Jaken, S., & Black, P. H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 246–250.
Kaltoft, K., Nielsen, L. S., Zeuthen, J., & Danø, K. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3720–3723.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Matsuo, O., Rijken, D. C., & Collen, D. (1981) *Nature (London) 291*, 590–591.
Mattsson, C., Nyberg-Arrhenius, V., & Wallén, P. (1981) *Thromb. Res. 21*, 535–545.
Nolan, C., Hall, L. S., Barlow, G. H., & Tribby, I. I. E. (1977) *Biochim. Biophys. Acta 496*, 384–400.
Ossowski, L., Biegel, D., & Reich, E. (1979) *Cell (Cambridge, Mass.) 16*, 929–940.
Reich, E. (1978) in *Molecular Basis of Biological Degradative Processes* (Berlin, R. D., Herman, H., Lepow, I. H., & Tanzer, J. M., Eds.) pp 155–169, Academic Press, New York.
Rifkin, D. B., Loeb, J. N., Moore, G., & Reich, E. (1974) *J. Exp. Med. 139*, 1317–1328.
Roblin, R., & Young, P. L. (1980) *Cancer Res. 40*, 2706–2713.
Skriver, L., Nielsen, L. S., Stephens, R., & Danø, K. (1982) *Eur. J. Biochem. 124*, 409–414.
Soberano, M. E., Ong, E. B., Johnson, A. J., Levy, M., & Schoellmann, G. (1976) *Biochim. Biophys. Acta 445*, 763–773.
Strickland, S., Reich, E., & Sherman, M. I. (1976) *Cell (Cambridge, Mass.) 9*, 231–240.
Unkeless, J. C., Tobia, A., Ossowski, J. P., Quigley, L., Rifkin, D. B., & Reich, E. (1973) *J. Exp. Med. 137*, 85–111.
Unkeless, J. C., Danø, K., Kellerman, G. M., & Reich, E. (1974a) *J. Biol. Chem. 249*, 4295–4305.
Unkeless, J. C., Gordon, S., & Reich, E. (1974b) *J. Exp. Med. 139*, 834–850.
Weber, K., & Osborn, M. (1975) *Proteins (3rd Ed.) 1*, 179–223.
Wilson, E. L., Becker, M. L. B., Hoal, E. G., & Dowdle, E. B. (1980) *Cancer Res. 40*, 933–938.

Glucose-6-phosphate Dehydrogenase from *Leuconostoc mesenteroides*. Isolation and Sequence of a Peptide Containing an Essential Lysine†

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ABSTRACT: Interaction of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* with pyridoxal 5′-phosphate and sodium borohydride leads to inactivation and modification of two lysine residues per enzyme dimer that are thought to bind glucose 6-phosphate [Milhausen, M., & Levy, H. R. (1975) *Eur. J. Biochem. 50*, 453–461]. The amino acid sequence surrounding this lysine residue is reported. Following tryptic hydrolysis of the modified enzyme, two peptides, each containing one pyridoxyllysine residue, were purified to homogeneity and subjected to automated Edman degradation. The sequences revealed that one of these, a heptapeptide, was derived from the other, containing 11 amino acids. Supporting evidence for the role of the modified lysine is provided in the following paper [Haghighi, B., & Levy, H. R. (1982) *Biochemistry* (second paper of three in this issue)]. End-group analysis of the native enzyme revealed that valine is the N-terminal and glycine the C-terminal amino acid and provides support for the identity of the enzyme's two subunits.

Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* was first isolated by DeMoss et al. (1953) and purified to homogeneity by Olive & Levy (1967). Its molecular weight is 103 700 (Olive & Levy, 1971), and the subunit molecular weight is 54 800 (Ishaque et al., 1974; Kawaguchi & Bloch, 1974). Kinetic studies suggested that a lysine residue in this enzyme may function in glucose 6-phosphate binding (Olive et al., 1971). Detailed studies on the interaction between the enzyme and pyridoxal 5′-phosphate (PLP)[1] showed that inhibition by PLP was competitive with respect to glucose 6-phosphate; in the presence of sodium borohydride this inhibition led to inactivation; N^6-pyridoxyllysine was identified after acid hydrolysis of the modified enzyme, and of the 74 lysine residues/mol of dimeric enzyme (Ishaque et al., 1974), only two were modified in the totally inactive enzyme (Milhausen & Levy, 1975). It was concluded from these studies that one unique lysine residue was pyridoxylated per subunit of *L. mesenteroides* glucose-6-phosphate dehydrogenase and that this lysine was probably involved in glucose 6-phosphate binding (Milhausen & Levy, 1975).

We now report the amino acid sequence surrounding this lysine residue. Two tryptic peptides, DIIA and DIIB, containing 7 and 11 amino acid residues, respectively, were isolated from pyridoxylated *L. mesenteroides* glucose-6-phosphate dehydrogenase and purified to homogeneity. Both DIIA and DIIB contain 1 mol of pyridoxyllysine/mol of peptide, and their amino acid sequence revealed that DIIA was derived from DIIB. This is the first report of the sequence of a peptide

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; PTH, phenylthiohydantoin; dansyl or Dns, 5-(dimethylamino)naphthalene-1-sulfonyl; Tris, tris(hydroxymethyl)aminomethane. For designation of flourescence excitation and emission wavelengths, see Haghighi & Levy (1982).

