

Genetic Variants of Glucose 6-Phosphate Dehydrogenase from Human Erythrocytes: Unique Properties of the A⁻ Variant Isolated from "Deficient" Cells*

(protein structure/sulfhydryl groups/enzyme inactivation/biochemical genetics)

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ABSTRACT The A⁻ type of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) has been isolated from human erythrocytes deficient in this enzyme. The specific activity of the purified protein is similar to that previously reported for the enzyme isolated from normal, nondeficient erythrocytes. During the purification procedure, a portion of the A⁻ enzyme converts spontaneously, from the native "fraction I", to a "fraction II" having different kinetic and chromatographic properties. The conversion of fraction I to II can be reproduced freely by treatment with iodosobenzoate, and fraction II can be converted back to fraction I by treatment with dithioglycol. We suggest that fraction II is an enzyme species in which one or more sulfhydryl groups have been oxidized to disulfide(s). The tendency to oxidation appears to be a property specific to the A⁻ variant and may represent the basis for its rapid rate of inactivation and consequent deficiency *in vivo*.

Deficiency in the activity of glucose 6-phosphate (Glu-6-P) dehydrogenase (EC 1.1.1.49), the most common genetically determined enzyme abnormality known in man (1), is associated with diverse forms of the enzyme in different populations (2-4). For instance, in the Mediterranean area a common variant associated with deficiency of activity has the electrophoretic mobility of the normal enzyme, designated B₁. In Africa and in populations of African ancestry the common variant associated with enzyme deficiency (A⁻) has the same electrophoretic mobility as another variant, having normal activity, designated A. In spite of some previous suggestions to the contrary, all forms of deficiency studied thus far are the result of a structural change in the enzyme, and not of a regulatory mutation (5-9). However, for both of the two deficient variants mentioned, the nature of the structural change remains unknown, largely due to the difficulties in obtaining the protein in a pure state. Also, it is not clear why the A⁻ variant (and perhaps the Mediterranean variant as well) is inactivated *in vivo* more rapidly than the normal enzyme (2, 7, 10, 11).

Abbreviations: DFP, diisopropylfluorophosphate; S₂ glycol, dithioglycol; S₂ threitol, dithiothreitol; (NH₂)Cap, ε-aminocaproic acid; Glu-6-P, glucose 6-phosphate; NEM, N-ethylmaleimide.

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‡ Throughout this paper we have followed the genotypic and phenotypic nomenclature recommended by the World Health Organization (1).

We report here the first purification of the A⁻ enzyme to a state of electrophoretic homogeneity. We found that this protein tends to undergo conversion from its native form to a form with distinctive properties in which two or more sulfhydryl groups have been oxidized to disulfide. This conversion may be the basis for the instability of the A⁻ variant *in vitro* and for the enzyme deficiency *in vivo*.

MATERIALS AND METHODS

Reagents. NADP, NADPH, and iodosobenzoate were purchased from Sigma Chemical Co., St. Louis, Mo; Glu-6-P from Boehringer, Mannheim, Germany; Sephadex products from Pharmacia, Uppsala, Sweden; DEAE-cellulose from Reeve & Angel; N-ethylmaleimide (NEM) and dithiothreitol (S₂ threitol) from California Biochemicals, Richmond, Calif.; and dithioglycol (S₂ glycol) from Eastman Kodak, Rochester, N.Y. All other reagents were of analytical grade from various commercial sources.

Enzyme Assay. For routine purposes and while following the purification, enzyme activity was assayed by standard procedures (1). In other cases, pH and other conditions of assay are specified. Activity is expressed in international units (1), unless otherwise specified.

Electrophoresis of Enzyme. Starch gel electrophoresis was used for "typing" the enzyme variants; the technique was as described (12).

For acrylamide gel electrophoresis, the apparatus used was that from Canalco, Rockville, Md. Unless otherwise specified, we used columns (4 × 50 mm) of 8% gel in 0.5 M Tris·HCl buffer (pH 8.0) containing 2.5 mM EDTA and 5 μM NADP (see ref. 13). The electrophoresis was for 15 hr at 4° and 160 V. With this technique, migration distance of the normal B enzyme was about 20 mm.

