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AMINO ACID SEQUENCE OF THE CARBOXY-TERMINAL END OF HUMAN ERYTHROCYTE GLUCOSE-6-PHOSPHATE DEHYDROGENASE +

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Human erythrocyte glucose-6-phosphate dehydrogenase was purified to homogeneity by a simplified procedure, consisting of 2',5'-ADP-Sepharose affinity chromatography, followed by Sephadex G-100 gel filtration. The carboxy-terminal region of the protein was identified by carboxypeptidase digestion: the sequence -Lys-Leu-COOH was found instead of the reported -Gly-COOH, thus showing identity with the carboxy-terminal sequence of glucose-6-phosphate dehydrogenase from human leukocytes and platelets. In addition, the carboxy-terminal peptide was isolated from a tryptic digest of the protein and sequenced. The sequence is: Trp-Val-Asp-Pro-His-Lys-Leu.

The importance of erythrocyte G6PD in human biochemical genetics, as well as the clinical implications of genetically determined G6PD deficiency (1,2), have attracted the interest of several laboratories.

The relatively small enzyme concentration in blood, 2-3 mg/l (3), while allowing detailed studies on the quaternary structure (4), had hindered extensive work on its primary structure. Until recently, an additional difficulty was the availability of very complex and lengthy purification methods (5). Preliminary structural studies have all been carried out with preparations obtained through such procedures; one report (6) indicates glycine as the carboxy-terminal residue.

In 1975, a simplified procedure was published, based on an affinity chromatography step (7,8), by which pure erythrocyte G6PD could be prepared in a very short time and in amounts suitable for structural studies.

An outline of the primary structure of the protein was recently communicated (9,10), although no details were given on the procedure used for purification.

+Dedicated to the memory of Luigi Casola.

Abbreviations: G6PD, glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49); G6P, glucose-6-phosphate; PMSF, phenylmethylsulfonylfluoride; DABITC, 4-NN-dimethylaminoazobenzene 4'-isothiocyanate; DABTH, 4-NN-dimethylaminoazobenzene 4'-thiohydantoin; PITC, phenylisothiocyanate; HPLC, high-performance liquid chromatography; CPA, carboxypeptidase A; CPB, carboxypeptidase B.

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According to Kahn et al. (11), G6PD purified from both human leukocytes and platelets showed a carboxy-terminal sequence, -Lys-Leu-COOH, different from that of the erythrocyte enzyme.

We have purified G6PD from human red blood cells by a simple and rapid procedure, essentially a modification of that described by De Flora et al. (7), which allows to obtain pure G6PD in 24 hours. Our results show that the carboxy-terminal sequence of the protein purified by this method is identical to that reported for human leukocyte and platelet G6PD (11).

EXPERIMENTAL

Materials. 2',5'-ADP-Sepharose and Sephadex G-50 and G-100 were from Pharmacia Fine Chemicals; NADP, G6P, PMSF-treated CPA were from Sigma; PMSF-treated CPB and trypsin, treated with L-1-tosylamide-2-phenylethylchloromethylketone, were from Worthington. Polyamide sheets were purchased from Schleicher and Schull; DABITC, PITC and methanesulfonic acid were from Pierce. Sequanal-grade trifluoroacetic acid was from Fluka; HPLC-grade acetonitrile was from Baker. All other reagents were of the highest purity commercially available.

Enzyme Purification. Outdated blood samples (within 30 days) were kindly provided by the Blood Bank of II Policlinico, University of Naples, and by the Blood Bank of Ospedale "Nuovo Pellegrini", Naples. The erythrocytes were washed three times with 0.15 M NaCl and the leukocyte layer was carefully drawn off. The packed erythrocytes were lysed in four volumes of 0.1% β-mercaptoethanol, 1 mM EDTA; after 15 min, the stromas were removed by centrifugation. 2',5'-ADP-Sepharose was added to the hemolysate in a ratio of 5 gm per liter. The gel was collected on a Buchner funnel, washed with 0.2% β-mercaptoethanol, 1 mM EDTA; then poured in a column and washed extensively with 50 mM phosphate buffer, pH 7.5, containing 25 mM NaCl, 1 mM EDTA, 0.2% β-mercaptoethanol. The enzyme was eluted in the void volume by adding 0.2 mM NADP in the same buffer. The active fractions were pooled and concentrated under pressure using an Amicon P 30 membrane. The pool was then applied on a Sephadex G-100 column (1 x 200 cm), equilibrated with the same buffer containing 10 µM NADP. The active fractions were pooled, extensively dialysed against distilled water and lyophilised.