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derived from any glucose-6-phosphate dehydrogenase (Levy, 1979). Support for our earlier suggestion that the lysine modified by PLP functions to bind glucose 6-phosphate is provided in the following paper (Haghighi & Levy, 1982). We also report the identification of N-terminal valine and C-terminal glycine in *L. mesenteroides* glucose-6-phosphate dehydrogenase, suggesting that the two subunits comprising the functional enzyme are identical.

Experimental Procedures

Materials. Leuconostoc mesenteroides (ATCC 12291) was obtained from the American Type Culture Collection. PLP was purchased from ICN Pharmaceuticals, Inc.; $NaBH_4$, Sephadex, Dns-Cl, protamine sulfate, Dns-amino acids, and carboxypeptidases A and B (treated with diisopropyl fluorophosphate) were obtained from Sigma Chemical Co.; trypsin (TPCK treated) was from Worthington Biochemical Co.; fluorescamine was from Roche Diagnostics, division of Hoffmann-La Roche Inc. For automated sequencing, Beckman reagents and solvents were used throughout. Reagents for HPLC were from Fisher (HPLC grade). All other chemicals were of the best grade commercially available.

Enzyme Isolation. L. mesenteroides was grown in 550-L culture according to Olive & Levy (1967). The cells were disrupted by eight passages through a Manton–Gaulin laboratory homogenizer operated at 9000 psi. Glucose-6-phosphate dehydrogenase was precipitated from the clarified extract with saturated ammonium sulfate. These steps were performed by the New England Enzyme Center. Removal of the nucleic acids with protamine sulfate and enzyme precipitation at 80–100% ammonium sulfate followed the procedure of Olive & Levy (1967), except that 0.2 M sodium chloride was added to the Tris buffer before removing nucleic acids. The final purification steps were modified from the original (Olive & Levy, 1967) and modified (Grove et al., 1976) procedures to accommodate the large quantities of protein in this preparation. The enzyme was applied to a DEAE-Sephadex column (26 × 1.6 cm), equilibrated with 0.15 M imidazole buffer, pH 7.0. The enzyme was eluted with a linear concentration and pH gradient of 150 mL each of 0.15 M imidazole, pH 7.0, and 0.5 M imidazole, pH 6.0. Further purification was performed on a column of Sephacryl S-200 (80 × 2.6 cm), equilibrated with 35 mM phosphate buffer, pH 7.6, containing 0.2 M sodium chloride and 1 mM EDTA. The enzyme was homogeneous as judged by polyacrylamide gel electrophoresis, with a specific activity identical with that reported previously (Olive & Levy, 1967). Protein concentration was measured by a modification of the biuret method (Zamenhof, 1957). Enzyme assays were routinely performed in 33 mM Tris-HCl, pH 7.8, as described by Olive & Levy (1967), but using 57 μM $NADP^+$ and 0.81 mM glucose 6-phosphate. All absorbance measurements were performed on a Gilford Model 240 spectrophotometer attached to a Gilford Model 6051 recorder. Fluorescence measurements were made with a Perkin-Elmer MPF3L fluorescence spectrophotometer.

End-Group Determination. N-Terminal amino acid analysis was performed by using the dansyl chloride method according to Gray (1972). Dansyl amino acids were identified by two-dimensional chromatography on polyamide sheets as described by Woods & Wang (1967). C-Terminal amino acid determination was conducted with carboxypeptidase B, using the procedure of Ambler (1972) but in 0.025 M sodium borate, pH 9.1, and a carboxypeptidase B to glucose-6-phosphate dehydrogenase ratio of 1:100 at room temperature. At various time intervals aliquots of the mixture were removed and immediately lyophilized to stop the reaction. Amino acids released were analyzed quantitatively, using an automatic amino acid analyzer, and qualitatively after dansylation by a modification of the method of Gray (1972). About 100 μg of protein, taken from a carboxypeptidase B digest, was dissolved in 15 μL of 0.025 M sodium bicarbonate, and 15 μL of Dns-Cl (0.65 mg/mL acetone) was added. The mixture was incubated at room temperature for 2 h and then evaporated to dryness in a vacuum desiccator. Dns-amino acids were extracted with water-saturated ethyl acetate and identified on polyamide sheets (7 × 7 cm) by using two-dimensional chromatography according to Woods & Wang (1967). Carboxypeptidase B was assayed by the spectrophotometric method of Folk et al. (1960).