Purification. The procedure incorporates several steps from published methods (14-18). Basically, blood units that had been stored between 7 and 28 days were screened by starch gel electrophoresis. Bottles of blood containing the A⁻ variant of the enzyme with more than 0.7 unit/g of hemoglobin were used. The erythrocytes were washed twice with normal saline (0.15 M). The enzyme was then concentrated by partial hemolysis (most of the activity in blood containing the A⁻ variant is concentrated in the younger cells; ref. 2, 7, 11). The erythrocytes were, therefore, treated with 0.06 M NaCl, to

TABLE 1. Purification of glucose 6-phosphate dehydrogenase variant A⁻, compared to the normal type B

Step*	A ⁻					B	
	Total units	Total mg	IU/mg	Purification	Yield (%)	IU/mg	Yield (%)
1. Hemolyzate	148	156,000	0.00095	1.6	95	0.0055	100
2. DEAE-cellulose (pH 6.4) followed by (NH ₄) ₂ SO ₄ precipitation	98	670	0.097	154	63	0.687	62
3. Calcium phosphate gel, followed by (NH ₄) ₂ SO ₄ precipitation	92	302	0.304	511	59	—	—
4. Sephadex G-100	66.5	44	1.52	2,560	43	2.87	34
5. DEAE-Sephadex (pH 7.0)	60.6	18.5	3.28	5,515	39	4.5	17
6. CM-Sephadex (pH 6)							
fraction I	6.8	0.08	85	143,100	4.4	109.5	17.6
fraction II	14.6	1.1	13.4	22,500	9.4	—	—

* All solutions contained 13 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM diisopropylfluorophosphate (DFP) or ϵ -aminocaproic acid [(NH₂)₂Cap] and 0.02 mM NADP, unless otherwise indicated. (1) Starting material was about 1700 ml blood for A⁻ and 3400 ml blood for B. Hemolysis was performed according to Rattazzi (17), except that for A⁻ hemolysis was preceded by elimination of the oldest cells (see text). Purification was 1.6-fold, referred to the specific activity that would have been obtained if all cells had been lysed at once. (2) Similar to that of Chung and Langdon (14), except that the resin slurry was poured into a column 5 cm in diameter, and the enzyme was eluted by a linear gradient of NaCl (0.1–0.6 M, in 0.05 M phosphate buffer, pH 6.4). The fractions with enzyme activity were pooled and brought to 60% saturation with (NH₄)₂SO₄. The precipitate was collected by centrifugation for 20 min at 18,000 × *g* and redissolved in a small volume of 0.05 M phosphate buffer. (3) According to Bonsignore *et al.* (21), followed by (NH₄)₂SO₄ precipitation and redissolution as in step (2). (4) As in ref. 17, except that our column was 7.5 × 90 cm and no recycling chromatography was used. Flow rate was between 60 and 90 ml/hr. (5) According to Luzzatto and Allan (6), except that the column was 3.5 × 18 cm. (6) According to ref. 17, with the following changes. (a) The enzyme solution from the previous step was desalted by dialysis against the appropriate buffer instead of passage through Sephadex G-25. (b) After the enzyme was applied, the column was washed with 200 ml of 20 mM phosphate buffer (pH 6.0), followed by a linear gradient of 100 ml of buffer and 100 ml of 0.03 M NaCl in buffer, before elution by 2 mM Glu-6-P. For A⁻, further elution was as described in the text and in the legend to Fig. 1.

hemolyze only the older cells. The remaining cells were then hemolyzed at lower ionic strength, and this second hemolyzate was used as the starting material for further purification, as summarized in Table 1.

Kinetic Constants. These were determined as described (12, 19).

Sucrose Gradients. Samples were applied to 5-ml gradients of 4–20% sucrose dissolved in 0.05 M Tris·borate (pH 8.0) containing 0.1 mM EDTA and 10 μ M NADP. Hemoglobin was used as a marker; it had a nominal sedimentation coefficient of 4.6 S. The tubes were centrifuged for 16 hr at 6° at 30,000 rpm in the SW 39 rotor of the Spinco ultracentrifuge, model L. At the end of the run, 36 fractions were collected from each tube and tested for enzyme activity.