The enzyme assays were performed as described (12); protein was determined by the BioRad protein assay (13).

Carboxypeptidase Digestion. The lyophilised protein, previously reduced and carboxymethylated, was dissolved in 0.3 ml of 0.2 M N-ethylmorpholine acetate, pH 8.0, 0.5% SDS; one drop of glacial acetic acid was then added, bringing the pH to 3.0-3.5 and precipitating the protein. The suspension was centrifuged; the precipitated protein was dried under a nitrogen stream, dissolved again in the same buffer and incubated with CPA (CPA/protein molar ratio, 1:20) for 3 h at 37°C. The protein was precipitated again with acetic acid and centrifuged; the dried precipitate was dissolved in the same buffer, incubated with CPB (CPB/protein molar ratio, 1:20) for 3 h at 37°C and precipitated. The supernatants after CPA and CPB digestion were dried and analysed for amino acids. Before digestion with each carboxypeptidase, an aliquot of protein solution was hydrolysed and analysed for protein determination. A blank without protein was run in parallel.

Tryptic Digestion. The tryptic digestion of the carboxymethylated protein was carried out in 1 ml of 1% NH_4HCO_3 for 4 h at 37°C (trypsin/protein ratio, 1:50 w/w). The clear solution was then directly loaded on a Sephadex G-50sf column (1.5 x 200 cm), equilibrated with 0.1 M NH_4HCO_3.

High-Performance Liquid Chromatography. All operations were performed on a Beckman 5 μ ultrasphere ODS, 100 Å pore size, reverse phase column (0.45 x 25 cm), attached to a Beckman model 324 gradient liquid chromatograph, equipped with a Beckman model 155 variable wavelength detector. The aqueous buffer (solution A) was 0.1% trifluoroacetic acid; the organic eluant (solution B) was 0.1% trifluoroacetic acid in acetonitrile. A linear gradient (5-70% B in 90 min) was run at a flow rate of 1 ml/min. Fractions were collected at 0.7-min intervals.

Peptide Sequencing. The peptide (2-5 nmoles) was sequenced by modified Edman degradation, using the DABITC-PITC double coupling method; the DABTH-amino acids were identified on polyamide thin layer plates (14).

Amino Acid Analysis. Analyses were performed on a Carlo Erba model 3A29 automatic analyzer, using the single column protein hydrolysate program.

RESULTS

Electrophoretically homogeneous G6PD was purified as described in Experimental. The average yield was approximately 1.5 mg per liter of blood; the average specific activities in the hemolysate, in the eluates from 2',5'-ADP-Sepharose and Sephadex G-100 were 0.006, 160 and 180 U/mg, respectively. The last value is in agreement with other reports (7,15,16).

Table 1 shows the results of carboxypeptidase digestion of G6PD. Previous acid precipitation of the protein was essential, since substantial amounts of free amino acids, mainly serine, glycine and alanine, were protein-bound and remained so even after urea denaturation and carboxymethylation, thus constituting a potential source of misleading results; they could be removed by a drastic pH change. Leucine and lysine were sequencially hydrolysed in stoichiometric amounts to the protein. Further prolonged incubation with CPA did not release any residue. The fact that leucine was the only residue released by CPA is in agreement with the results reported for the leukocyte enzyme (11).

Table 1. Carboxypeptidase digestion of G6PD (1 mg). Conditions are outlined in Experimental. Values are expressed after correction for blank.

Amino acid	CPA supernatant	CPB supernatant
	mol/mol of protein subunit	
Leucine	0.75	0.2
Lysine	--	0.8