PLP Modification. Glucose-6-phosphate dehydrogenase was treated with PLP by using a modification of the method described by Milhausen & Levy (1975). Two hundred milligrams of the enzyme (20 mg/mL) was incubated with 1 mM PLP in 0.03 M potassium phosphate, pH 7.5, at 25 °C. Aliquots were removed at different times and assayed immediately for enzyme activity. A control sample (5 mg) was subjected to an identical procedure, but without PLP. After 30 min, acetic acid (0.5M) was added to bring the pH to 6.0. A fresh, aqueous solution of 5 M $NaBH_4$ was added in portions of 50 μL, until a concentration of 0.19 M $NaBH_4$ was achieved in the enzyme solutions; 1-octanol (50 μL) was added to prevent foaming. The reaction mixtures were maintained at pH 6.0 during the reduction, which was allowed to proceed for 10 min at room temperature, and then the enzyme was dialyzed for 2 days against several changes of 0.03 M potassium phosphate, pH 7.5. The procedure of modification and subsequent reduction were carried out 3 times sequentially. The reduced product showed a major absorption band at 325 nm, characteristic for the reduced Schiff base (Fischer et al., 1963). Since the PLP modification is light sensitive (Ritchey et al., 1977), all procedures were performed in the dark. The stoichiometry of pyridoxylation was determined following reduction of the Schiff base with $NaBH_4$ by using a molar extinction coefficient of 8600 M^{-1} cm^{-1} at 316 nm (Forrey et al., 1971b).

Tryptic Digestion of PLP-Modified Enzyme. The dialyzed, modified enzyme was denatured in 6 M guanidine hydrochloride (Tanford et al., 1967) by gently stirring for 3 h at room temperature and then dialyzed against distilled water for 3 days with several changes. A heavy precipitate that formed was collected by centrifugation and resuspended in 0.1 M NH_4HCO_3 (pH adjusted to 8.1 with ammonia or CO_2). The supernatant was lyophilized, dissolved in 0.1 M NH_4HCO_3, and added to the suspension. The final volume was 20 mL. The control sample, after denaturation, was lyophilized and dissolved in NH_4HCO_3. Aliquots of freshly prepared solutions of TPCK–trypsin were added in two stages to pyridoxylated and control enzyme (10 mg/mL) at a trypsin to glucose-6-phosphate dehydrogenase ratio of 1:50 (w/w). The digestion was allowed to proceed at 37 °C, with gentle mixing, for 6 h. The pH of the reaction was monitored continuously during the digestion and kept at 8.1. The solution became clear within a few seconds after trypsin was added. Although the digests appeared to be clear, they were centrifuged to remove any remaining protein core or particles and lyophilized to stop the digestion (Allen, 1980).

Thin-Layer Chromatography and Electrophoresis. For preparative peptide purification peptides were either chromatographed or electrophoreses on 20 × 20 cm cellulose thin-layer sheets (Eastman) using the solvent systems described by Garcy (1977). For determinations of peptide purity two-

dimensional chromatography and electrophoresis were conducted on the cellulose sheets. All TLC plates were washed with both electrophoresis and chromatography solvents before use. For visualization of the peptides, the plates were washed with acetone and subsequently sprayed with 1% triethylamine in acetone and finally with 0.03% (w/v) fluorescamine in acetone. The fluorescent peptides were viewed under a UV lamp. Peptides containing the pyridoxyl group were observed before spraying with fluorescamine as distinctly blue fluorescent spots.

Amino Acid Analysis. Each peptide was hydrolyzed for 24 h at 110 °C with 1 mL of 6 N HCl. The hydrolysates were dried under N_2 gas. The dried hydrolysates were dissolved in 1 mL of 0.2 M sodium citrate buffer, pH 2.2, and aliquots were taken for analysis either on a Beckman 120C automatic amino acid analyzer, modified for single column analysis, or on a Durrum 500 amino acid analyzer. For analysis of pyridoxyl-ε-lysine in hydrolysates the third buffer in the Beckman system was 0.2 M sodium citrate, pH 7.2, where the modified amino acid eluted after lysine and before histidine, and in the Durrum system the final buffer was 0.2 M sodium borate, pH 9.0, where the elution order was histidine, pyridoxyl-ε-lysine, and lysine.

Peptide Sequencing. Each peptide was subjected to sequencing by automated Edman degradation in a Beckman 890C automatic protein–peptide sequencer. A single cleavage program with double coupling on the first cycle using 0.33 M Quadrol [a modification of the Beckman dilute Quadrol (0.1 M) program no. 12. 11. 78] was used with 0.0015% (w/v) dithiothreitol in the extracting solvent, *n*-butyl chloride. Prior to sequencing the peptide, the program was run for four cycles with 3 mg of polybrene and 100 nmol of glycylglycine in the cup. The peptide (25–36 nmol) dissolved in 600 μL of 50% acetic acid was then placed in the cup and subjected to 15 successive cycles of Edman degradation. PTH-amino acids were analyzed by HPLC on a Beckman HPLC instrument with a Zorbax (Du Pont) cyanopropyl column according to the method of Johnson et al. (1979). PTH-amino acids were also identified by TLC on polyamide sheets (Summers et al., 1973).