RESULTS

Behavior of the A⁻ variant during purification

In all but the last step of the purification procedure, Glu-6-P dehydrogenase type A⁻ and the normal B type behaved similarly. At this point the purification of A⁻ was almost seven times greater than that of B (Table 1), in other words, the specific activities of A⁻ and B were about the same. This result suggests that enzyme deficiency in the A⁻ hemolyzate is essentially due to a smaller number of active enzyme molecules (20) and that the molecular enzymatic activity of A⁻ and B is similar.

In the last purification step, the behavior of the two variants was sharply different. Variant B was effectively eluted from the CM-Sephadex column by 2 mM Glu-6-P as a single peak with high recovery (60–100%), as originally described by Rattazzi (17). In contrast, the same concentration of Glu-6-P eluted only 10% of A⁻. However, a further 25% of the enzyme

activity applied to the column could be recovered by increasing the pH to 7§ (Fig. 1).

Properties and interconversion of the two forms of A⁻

Since the resolution of two peaks of enzyme activity appeared to be a peculiarity of the A⁻ variant, a study of these peaks might give clues to the nature of the basic abnormality in A⁻. We assumed that the two peaks consisted of two different molecular forms of the enzyme, and that one had arisen from the other during purification. In order to prove these points, we studied some physical and enzymic properties of the fractions, and the conditions required for their interconversion.

Physicochemical Properties. Electrophoresis on polyacrylamide gels was performed in low and high NADP (Fig. 2). In both cases there was a significant difference of mobility between the two fractions. The difference appeared too small to be compatible with a dimerization process. (This was confirmed by sucrose gradient analysis, in which a sedimentation coefficient of about 7 S was found for both fractions.) Rather, we attribute the difference in electrophoretic migration to a change in charge or in conformation. The effect of NADP can be similarly interpreted, and may be related to the transition of the enzyme from a state of low affinity to a state of high affinity for NADP, as previously demonstrated by kinetic experiments (9, 19) and by binding experiments (21). The NADP-induced change in mobility is much less for fraction

§ The relative and absolute amounts of activity recovered in the two peaks have varied. In one case, the two peaks have not been clearly resolved. The variability is likely to depend on the extent of spontaneous oxidation of the native A⁻ enzyme that takes place before the last purification step.

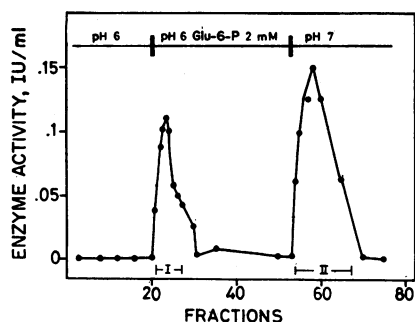


FIG. 1. Elution of purified Glu-6-P dehydrogenase variant A^- from a CM-Sephadex column (see step 6 in Table 1). Since the enzyme activity eluted by Glu-6-P was only 5% of that applied, elution was continued with 0.05 M phosphate buffer (pH 7.0) and a second peak of activity was obtained. In a similar experiment with the normal enzyme B, 60–100% of activity would be eluted in the position indicated as fraction I.

II than for fraction I, suggesting that its conformation is less affected by NADP. Consistent with this, we found that the thermostability of this fraction, while similar to that of fraction I, is almost independent of [NADP] over a concentration range from 10^{-7} to 10^{-3} M (Fig. 3).

Enzymic Properties. The Michaelis constant for Glu-6-P of fraction I was within the range of previously reported values for less-pure preparations (1); however, for fraction II it was over three times higher (Table 2). The finding probably explains why this fraction was not eluted by the substrate from CM-Sephadex. Analysis of double-reciprocal plots always revealed a single straight line for peak I, but sometimes a two-branched line for peak II, suggesting that it is contaminated by some of the enzyme in peak I (Fig. 4). With respect to NADP-binding, fraction I exhibits sigmoid kinetics, with a transition at very low substrate concentration, as reported (9), whereas fraction II exhibits hyperbolic kinetics, with a K_m of $4.4 \mu\text{M}$.

