*A SINGLE AMINO ACID SUBSTITUTION (ASPARAGINE TO ASPARTIC ACID) BETWEEN NORMAL (B+) AND THE COMMON NEGRO VARIANT (A+) OF HUMAN GLUCOSE-6-PHOSPHATE DEHYDROGENASE**

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Extensive work with human hemoglobins indicates that the typical variant hemoglobin differs from normal hemoglobin by a single amino acid substitution. Mutational studies with *E. coli* tryptophan synthetase have shown that variants of this protein also owe their origin to single replacement of an amino acid. Using biochemical and genetic recombination techniques, it has been shown that the site of such replacements in the amino acid of the enzyme bears a linear relationship to the site of the mutation within the gene specifying the structure of this enzyme.[1] Based on this type of evidence, there is general belief among biochemical geneticists that most mutationally altered enzymes are produced by single amino acid substitutions. Since it is difficult to obtain the necessary quantities of pure enzyme proteins for the required biochemical analyses, proof for this hypothesis for mutant enzymes from mammals is not yet available.

About 20 variants of human red cell glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, E.C.1.1.1.49) are known. A common variant found in about 18 per cent of American Negro males manifests with rapid electrophoretic mobility and is not associated with enzyme deficiency.[2-4]

Normal human glucose-6-phosphate dehydrogenase (B+) and the Negro-type variant with normal activity (A+) have been isolated in homogeneous form, and their molecular weights, subunit molecular sizes, amino acid compositions, enzymatic properties, and serological characteristics have been compared.[5, 6] The results indicated that the two enzymes were very similar and that any structural difference between them would be very small. Further studies indicate that the two enzymes differ in a single amino acid–asparagine to aspartic acid.

Materials and Methods.—Glucose-6-phosphate dehydrogenase: The normal (B+) and the Negro-type variant (A+) enzymes were prepared from blood by methods described previously.[5, 6] The preparations were homogeneous by physicochemical criteria. Trypsin (EC.3.4.4.4, salt-free, 3 × cryst.), leucine amino peptidase (EC.3.4.1.1, diisopropylfluorophosphate, DFP treated, 80 units/mg), carboxypeptidase A (EC.3.4.2.1, 2× cryst., 40 units/mg), and carboxypeptidase B (EC.-3.4.2.2, 100 units/mg) were purchased from Worthington Biochemical Corporation. Leucine amino peptidase was activated by $MgCl_2$ before use. Carboxypeptidase A and B were treated with DFP before use.[7]

Urea was deionized by recrystallization according to the method described by Benesch *et al.*[8]

Iodoacetic acid was recrystallized from ether and kept at −20°. The procedures used for reduction and S-carboxymethylation of the enzyme were essentially those described by Crestfield *et al.*,[9] and the details have been reported.[10]

Trypsin digestion: The lyophilized powder of the reduced S-carboxymethylated protein was digested by trypsin at room temperature for four hours. During the

digestion, the pH of the reaction mixture was maintained at pH 8.0–8.2 by adding 0.01 *M* $(NH_4)HCO_3$. The reaction mixture was acidified to pH 3–4 with acetic acid and lyophilized.

Fingerprinting: Tryptic hydrolysates (lyophilized powder), 2.5–3 mg, were applied to a Whatman no. 3 MM (57 × 46 cm paper). The method of fingerprinting of the tryptic hydrolysates was essentially that described by Katz *et al.*[11] Peptide spots were developed by heating at 60° for 30 minutes after spraying 0.025 per cent of ninhydrine solution in acetone.

Results.—Fingerprints: The fingerprints of tryptic peptides obtained from the normal (B+) and the Negro-type variant (A+) of glucose-6-phosphate dehydrogenase are shown in Figure 1. Among approximately 40 peptide spots appearing in each fingerprint, only one peptide spot (marked I) of the normal enzyme was replaced by a peptide (marked II) in the variant enzyme; all other peptide spots corresponded to each other in both enzymes. The results strongly suggested a single amino acid substitution between the normal and the variant enzyme. Since peptide I moved more rapidly than peptide II on electrophoresis at pH 3.6–3.7, it was likely that an amino acid residue of peptide I might be substituted by a more acidic amino residue of peptide II.