For C-terminal amino acid determination, the peptides were dissolved in 0.4 mL of 0.2 M *N*-ethylmorpholine–acetate, pH 8.5, containing 1% sodium dodecyl sulfate and 20 nmol of norleucine as internal standard. A mixture of carboxypeptidases A and B was added to give an enzyme to substrate ratio of 1:40, and the amino acids released were analyzed quantitatively as described above for the end-group determinations.

High-Performance Liquid Chromatography (HPLC). All separations were performed on a Beckman 5-μm ultrasphere ODS semipreparative (0.7 × 25 cm) reverse-phase column attached to a Beckman Model 420 liquid chromatograph. The aqueous buffer (solution A) consisted of 10% triethylamine formate in water while the organic eluate (solution B) was 10% triethylamine formate in 2-propanol. A linear gradient (0.5% B/min) was run at a constant flow rate of 1 mL/min. Fractions were collected at 0.5-min intervals.

Results

End-Group Determination. N-Terminal amino acid analysis showed that Dns-valine was the only amino acid released from the N terminus. Carboxypeptidase B treatment followed by either dansylation or direct amino acid analysis indicated that glycine was released first, demonstrating that it is the C terminus. These data support the idea that the enzyme consists of two identical subunits.

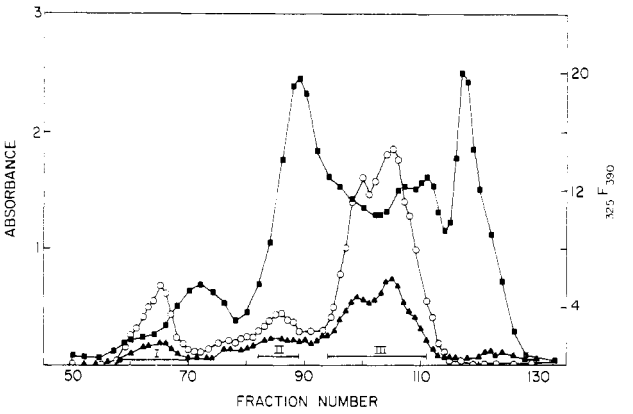

FIGURE 1: Isolation of pyridoxyl peptides from tryptic digest of pyridoxylated glucose-6-phosphate dehydrogenase on Sephadex G-50. The flow rate was 4.8 mL/h. Fractions (2.3 mL) were collected and monitored for absorbance at 280 nm (■), absorbance at 315 nm (▲), and for the fluorescence at 390 nm (○) as indicated under Experimental Procedures. The bars represent the fractions pooled. For experimental details see the text. For an explanation of fluorescence symbols in this figure and Figure 2, see footnote 1.

Stoichiometry of Pyridoxylation. The stoichiometry of pyridoxylation was determined following reduction of the Schiff base with $NaBH_4$ as described under Experimental Procedures. The number of moles of pyridoxyllysine per mole of enzyme was 0.62, 0.98, and 1.26 while enzymatic activity dropped to 23, 6.7, and 1.6% of the control value, after the first, second, and third modification cycle, respectively. Control enzymes (see Experimental Procedures) lost no activity during these incubations. Milhausen & Levy (1975) reported that a maximum of 2 of the 74 lysine residues per enzyme dimer could be pyridoxylated after several modification cycles to obtain 100% inactivation.

Purification of Phosphopyridoxyl Peptides. The use of trypsin to cleave *L. mesenteroides* glucose-6-phosphate dehydrogenase generates a large number of peptides; there are 37 lysines and 18 arginines per subunit (Ishaque et al., 1974). Extensive purification was therefore required to generate the pure pyridoxyl peptides. Preliminary experiments indicated that the absorbance and fluorescence of the pyridoxyl peptides were sufficiently intense to monitor their elution from columns and on thin-layer chromatograms. Not all fluorescent, 315-nm-absorbing fractions proved to contain pyridoxyllysine, however; this was always tested by direct amino acid analysis. The tryptic digest was passed over a 160 × 1.5 cm column of Sephadex G-50 (superfine) equilibrated with 50 mM NH_4HCO_3. All fractions were monitored by absorbance at 280 nm. Pyridoxyl-containing peptides were detected by their absorbance at 315 nm (where the absorbance is pH independent) and their characteristic fluorescence emission at 390 nm upon excitation at 325 nm (Forrey et al., 1971b). The elution pattern of this column is shown in Figure 1. Two minor (I, II) and one major (III) fluorescent peaks were detected which contained 12, 14, and 74%, respectively, of the total 315-nm-absorbing material recovered. No further purification was performed for peptides I and II, but amino acid analysis revealed no pyridoxyllysine. Fractions comprising peak III were combined and lyophilized several times to remove NH_4HCO_3. The material was then dissolved in 0.02 M NH_4HCO_3 and applied to a 28 × 1.6 cm column of DEAE-Sephadex (40–120 mesh) equilibrated with 0.02 M NH_4HCO_3. The column was developed with a linear gradient composed of 250 mL each of 0.20 M and 1 M NH_4HCO_3. The elution profile is illustrated in Figure 2. As monitored by fluorescence and absorbance at 315 nm, two major (DI, DII) and two minor (S-1,

