the blood oxygen tension at 50% oxygen saturation), for both hemoglobins A and Rahere are similar. Nevertheless, there may be a small Bohr effect difference between the two hemoglobins. As shown in Fig. 3, the Bohr effect of hemoglobin Rahere is greatly reduced relative to that of hemoglobin A when compared in the presence of 2,3-DPG. A distinctive feature of the substitutions of a neutral

residue for the β82 lysine is the fact that the variant is the low affinity molecule even in the absence of cofactors. This is shown in Table II, where the effect of 0.1 M NaCl on Hb Rahere at pH 7.4 is seen. While stripped hemoglobin Rahere has less total NaCl-induced change in ligand affinity than hemoglobin A, its oxygen affinity in 0.1 M NaCl is essentially the same at pH 7.4. Both hemoglobin Rahere and hemoglobin A in 0.05 M bis-Tris or Tris buffers show a fairly normal degree of cooperativity in oxygen binding. The *n* values at half (50%) saturation point (n½) are typically 2.8 to 3.0 from pH 6 to pH 9.

Fig. 4 shows the effects of 2,3-DPG and bezafibrate on purified hemoglobin Rahere and hemoglobin A in the presence of 0.1 M NaCl at pH 7.4 and 37°C. Bezafibrate lowers the oxygen affinity of hemoglobin Rahere as strongly as that of hemoglobin A. This contrasts with the finding that 2,3-DPG has no effects on the oxygen dissociation curves of hemoglobin Rahere under the same conditions.

Oxygen dissociation curves of the patient's erythrocytes at 37°C are compared with a normal curve in Fig. 5. The blood oxygen tension at 50% oxygen saturation (P_{50}) is 22 mmHg compared with the normal value of 31 mmHg. 30 min after incubation with 5 mM bezafibrate in Hepes buffer at pH 7.2 and 37°C, the P_{50} value for patient's erythrocytes containing both hemoglobin Rahere and hemoglobin A increased from 31.6 to 44.6 mmHg, and that for normal erythrocytes increased from 39.8 to 58.6 mmHg, respectively. Thus, in the absence or presence of 5 mM bezafibrate, the log P_{50} values for normal erythrocytes differ by +0.17, and the values for patient's erythrocytes differ by +0.15, an equal degree of curve shifts being observed.

Discussion

The β82 (EF6) lysine residues in human hemoglobin are involved in the binding of anionic cofactors (3, 4). When a cofactor binds to this site the allosteric equilibrium between the high affinity (R) and low affinity (T) conformations is shifted toward the T state (3). This residue is replaced by a threonine residue in hemoglobin Rahere (5), by methionine in hemoglobin Helsinki
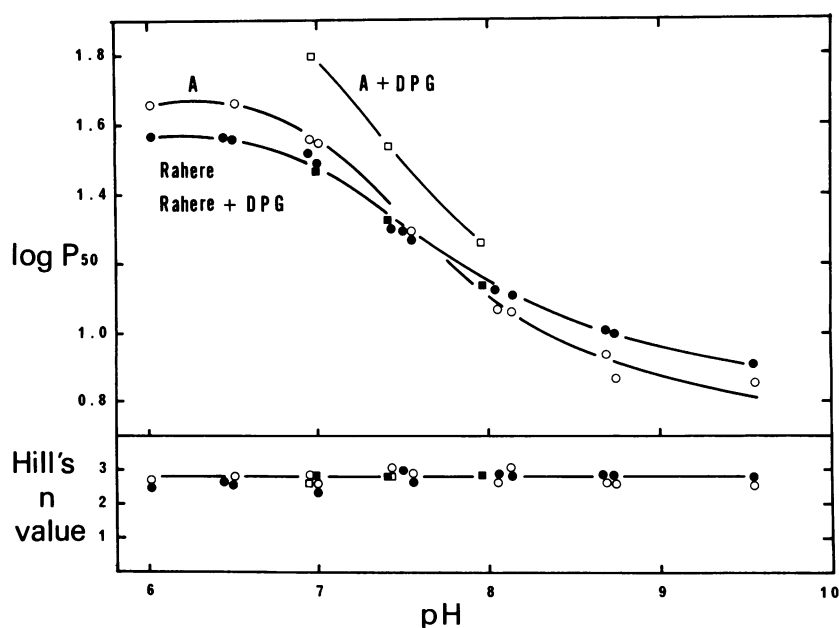

Figure 3. Effects of pH and 2,3-DPG on the oxygen affinity of 62 μM (heme) solutions of stripped hemoglobins A (o) and Rahere (•) at 37°C in 0.05 M bis-Tris (or Tris) buffer containing 0.1 M NaCl and 1 mM EDTA. Open and closed squares denote data obtained in the presence of 1.55 mM 2,3-DPG. The effect of 2,3-DPG on the Hill constant (n) is shown in the lower part of the figure.
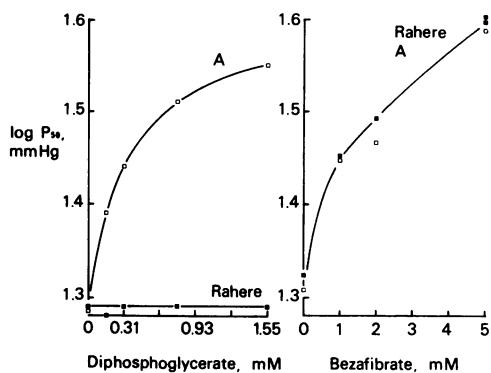
Figure 4. Effects of increasing concentrations of 2,3-DPG and bezafibrate at pH 7.4 on the oxygen affinity of hemoglobin A (□) and Rahere (■). Experimental conditions are the same as in Fig. 2.