Amino acid substitution: The peptide spots marked I and II were cut and eluted. Amino acid analysis was carried out with a Technicon amino acid analyzer.

The amino acids found in peptides I and II are shown in Table 1. Both peptides had the same amio acid composition after acid hydrolysis.

Considering the difference of electrophoretic mobility, asparagine or glutamine in peptide I should be replaced by aspartic acid or glutamic acid in peptide II, since asparagine and glutamine are converted to aspartic acid and glutamic acid by acid hydrolysis.

To distinguish between the two possibilities, peptides I and II were digested by peptidases, and the amino acids that appeared were identified by high-voltage electrophoresis and paper chromatography.

Each peptide (I or II) was dissolved in 0.25 ml of 0.1 *M* triethanolamine–acetic acid buffer (pH 7.9) containing 0.005 *M* $MgCl_2$ and was digested by leucine amino peptidase (0.021 mg) at 30° for 20 hours (toluol was added to prevent bacterial growth). Subsequently, the peptide was digested with carboxypeptidase B (0.05 mg) and carboxypeptidase A (0.01 mg) for another 20 hours at 30°. As a control, the reaction mixture without the peptide was incubated under the same conditions. After digestion, the reaction mixture was acidified to pH 3–4 with acetic acid and treated with about 0.3 ml (bed volume) of Dowex-50, ×12 (H+ form, 20–50 mesh). The amino acids absorbed by the resin were extracted with 4 *N* NH_4OH. The eluents were evaporated to dryness under reduced pressure in the presence of sulfuric acid and used for identification of amino acids.

The analysis was carried out using the following system: first dimension: paper electrophoresis, Whatman no. 3MM (57 × 46 cm), formic acid–pyridine–water (4:03:96, V/V, pH 2.2), 2000 volts for 60 min; second dimension: paper chromatography, ethanol-water (77:23, V/V) for 16 hours. This system gave a good separation of all amino acids which might be contained in the peptides I and II. Thus, it was possible to distinguish asparagine, aspartic acid, glutamine, and glutamic acid.