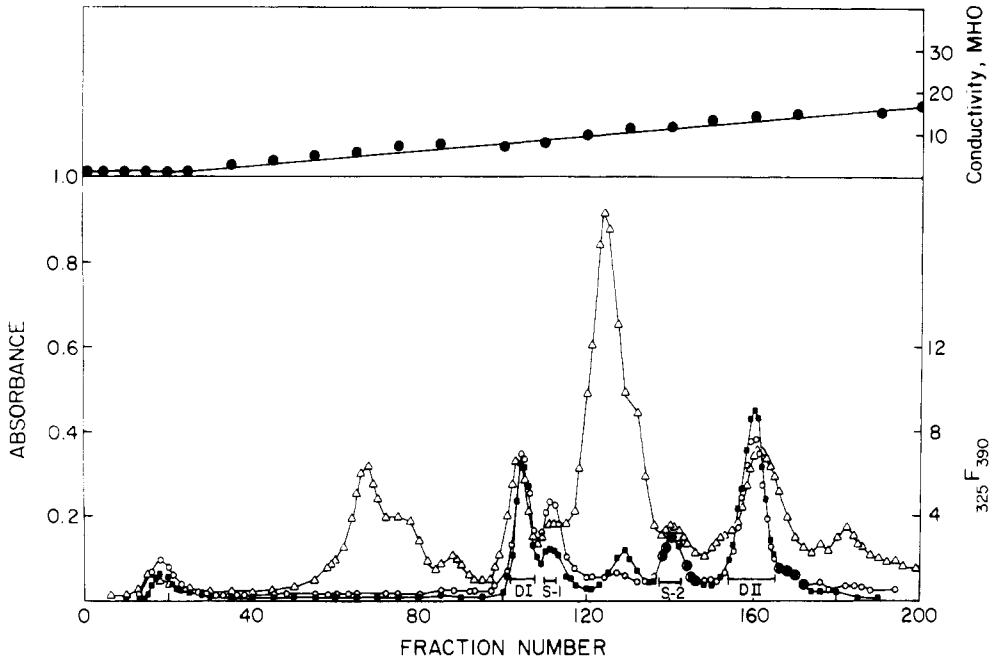

FIGURE 2: DEAE-Sephadex anion-exchange chromatography of pyridoxyl peptide peak III from Sephadex G-50 column. Fractions of 1.8 mL were collected at a flow rate of 14.4 mL/h. The fractions were monitored for absorbance at 280 nm (Δ), absorbance at 315 nm (○), conductivity (●), and fluorescence at 390 nm (■). The bars represent the fractions pooled. For additional experimental details see the text.

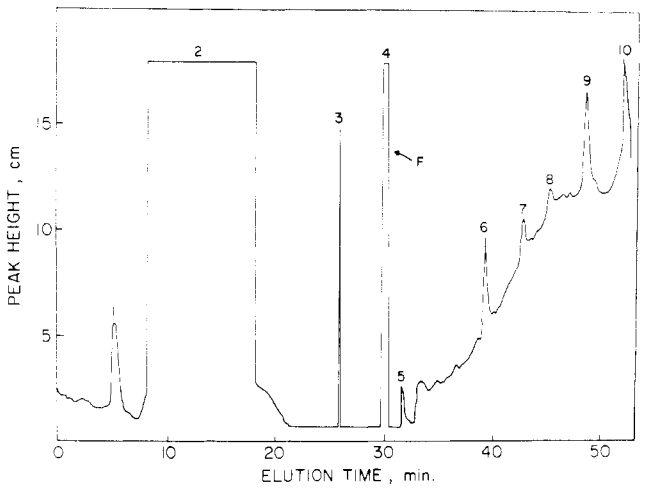

FIGURE 3: Preparative HPLC chromatogram of peptide DIIA. Peak 4, containing pyridoxyl peptide (F), was collected. Chromatographic conditions: column, C_{18} reverse phase; detector, UV at 210 nm; chart speed, 0.5 cm/min; solvent A, 10% triethylamine formate in water; solvent B, 10% triethylamine formate in 2-propranol; gradient, increasing B (0.5%/min) in A, starting with 5% B in A.

S-2) peaks were observed. For further purification of DI, it was applied onto an Aminex AG50 column (28 × 1.6 cm) equilibrated with 0.2 M formic acid containing 0.02 M pyridine (pH 2.8) and eluted with 400 mL of a 0.02–1 M pyridine gradient in 0.2 M formic acid. Only one small 315-nm-absorbing peak was eluted, suggesting that some material may have remained bound to the column. Amino acid analysis of this peptide showed that it did not contain pyridoxyllysine. Peptides S-1 and S-2 were further purified by HPLC, amino acid analysis indicated no detectable pyridoxyllysine.