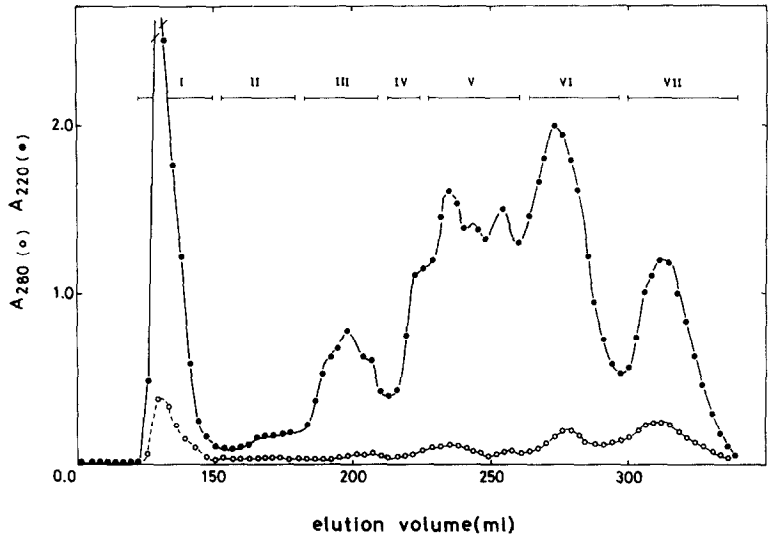

Fig. 1. Gel filtration of a tryptic digest of G6PD (12 mg) on Sephadex G-50sf. The column (1.5 x 200 cm) was equilibrated with 0.1 M NH_4HCO_3. Fractions of 1.5 ml were collected at a flow rate of 10 ml/h. The horizontal bars indicate the fractions pooled after elution.

The isolation of the carboxy-terminal tryptic peptide was then attempted, in order to obtain more information about the carboxy-terminal end. G6PD was digested with trypsin and run on a Sephadex G-50sf column (Fig. 1); pool VII was subsequently fractionated by HPLC (Fig. 2) and a peptide was purified, VII-4, having the amino acid composition shown in Table 2. The peptide yield was 33%.

The peptide VII-4 was subjected to Edman degradation, modified by Chang et al. (14); five cycles yielded clearly identifiable residues, allowing to determine the following sequence: Trp-Val-Asp-Pro-His-(Lys,Leu). The dipeptide (Lys,

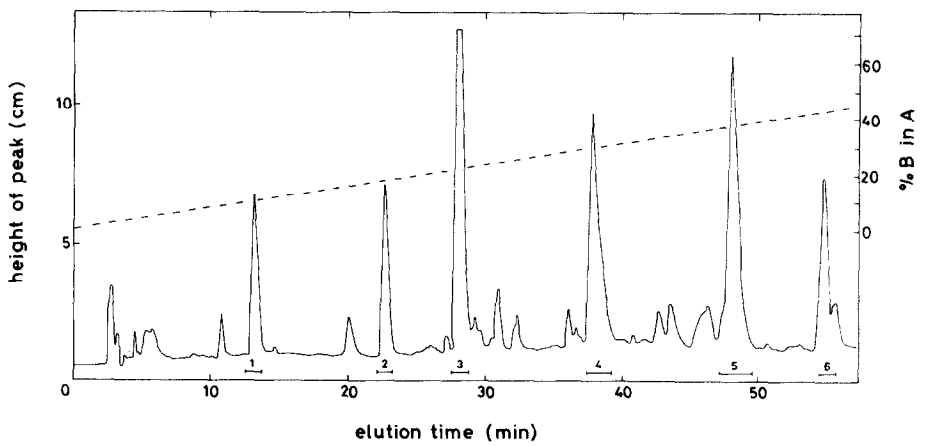

Fig. 2. HPLC separation of pool VII from the Sephadex G-50sf column. Conditions are outlined in Experimental. The horizontal bars indicate the fractions pooled after elution.

Table 2. Amino acid composition of peptide VII-4.

Amino acid	Amount
	mol/mol
Aspartic acid	0.80
Proline	1.21
Valine	1.06
Leucine	0.95
Histidine	1.03
Lysine	1.06
Tryptophan+	0.89

+determined by hydrolysis with methanesulfonic acid (17).

Leu) was probably lost during the extraction in the next cycle, because of its hydrophobic nature, enhanced by the introduction of an extra DABTC on the ϵ-amino group of Lys. However, the carboxy-terminal sequence was determined by digestion with CPA and CPB. CPA hydrolysed only leucine in stoichiometric amount to the peptide, while CPB alone, or in combination with CPA, released both leucine and lysine, supporting the results obtained when digesting the whole protein.

The complete sequence was thus established: Trp-Val-Asp-Pro-His-Lys-Leu.

DISCUSSION

In an effort to obtain pure human erythrocyte G6PD in amounts suitable for structural studies and as close as possible to the native conditions, a simple procedure has been developed, requiring a minimum of manipulation and time. We believe that the protein purified by this method has had very little chance of undergoing non-physiological structural alterations.

The carboxy-terminal sequence of human erythrocyte G6PD has thus been clarified. Unlike previous results (6,11), we have not found glycine at the carboxy-terminal end: we have found the sequence -Lys-Leu-COOH, identical to the carboxy-terminal sequence reported for human leukocytes and platelets (11). This conclusion does not support the hypothesis that post-translational partial proteolysis is responsible for the difference between the two enzyme forms (11). Such a difference, on the other hand, may be explained on the basis of an alteration of the erythrocyte enzyme occurring in the course of the purification.