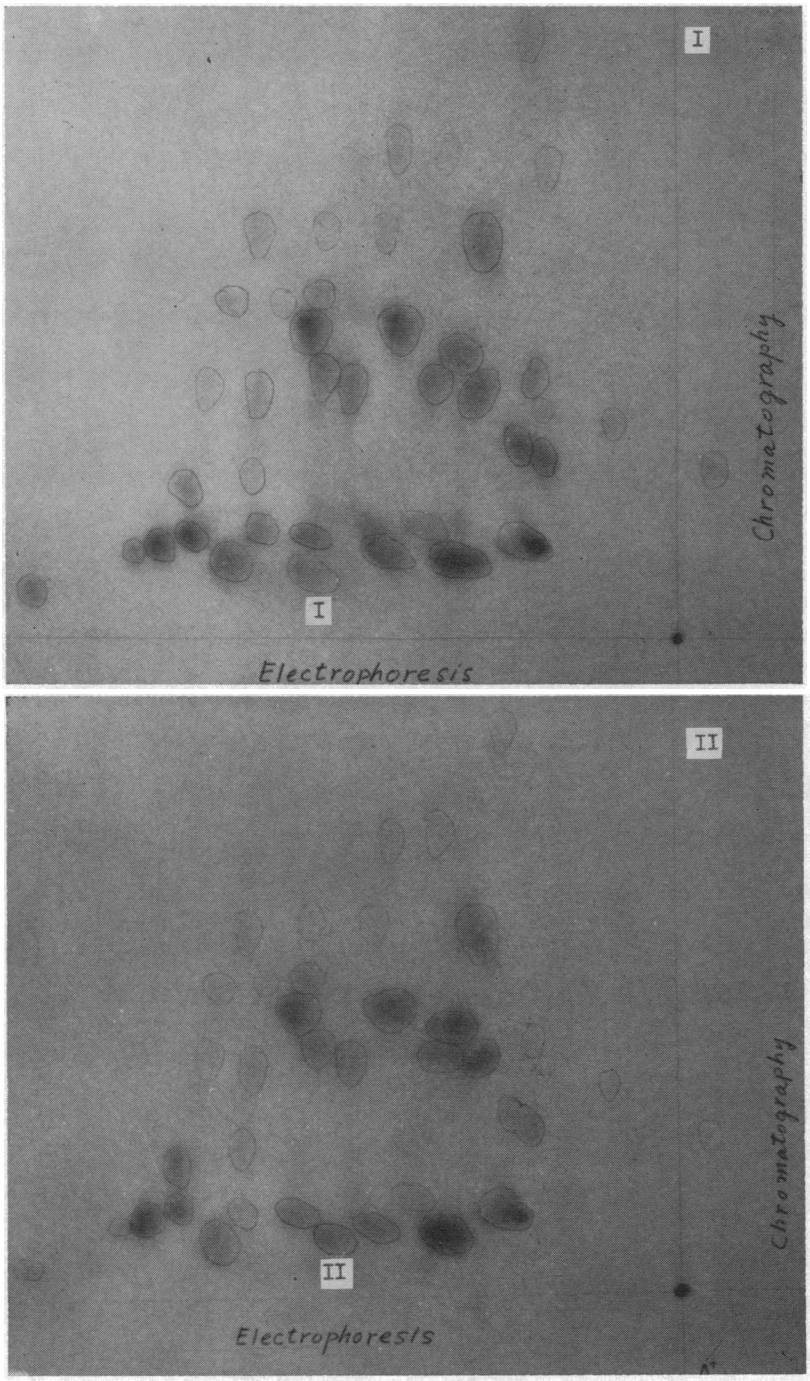

FIG. 1.—Fingerprint of tryptic digest of reduced, S-carboxymethylated human glucose-6-phosphate dehydrogenase. I, Normal enzyme; II, Negro-type variant. First dimension, chromatography, n-butanol–acetic acid–water (4:1:5, upper layer), for 18 hr. Second dimension, electrophoresis, pyridine–acetic acid–water (1:10:289, pH 3.6–3.7), 2000 volts for 60 min.

The hydrolysates of peptide I contained very little aspartic acid (apparently in the same order of the control) and a large quantity of asparagine. Peptide II contained both asparagine and aspartic acid in apparently the same quantity. Both peptide hydrolysates contained glutamine instead of glutamic acid.

From these findings, together with the results of amino acid composition of acid hydrolysates of the peptides (Table 1), the following amino acid compositions for the peptides I and II were established:

Peptide I (from the normal enzyme): Asn_2, Ser_1, Gln_1, Gly_1, Ala_2, Leu_2, His_2, Arg_1.

Peptide II (from the Negro-type variant): Asn_1, Asp_1, Ser_1, Gln_1, Gly_1, Ala_2, Leu_2, His_2, Arg_1.

Accordingly, the amino acid substitution between the two enzymes is asparagine (normal enzyme) to aspartic acid (Negro-type variant).

Discussion.—About 40 peptide spots appeared (Fig. 1) in the fingerprints of the normal or the Negro-type variant of human glucose-6-phosphate dehydrogenase. From the number of peptide spots and the known molecular weight, as well as the content of lysine and arginine in the protein, one can estimate the number of subunits if the following reasonable assumptions are met: (1) nearly all the trypsin-susceptible peptide bonds of the denatured, reduced and S-carboxymethylated protein are hydrolyzed under the conditions used; (2) digestion by other proteolytic enzymes which may contaminate the trypsin or by chymotrypsin-like activity of trypsin itself is negligible; (3) the resolution of the fingerprint is high enough to separate nearly all tryptic peptides.