Interconversion of the Two Forms. 0.5 ml of fraction II, containing 0.5 unit of enzyme, was dialyzed overnight at 4° against three changes, 300 ml each, of 50 mM Tris-borate buffer (pH 8.0)–0.1 mM EDTA– $10 \mu\text{M}$ NADP. S_2 threitol (Cleland's reagent) (22) was then added at a final concentration of 10 mM and incubation was performed at 25° for 30 min. The properties of fraction II now became indistinguishable from those of fraction I (which in contrast was not significantly affected by identical treatment). Not only the electrophoretic mobility was now the same, but the behavior with respect to Glu-6-P (Table 2) and NADP was also restored to normal. The conditions for the reaction with S_2 threitol appear to be critical; for instance, full conversion was not obtained if we started from a mixture of fractions I and II of the enzyme. In contrast, using the K_m for Glu-6-P as a marker of the conversion, we found that another sulfhydryl group reagent, S_2 glycol, can convert fraction II to fraction I reproducibly and at lower concentrations (1–5 mM; see Table 2 and Fig. 4).

These results suggest that peak II is an oxidized form of the enzyme. We attempted to obtain the oxidation artificially by incubating peak I (or a mixture of peaks I and II; Fig. 4) with 2 mM iodosobenzoate for 90 min at 25° . After dialysis,

the K_m for Glu-6-P was now $230 \mu\text{M}$, like that of peak II. In order to prove that iodosobenzoate acts by attacking sulfhydryl groups (23), we pretreated peak I with NEM. This alkylating agent causes some inhibition (12) and a slight increase in K_m ; however, once the sulfhydryl groups are alkylated, iodosobenzoate can no longer produce the marked increase in K_m characteristic of fraction II. Finally, the entire conversion cycle was performed by treatment of peak I first with iodosobenzoate and then with S_2 glycol; the K_m went from 78 to $230 \mu\text{M}$, and then back to $120 \mu\text{M}$ (Table 2).

In control experiments, S_2 threitol, S_2 glycol, and iodosobenzoate failed to produce any significant change in the K_m for Glu-6-P of variants A and B of Glu-6-P dehydrogenase.

DISCUSSION

Thus far, only three variants of human erythrocyte Glu-6-P dehydrogenase have been purified almost to homogeneity: B (14–18), A(24), and Hektoen (25). Of these, B is considered the normal enzyme type; A is a variant common in Africa with nearly normal activity (26); Hektoen is a rare variant with increased activity. Here the purification of a variant with markedly decreased enzyme activity is described. The low yield of enzyme contrasts with the higher yield of B obtained previously by others (15, 17) and by us in parallel; it undoubtedly reflects an intrinsically lower stability of A^- ; and it might perhaps be increased by protection of sulfhydryl groups with S_2 threitol or S_2 glycol.

Although the amount of pure protein obtained is very small, it should become possible, in areas like Nigeria where the incidence of A^- is high (27), to accumulate sufficient material for direct structural analysis. Meanwhile, our studies have been based on enzymatic activity. The results show directly that the specific activities of pure A^- and B are comparable (see Table 1; the accuracy of protein determination in the very dilute solution of A^- is not sufficient to state that they are identical). Thus, enzyme "deficiency" *in vivo* is due mainly to a loss of active enzyme molecules, rather than a decreased activity of each molecule. Consistent with this interpretation is the loss of activity that can be demonstrated in erythrocytes containing the A^- variant in the course of their ageing (2, 7, 10, 11).