Purification of peak DII by preparative chromatography and electrophoresis on TLC plates yielded two fluorescent bands (DIIA, DIIB). A peptide map of DIIA showed only one fluorescent pyridoxyl peptide, corresponding exactly to the peptide visualized by fluorescamine. HPLC analysis, however, revealed that the peptide was not pure, and it was subjected

Table I: Amino Acid Composition of Peptides Isolated from Tryptic Digest of Pyridoxylated *L. mesenteroides* Glucose-6-phosphate Dehydrogenase

amino acid	mol/mol of peptide[a] DIIA	DIIB
Phe		1.15 (1)
Leu	1.28 (2)[b]	1.37 (2)[b]
Lys(Pxy)[c]	0.99 (1)	1.19 (1)
Ser	1.51 (2)	1.43 (2)
Pro	1.03 (1)	1.24 (1)
Tyr	1.00 (1)	1.00 (1)
Asp		1.24 (1)
Val		1.18 (1)
Lys		0.89 (1)

[a] The numbers in parentheses are the nearest integer values and are consistent with the values determined by sequence analysis. [b] Sequence determination revealed the presence of two leucine residues. [c] Pyridoxyllysine.

to preparative HPLC on a reverse-phase column. The elution profile of this column (Figure 3) showed the presence of several 210-nm-absorbing peaks, of which only one contained fluorescence characteristics of a pyridoxyl peptide. A number of other peaks (notably peak 2 which was ninhydrin negative) are probably impurities extracted from TLC cellulose sheets, although these had been washed (see Experimental Procedures). The fluorescent peak was collected, lyophilized, and rechromatographed on the same HPLC column, and the major peak was collected. HPLC analysis of a small portion of this peak revealed only one single pyridoxyl peptide peak.

Peptide DIIB was also purified by HPLC techniques as described for DIIA.

Amino Acid Analysis. Amino acid compositions of peptides DIIA and DIIB are shown in Table I. The low yield of leucine may be attributable to incomplete hydrolysis of Leu–Leu bonds. incomplete hydrolysis of peptide bonds linking two aliphatic residues has been observed previously (Mahoney et al., 1981). Peptide DIIB possesses an amino acid composition that corresponds to that of DIIA plus four extra amino acid residues. Both peptides contained 1 mol of pyridoxyllysine/mol

Table II: Automated Sequence Analysis of Peptides DIIA and DIIB

	peptide DIIA		peptide DIIB	
cycle	residue identified	yield of PTH-amino acid (nmol)	residue identified	yield of PTH-amino acid (nmol)
1	Leu	7.84	Phe	17.5
2	Leu	4.57	Leu	14.3
3	Lys(Pxy)[a]	4.61	Leu	18.9
4	Ser	0.30	Lys(Pxy)[a]	9.8
5	Pro	2.51	Ser	1.7
6	Ser[b]		Pro	6.3
7	Tyr[c]		Ser	0.3
8			Tyr[d]	
9			[e]	
10			[e]	
11			Lys[f]	

[a] Pyridoxyllysine. [b] Amount of PTH-Ser was sufficient for positive identification but less than 0.1 nmol. [c] Inferred from amino acid composition. [d] Inferred by alignment with DIIA and from amino acid composition. [e] Not identified. [f] Identified by amino acid analysis following digestion of DIIB with carboxypeptidases A and B.

Table III: Alignment of Peptides and Sequence of PLP Binding Site in *L. mesenteroides* Glucose-6-phosphate Dehydrogenase[a]

sequence:	Phe-Leu-Leu-Lys(Pxy)-Ser-Pro-Ser-Tyr-(Asp,Val)-Lys
DIIA:	Leu-Leu-Lys(Pxy)-Ser-Pro-Ser-Tyr
DIIB:	Phe-Leu-Leu-Lys(Pxy)-Ser-Pro-Ser-Tyr-(Asp,Val)-Lys

[a] Arrows below the sequence, (→) and (←), show the results of Edman degradation and carboxypeptidase treatment of peptides, respectively. Lys(Pxy) is pyridoxyllysine.

of peptide. The absence of tryptophan was confirmed with the Ehrlich reagent.

Sequence Determination of Phosphopyridoxyl Peptides. The Edman degradation of peptides DIIA and DIIB was carried out on an automatic amino acid sequencer (see Experimental Procedures). The sequence from the N terminus was established by the identification of each residue as its PTH derivative using the HPLC technique. Fifteen cycles of the Edman degradation revealed that DIIA is composed of only seven amino acid residues (Table II). Although a larger amount of DIIB (28 nmol of DIIB compared to 18 nmol of DIIA) was available for sequencing, and both DIIA and DIIB were sequenced in an identical manner, DIIB appeared not to be retained in the spinning cup after cycle 7. The reason for this is not known. However, the amino acid sequence of the first seven residues as well as the C-terminal lysine was determined (Table II). Insufficient material was available for sequencing DIIB from the C terminus, but the presence of stoichiometric amounts of aspartate and valine in the HPLC-purified peptide (Table I) precludes the possibility that these amino acids are impurities. Alignment of the two sequences suggests that DIIA is derived from DIIB by further degradation (Table III). This requires cleavage by TPCK-treated trypsin at tyrosine and phenylalanine residues (see Discussion).