The molecular weight of human glucose-6-phosphate dehydrogenase is about 230,000–240,000, and the content of lysine residue is 6.20 ± 0.01 per cent (110–115 residues per molecule) and that of arginine residues is 8.04 ± 0.30 per cent (125–130 residues per molecule).[5, 6] From the number of peptide spots observed on the fingerprint, it can be concluded that the protein consists of six repeating units (mol wt = 40,000) since the expected number of tryptic peptides in a protein of molecular weight of 40,000 is about 40 (about 19 lysine residues and about 22 arginine residues per subunit).

It has been found that the enzyme dissociated into smaller subunits with a molecular weight of about 43,000 in the presence of 4 *M* guanidine-HCl and mercaptoethanol.[5, 6] Therefore, the molecular size of the repeating unit found by fingerprinting is consistent with the molecular size of these subunits found by the sedi-

TABLE 1
AMINO ACID COMPOSITION OF TRYPTIC PEPTIDE OF HUMAN GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Amino acid	Normal enzyme, peptide I (μmole)*	Negro-type variant, peptide II (μmole)*	Ratio of amino acid residues†
Aspartic acid	0.043	0.040	2
Serine	0.021	0.020	1
Glutamic acid	0.019	0.016	1
Glycine	0.020	0.018	1
Alanine	0.032	0.032	2
Leucine	0.039	0.038	2
Histidine	0.036	0.033	2
Arginine	0.021	0.018	1

* Amount of amino acid found in peptide I and peptide II eluted from one fingerprint each.
† Closest integral number of amino acid residues, taking arginine as 1.

mentation equilibrium method after dissociation of the enzyme. It can therefore be concluded that both the normal enzyme and the A+-type variant enzyme consist of six identical (or closely identical) subunits of molecular weight of about 40,000. This finding suggests that the structure of the enzyme is specified by single gene only.

An amino acid substitution, i.e., asparagine in the normal enzyme by aspartic acid in the Negro-type variant, has been found in this work. Considering the similarity of peptide spots on the fingerprints (Fig. 1), this substitution probably is the only structural difference between the normal and variant (A+) enzymes. Although another substitution, which could not be detected by the fingerprinting system used, might exist, this possibility is highly unlikely on genetic grounds.

The substitution of aspartic acid for asparagine is frequently found in mammalian hemoglobins.[12] Since the mRNA codons for asparagine are AAU or AAC, and those for aspartic acid are GAU or GAC, this conversion is associated with a single-step transition from $\frac{A}{T}$ to $\frac{G}{C}$ in the gene.

It has been reported that 15–20 per cent of chimpanzees also have an electrophoretic variant of glucose-6-phosphate dehydrogenase which corresponds in electrophoretic mobility to the human A+ variant.[11] Microcomplement fixation tests (done by Drs. V. Sarich and A. C. Wilson, University of California, using our enzyme preparations and antiserum which was obtained by immunizing rabbits with crystalline human normal glucose-6-phosphate dehydrogenase) indicated that the "normal" enzyme from chimpanzees can be distinguished from both the normal (B+) and the variant (A+) human enzymes which could not be distinguished from each other with this technique. It is therefore most likely that the human A+ and the chimpanzee "A+" mutations are not structurally identical, but represent independent single mutations from their respective normal enzyme.

The finding of a single amino acid substitution in the Negro-type variant (A+) suggests that other genetic variants of human glucose-6-phosphate dehydrogenase are probably caused by single amino acid substitutions. For example, the common glucose-6-phosphate dehydrogenase variant found in many Mediterranean populations has very weak activity for D-glucose-6-phosphate but has increased activity for D-galactose-6-phosphate or 2-deoxy-glucose-6-phosphate as a substrate. A single amino acid substitution, therefore, presumably causes alteration of specificity in the enzyme. Such mutations might play an important role in the differentiation and evolution of enzymes [10] Elucidation of the structural difference of such variants may supply useful knowledge about the factors implicated in enzyme specificity and activity.

Summary.—The structural difference between the normal (B+) and the common Negro variant (A+) of human glucose-6-phosphate dehydrogenase was elucidated by fingerprinting of their tryptic peptides. A single amino acid substitution, i.e., asparagine in the normal enzyme to aspartic acid in the variant enzyme, was found.

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