Reproducible and stoichiometric results were only obtained when G6PD was acid-precipitated before carboxypeptidase digestion, since protein-bound amino acids were a source of artifacts and seriously affected the meaning of the analyses. For instance, in one acid supernatant, serine, glycine and alanine were found in amounts of 4.9, 2.8 and 1.2 nmoles, respectively, per nmoles of G6PD subunit.

CPA and CPB were only able to release two residues (Leu and Lys) from the whole protein. However, a seven-residue peptide was isolated and sequenced, considered to be the carboxy-terminal one on the basis of the following considerations: 1; the two terminal residues coincided with those of the protein; 2, the rest of its sequence is in accordance only with that reported for the carboxy-terminal end (9,10), once -Gly-COOH is substituted by -Lys-Leu-COOH; 3, the presence of -Pro-His- preceding Lys explains the lack of further carboxypeptidase digestion

It seemed rather surprising for a tryptic peptide to have a carboxy-terminal residue different from Lys. However, the same peptide was also obtained following overnight tryptic digestion; furthermore, no chymotryptic cleavages were observed during the isolation and sequence determination of several other peptides from the same tryptic digest. For these reasons, the possibility of dealing with a chymotryptic peptide was ruled out. On the other hand, the lysyl bond is known to be resistant to trypsin when there is a free contiguous carboxyl group, thus strengthening our conclusion that this peptide is indeed located at the carboxy-terminal end.

It should be noted that the specificity of each carboxypeptidase and the carboxy-terminal sequence of G6PD constituted a favourable situation for a simple digestion procedure of the whole protein: Leu was first cleaved, almost quantitatively, and identified in the supernatant following precipitation of the protein, from which Lys was then released in the next digestion.

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REFERENCES

1. Beutler, E. (1978) in The Metabolic Basis of Inherited Disease, 4th edn (Stanbury, J.B., Wyngaarden, J.B. and Fredricson, D.S., eds) pp. 1430-1451, McGraw-Hill, New York.
2. Luzzatto, L. and Testa, U. (1978) in Current Topics in Hematology (Piomelli S. and Yachnin, S., eds) vol 1, pp. 1-70, Alan R. Liss, Inc., New York.
3. Yoshida, A. (1977) Acta Biol. Med. Germ. 36, 698-701.
4. Bonsignore, A. and De Flora, A. (1972) in Current Topics in Cellular Regulation (Horecker, B.L. and Stadtman, E.R., eds) vol 6, pp. 21-62, Academic Press, New York.

5. Levy, H.R. (1979) Adv. Enzymol. 48, 97-192.
6. Yoshida, A. (1968) Biochem. Genetics 2, 237-243.
7. De Flora, A., Morelli, A., Benatti, U. and Giuliano, F. (1975) Arch. Biochem. Biophys. 169, 362-363.
8. Morelli, A., Benatti, U., Gaetani, G.F. and De Flora, A. (1978) Proc. Natl. Acad. Sci. USA 75, 1979-1983.
9. Beutler, E. (1982) in The Metabolic Basis of Inherited Disease, 5th edn (Stanbury, J.B., Wyngaarden, J.B. and Fredricson, D.S., eds) pp. 1634-1635, McGraw Hill, New York.
10. Huang, I-Y. and Yoshida, A. (1982) Fed. Proc. 41, 1181.
11. Kahn, H., Bertrand, O., Cottreau, D., Boivin, P. and Dreyfus, J.-C. (1977) Biochem. Biophys. Res. Commun. 77, 65-72.
12. Camardella, L., Romano, M., di Prisco, G. and Descalzi-Cancedda, F. (1981) Biochem. Biophys. Res. Commun. 103, 1384-1389.
13. Bradford, M. (1986) Anal. Biochem. 72, 248-254.
14. Chang, J.Y., Brauer, D. and Wittman-Liebold, B. (1978) FEBS Letters 93, 205-214.
15. Yoshida, A. (1966) J. Biol. Chem. 241, 4966-4976.
16. Cohen, P. and Rosemeyer, M.A. (1969) Eur. J. Biochem. 8, 1-7.
17. Simpson, R.J., Neuberger, M.R. and Liu, T.-Y. (1976) J. Biol. Chem. 251, 1936-1940