Discussion

Subunit Identity. L. mesenteroides glucose-6-phosphate dehydrogenase denatured in 0.1% sodium dodecyl sulfate (Milhausen, 1974) or 8 M urea (Haghighi, 1982) migrates as a single band on polyacrylamide gel electrophoresis. Analysis of end-group residues revealed glycine as the sole C terminus and valine as the only N terminus of the enzyme. These observations, together with the fact that the enzyme has an average molecular weight of 103 700 (Olive & Levy, 1971) and a subunit molecular weight of 54 800 (Ishaque et al., 1974; Kawaguchi & Bloch, 1974), are consistent with the enzyme containing two identical subunits.

Amino Acid Sequence around PLP Binding Site. Loss of enzyme activity was not linear with pyridoxylation of lysine residues, as noted and discussed previously (Milhausen & Levy, 1975): over 98% inactivation resulted from the covalent incorporation of 1.26 mol of pyridoxyl groups/mol of enzyme dimer. Although 2 mol of pyridoxyl groups/mol of enzyme can be incorporated after several cycles of treatment with PLP and sodium borohydride (Milhausen & Levy, 1975), this was not done in order to avoid any possible nonspecific lysine modification.

Separation of the tryptic peptides from pyridoxylated enzyme by gel filtration led to the recovery of 74% of the 315-nm-absorbing material in a broad peak (Figure 1, peak III). This fraction was further purified by ion-exchange chromatography (Figure 2) from which pyridoxyl-containing peak DII appeared as the major peak, containing 45% of the total 315-nm-absorbing material. Other small, fluorescent peaks (DI, S1, S2) were also observed. These were further purified and shown to be devoid of detectable pyridoxyl peptides. Peak DII was further purified, and peptides DIIA and DIIB were derived as described under Results.

After these peptides were purified by preparative TLC and electrophoresis, peptide maps showed that they appeared to be essentially pure. Subsequent HPLC analysis, however, indicated that other, nonpyridoxyl peptides and impurities were still present. Both DIIA and DIIb were further purified by preparative HPLC and shown to be homogeneous by analytical HPLC.

The observed overlap between the amino acid sequence of DIIA and DIIB (Table III) suggests that DIIA has been derived from DIIB by nonspecific cleavage by trypsin at tyrosine and phenylalanine residues. Although trypsin is known to catalyze preferentially the hydrolysis of peptide bonds at lysine or arginine residues, α-chymotrypsin-like activity has also been observed [for review, see Keil (1971)]. Kostka & Carpenter (1964) reported that treatment of commercial, crystalline trypsin with TPCK virtually eliminates its chymotryptic activity toward the B chain of insulin; however, appreciable activity (50%) toward acetyl-L-tyrosine ethyl ester still remains. Pure TPCK–treated trypsin displayed chymotryptic-like activity toward the C chain of α-chymotrypsin and glucagon, but not the B chain of insulin, suggesting that this activity is displayed selectively toward only some high molecular weight peptides (Maroux et al., 1966). Studies of PLP-binding sites of glutamate decarboxylase (Strausbauch & Fischer, 1970) and glycogen phosphorylase (Forrey et al., 1971a) also showed that TPCK-treated trypsin still cleaves pyridoxyl peptides at phenylalanine residues. Although one might expect two other peptides, resulting from cleavage at only one of the two susceptible bonds in peptide DIIB, we did not locate these peptides. Either they were retained on the Aminex AG50 column or their yield was too low to permit detection, possibly because cleavage at one of the susceptible bonds greatly facilitates cleavage at the other.

Minchiotti et al. (1981) have compared the amino acid sequences around the PLP-binding sites of 38 peptides from different enzymes. The most common feature of these peptides is the presence of the basic amino acids arginine or lysine and/or the hydroxylated amino acid residues serine or threonine at a well-defined distance (three or four residues)

from the pyridoxyllysine residue. These authors believe that the labeled lysine in these enzymes is not essential for catalytic activity but that binding of PLP to this residue makes a phosphate (or anion) binding site inaccessible to a natural, anionic substrate. Also, these peptides generally are rich in nonpolar amino acids. The presence of serine, three residues removed from pyridoxyllysine, on the C-terminal side and, presumably, a lysine or arginine (cleavage site) on the N-terminal side of the *L. mesenteroides* glucose-6-phosphate dehydrogenase pyridoxyl peptide is in full agreement with the general feature of these peptides described by Minchiotti et al. (1981). This sequence also contains nonpolar residues, suggesting a hydrophobic environment for this region which, in turn, could facilitate binding of PLP to this part of the protein. Hydrophobicity of the PLP-binding site has been suggested for lysine decarboxylase (Sabo & Fischer, 1974), glutamate dehydrogenase (Moon et al., 1972), and glyceraldehyde-3-phosphate dehydrogenase (Forcina et al., 1971). In addition, the *L. mesenteroides* glucose-6-phosphate dehydrogense pyridoxyl peptide contains one serine residue next to the labeled lysine. Although, according to Minchiotti et al. (1981), none of the 38 peptides investigated possesses such a residue, in fact, two of the six PLP-modified peptides isolated from *N. crassa* glutamate dehydrogenase do have a serine adjacent to the pyridoxyllysine (Austen et al., 1977).