The existence of the two interconvertible forms of the enzyme described in this report suggests a specific mechanism

TABLE 2. Interconversion of two forms of glucose 6-phosphate dehydrogenase type A^-

Pretreatment	K_m for Glu-6-P (μM)	
	Peak I	Peak II*
None	78 ± 8	240 ± 20
S_2 threitol (10 mM)	75	70†
S_2 glycol (5 mM)	—	81
Iodosobenzoate (2 mM)	230	242
Iodosobenzoate (2 mM) followed by S_2 glycol (2 mM)	120	—
NEM (0.1 mM)	110	—
NEM (0.1 mM) followed by iodosobenzoate (1.2 mM)	119	—

* Sometimes yields a two-branched double-reciprocal plot (see Fig. 4), suggesting contamination by fraction I.

† Double-reciprocal plot also yielded another abscissa intercept at $270 \mu\text{M}$.

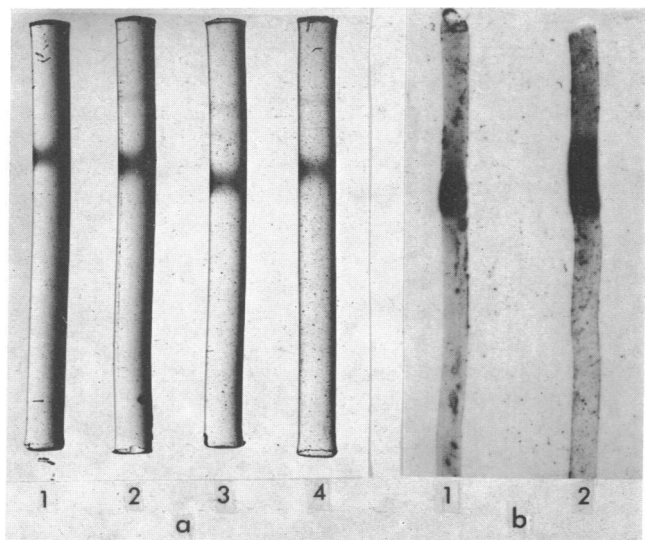


FIG. 2. Polyacrylamide gel electrophoresis of purified fractions of the A⁻ variant of Glu-6-P dehydrogenase (see *Methods*). The vessel buffer always contained NADP. (a) Columns 1 and 3, fraction I; columns 2 and 4, fraction II. Columns 1 and 2, but not columns 3 and 4, had 10 μM NADP in the gel. All columns were stained for enzyme activity. (b) Electrophoresis of fraction I on microcolumns (1 × 40 mm) for 1.2 hr at 0.5 mA/column. Column 1 was stained for protein with Coomassie blue; column 2 was stained for enzyme activity.

for inactivation. Fraction I probably represents the “native” form; its kinetics (Table 2) and thermostability (Fig. 3) correspond to those previously described in crude prepara-

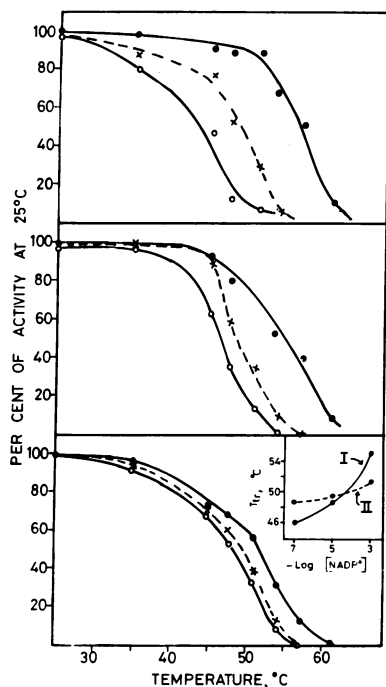


FIG. 3. Thermostability of Glu-6-P dehydrogenase variant A⁻, compared to that of the normal B type. (Top) B; (middle) A⁻, fraction I; (bottom) A⁻, fraction II. O, 10⁻⁷ M [NADP]; ●, 10⁻³ M [NADP]; ×, 10⁻⁵ M [NADP]. The inset in the bottom panel shows the transition temperature (at which 50% of the enzyme is inactivated in 7 min) as a function of [NADP]. Technique as in ref. 6.