(8), and by asparagine in hemoglobin Providence (9). As a result, the heterotropic (pH and anions) interactions due to alteration of the binding site are reduced substantially, though without concomitant change in the degree of homotropic (heme-heme) interactions.

In the absence of anionic cofactors, hemoglobin Rahere shows a decreased oxygen affinity, which implies that the deoxy-(T) state is stabilized or the oxy- (R) state is destabilized. Chloride ions can cause significant changes in hemoglobin's oxygen affinity even when position $\beta 82$ is not occupied by a positively charged group (7). The binding of chloride to the $\beta 82$ lysine residue would effectively reduce the net positive charge for the anion binding site in hemoglobin A, and it has been suggested that this effect may be a significant factor in the NaCl-induced reduction of the oxygen affinity of hemoglobin A (17). It would follow that the reduced oxygen affinity of the stripped hemoglobin Rahere is partially due to a decrease in positive charge density of the anion binding site. The effect of chloride ions on hemoglobin Rahere may then be related to a further decrease in net positive charge in this region when chloride binds to other members of the net charge cluster that form the binding site.

Bezafibrate greatly lowers the oxygen affinity of hemoglobin Rahere to a degree similar to that of hemoglobin A in 0.1 M NaCl solution at pH 7.4, where chloride ions may effectively mask the positive charges of the 2,3-DPG binding site in hemoglobin Rahere, implying that bezafibrate and 2,3-DPG com-

bine with different binding sites in the hemoglobin molecule. It is relevant to this discussion that formation of the T state is opposed by the positive charges of $\beta 82$ lysine residues and the other members of the charge cluster that form the 2,3-DPG binding site (9). Also, a pair of charged side chains that form the bezafibrate binding site (10) may contribute to this effect, since it suggests a basis for the additional decrease in oxygen affinity, which is observed when both cofactors are bound to the hemoglobin molecule. Bezafibrate also enhances the Bohr effect for hemoglobin Rahere. X-ray crystallographic analyses have shown that bezafibrate binds to a pair of sites between the two α-chains in the central cavity of the hemoglobin A molecule (10). In view of this evidence, bezafibrate may be thought to combine with the site formed between the amino and carboxyl termini of opposite α-chains of deoxyhemoglobin. Perutz and TenEyck (22) and Fermi (23) reported that there were four salt bridges, all involving terminal residues, in deoxyhemoglobin that were broken upon oxygenation. The presence of three of these has been confirmed by x-ray crystallography at high resolution (23). Also, the first two residues of the amino terminus of the α-chains might, under physiologic conditions, form salt bridges between opposing α-chain termini, but these are broken at high salt concentration (23). These salt bridges between the NH_2 terminal and COOH-terminal residues of the α-chains facing the central cavity in deoxyhemoglobin might be stabilized by the bezafibrate molecule held in place by polar interactions with these groups.

The results in this paper support the previous observation that bezafibrate and 2,3-DPG enhance each other's effect additively on oxygen dissociation curves for normal hemoglobin (10). While the natural allosteric effector influences only hemoglobin A, but not hemoglobin Rahere in erythrocytes, bezafibrate combines with both hemoglobins, thereby shifting the oxygen equilibrium curves for those hemoglobins toward the low affinity forms. The erythrocyte membrane is permeable to bezafibrate. Although the results described herein are encouraging from a clinical viewpoint, it remains to be seen whether a bezafibrate dose (450 mg–600 mg/d) as used in the therapy of hyperlipidemia patients (24) can build a concentration of this drug in erythrocytes sufficient to benefit the oxygen transport capacity. It is important to realize that the oral dose of bezafibrate employed in the therapy of hyperlipidemia patients will yield a plasma concentration at least an order of magnitude below that employed in the in vitro work presented in this paper.
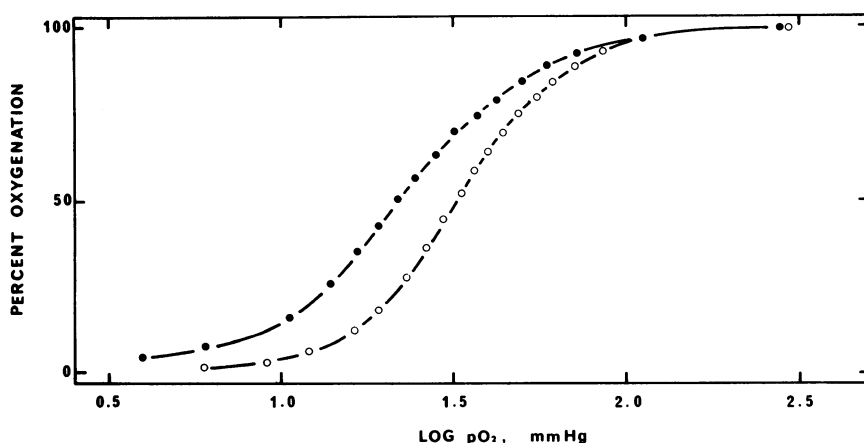

Figure 5. Oxygen equilibrium curves of the erythrocytes at 37°C in 0.11 M phosphate buffer, pH 7.4. Open symbols (o) represent the normal control, and closed symbols (●), the patient's erythrocytes.

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