The demonstrated absence of pyridoxyllysine in all fractions analyzed except in peptides DIIA and DIIB, which were shown to be homogeneous, together with the overlap in amino acid sequence of these two peptides and evidence for subunit identity, provides strong evidence that we have modified a unique lysine residue and determined the sequence of amino acids surrounding it. This is the first report of the sequence of a peptide containing an essential amino acid from a glucose-6-phosphate dehydrogenase (Levy, 1979). Support for previous evidence that the lysine modified by PLP functions to bind glucose 6-phosphate (Milhausen & Levy, 1975) is provided in the following paper (Haghighi & Levy, 1982).

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References

Allen, G. (1980) *Biochem. J. 187*, 545–563.
Ambler, R. P. (1972) *Methods Enzymol. 25*, 143–154.
Austen, B. M., Haberland, M. E., Nyc, J. F., & Smith, E. L. (1977) *J. Biol. Chem. 252*, 8142–8149.
DeMoss, R. D., Gunsalus, I. C., & Bard, R. C. (1953) *J. Bacteriol. 66*, 10–16.
Fischer, E. H., Forrey, A. W., Hedrick, J. L., Hughes, R. C., Kent, A. B., & Krebs, E. G. (1963) *Proc. Int. Symp. Chem. Biol. Aspects Pyridoxyl Catal., 1st*, 543.
Folk, J. E., Piez, K. A., Carroll, W. R., & Gladner, J. A. (1960) *J. Biol. Chem. 235*, 2272–2277.
Forcina, B. C., Ferri, G., Zapponi, M. C., & Ronchi, S. (1971) *Eur. J. Biochem. 20*, 535–540.
Forrey, A. W., Sevilla, C. L., Saari, J. C., & Fischer, E. H. (1971a) *Biochemistry 10*, 3132–3140.
Forrey, A. W., Olsgaard, R. B., Nolan, C., & Fisher, E. H. (1971b) *Biochimie 53*, 269–281.
Garcy, R. W. (1977) *Methods Enzymol. 47*, 195–204.
Gray, W. R. (1972) *Methods Enzymol 25*, 121–138.
Grove, T. H., Ishaque, A., & Levy, H. R. (1976) *Arch. Biochem. Biophys. 177*, 307–316.
Haghighi, B. (1982) Ph.D. Dissertation, Syracuse University.
Haghighi, B., & Levy, H. R. (1982) *Biochemistry* (second paper of three in this issue).
Ishaque, A., Milhausen, M., & Levy, H. R. (1974) *Biochem. Biophys. Res. Commun. 59*, 894–901.
Johnson, N. D., Hunkapiller, M. W., & Hood, L. E. (1979) *Anal. Biochem. 100*, 335–338.
Kawaguchi, A., & Bloch, K. (1974) *J. Biol. Chem. 249*, 5793–5800.
Keil, B. (1971) *Enzymes, 3rd Ed. 3*, 249–275.
Kostka, V. K., & Carpenter, F. H. (1964) *J. Biol. Chem. 239*, 1799–1803.
Levy, H. R. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 48*, 97–192.
Mahoney, W. C., Hogg, R. W., & Hermodsen, M. H. (1981) *J. Biol. Chem. 256*, 4350–4356.
Maroux, S., Rovery, M., & Desnuelle, P. (1966) *Biochim. Biophys. Acta 122*, 147–150.
Milhausen, M. (1974) Ph.D. Dissertation, Syracuse University.
Milhausen, M., & Levy, H. R. (1975) *Eur. J. Biochem. 50*, 453–461.
Minchiotti, L., Ronchi, S., & Rippa, M. (1981) *Biochim. Biophys. Acta 657*, 232–242.
Moon, K., Piszkiewicz, D., & Smith, E. L. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1380–1383.
Olive, C., & Levy, H. R. (1967) *Biochemistry 6*, 730–736.
Olive, C., & Levy, H. R. (1971) *J. Biol. Chem. 246*, 2043–2046.
Olive, C., Geroch, M. E., & Levy, H. R. (1971) *J. Biol. Chem. 246*, 2047–2057.
Ritchey, J. M., Gibbons, I., & Schachman, H. K. (1977) *Biochemistry 16*, 4584–4590.
Sabo, D. L., & Fischer, E. H. (1974) *Biochemistry 13*, 670–676.
Strausbauch, P. H., & Fischer, E. H. (1970) *Biochemistry 9*, 233–238.
Summers, M. R., Smythers, G. W., & Oroszlan, S. (1973) *Anal. Biochem. 53*, 624–628.
Tanford, C., Kawahara, K., & Lapanje, S. (1967) *J. Am. Chem. Soc.* 89, 729–736.
Woods, K. R., & Wang, K. T. (1967) *Biochim. Biophys. Acta 133*, 369–370.
Zamenhof, S. (1957) *Methods Enzymol. 3*, 696–704.