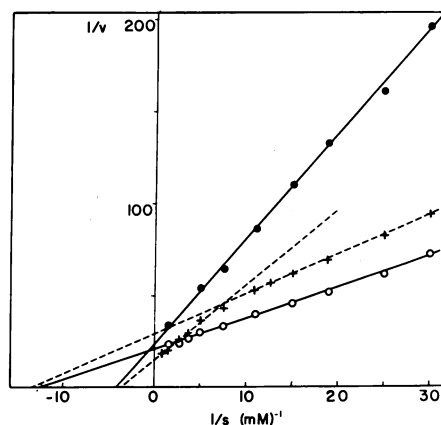


FIG. 4. Dependence of reaction velocity on Glu-6-P concentration. (Abscissa) reciprocal of Glu-6-P concentration; (ordinate) reciprocal of reaction velocity, expressed as ΔA₃₄₀/min. ×---×, peak II (Fig. 1): the experimental points extrapolate to two different K_m values, 75 and 270 μM. O—O, peak II treated with S₂ glycol (see Table 2): the abscissa intercept yields a K_m value of 81 μM. ●—●, peak II, treated with iodosobenzoate (Table 2): the abscissa intercept yields a K_m value of 242 μM.

tions of A⁻ (1, 9, 28). Furthermore, this fraction, once isolated, is very stable (no loss of activity in 1 month at 4°). Fraction II was not demonstrable by polyacrylamide electrophoresis at any stage before elution from CM-Sephadex (Fig. 1), and probably arose from the native form. Fraction II has abnormally low affinity for both substrates, Glu-6-P and NADP, and is unstable (loss of 50% of activity at 4° in 1 week). The capacity of iodosobenzoate to change the properties of fraction I to those of fraction II, and of S₂ threitol or S₂ glycol to restore to fraction II the properties of fraction I, strongly suggests that oxidation-reduction of sulfhydryl groups is involved in the interconversion. Since production of fraction II is not associated with a major change in molecular size, we infer that a disulfide bridge is formed within the oligomeric molecule (Fig. 5). This model can explain the observed differences in the properties of the two forms of A⁻. Glu-6-P dehydrogenase requires NADP for stability (28, 29), and this

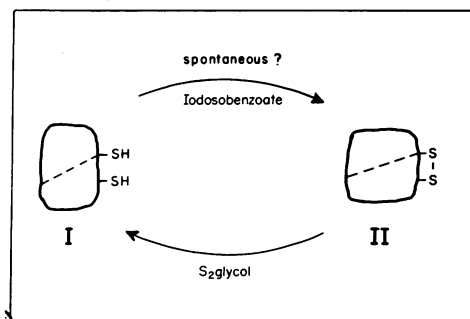


FIG. 5. Model for the production of an oxidized form of Glu-6-P dehydrogenase type A⁻. The diagonal broken line across the enzyme molecule symbolizes the contact between the two chains of the dimer. The disulfide bridge, shown near that line, might be either inter-chain or intra-chain. The oxidation is reversible *in vitro*; it may or it may not be reversible *in vivo*, but fraction II tends to become inactive.

effect is concentration dependent (6). Furthermore, NADP can cause a transition of the enzyme from a state of low affinity for NADP to a state of high affinity for NADP (19, 21). All these properties hold for A⁻ in the native form (see ref. 9 and this report). In contrast, the second (unstable) form of A⁻ shows very little dependence on NADP concentration in either electrophoretic mobility (Fig. 2) or thermostability (Fig. 3, *inset*) and has a relatively low affinity for NADP. We, therefore, suggest the possibility that the second form of A⁻ cannot undergo the transition from a state of low affinity to a state of high affinity for NADP. Presumably, the extra disulfide bridge imposes novel constraints on the possible conformations of the molecule, which is locked in the state of low affinity for NADP. Thus, the findings specifically suggest that the structural change (probably a single amino-acid substitution) in A⁻ is one that brings two cysteine residues in a favorable position for disulfide bridge formation. If the disulfide form is labile, such a change could explain all observed properties of A⁻, including the biological abnormality of decreased stability *in vivo* that leads to enzyme deficiency in the erythrocyte.

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