# ON THE ABSENCE OF CYSTEINE IN GLUCOSE 6-PHOSPHATE DEHYDROGENASE FROM LEUCONOSTOC MESENTEROIDES

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Glucose 6-phosphate dehydrogenase from Leuconostoc mesenteroides has a subunit molecular weight of 55,000, determined by SDS gel electrophoresis. The amino acid composition of the enzyme is reported. An unusual feature which has not been reported for any other dehydrogenase, is the complete absence of cystine or cysteine. This establishes the fact that cysteine is not an obligatory participant in the mechanism of action of NAD(P)-linked dehydrogenases.

Glucose 6-phosphate dehydrogenase from Leuconostoc mesenteroides was first isolated by DeMoss et al. (1) who showed that the enzyme could utilize either $NAD^+$ or $NADP^+$ as the hydrogen acceptor. We have reported on the isolation of the crystalline enzyme (2), some of its physical properties (3) and the kinetic mechanism (4). The present report deals with the amino acid composition and subunit molecular weight of this enzyme.

## Experimental Procedures

Enzyme. The commercial preparations of glucose 6-phosphate dehydrogenase obtained from Worthington Biochemicals or P. L. Laboratories, were generally approximately 50% pure. They were further purified by chromatography on hydroxylapatite and several crystallizations, as previously described (2). Enzyme assays and definition of units were described previously (2). Protein concentration was measured by the methods of Warburg and Christian (5) and Lowry et al. (6).

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed on 7.5% acrylamide gels at pH 9.3 according to the methods outlined by Gabriel (7). Ammonium persulfate was used for polymerization. Pre-

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electrophoresis to rid the gels of persulfate ions, was performed routinely. Staining for proteins was accomplished by the method of Chrambach et al. (8) and for enzyme activity by the method of Dewey and Conklin (9) but omitting KCN from the staining solution. All gels were scanned at 600 nm. Polyacrylamide gel electrophoresis of the purified enzyme revealed one major and two minor protein bands which stained for enzyme activity and one minor band (less than 2% of the total) which was inactive. When electrophoresis was conducted in the presence of 5 μM $NADP^+$ a single, symmetrical protein band was seen which stained for enzyme activity.

Subunit molecular weight. Enzyme was treated with Na dodecyl sulfate and mercaptoethanol and run on polyacrylamide gels in the presence of Na dodecyl sulfate according to the procedure of Weber and Osborn (10). Standard proteins run simultaneously included bovine serum albumin, bovine liver catalase, bovine liver glutamate dehydrogenase, pig heart fumarase and egg white ovalbumin. The gels were treated exhaustively with methanol-acetic acid to remove Na dodecyl sulfate, stained with Coomassie Blue and excess stain removed with methanol-acetic acid. They were scanned in a Gilford model 240 spectrophotometer with a linear transport attachment.

Amino acid composition. The total amino acid analysis was performed according to the methods of Moore et al. (11), Spackman et al. (12) and Spackman (13) in a Beckman-Spinco model 116 amino acid analyzer. Duplicate analyses were performed on samples hydrolyzed for 24, 48 and 72 hours. Portions of the enzyme were subjected to performic acid oxidation according to the procedure of Hirs (14). For this purpose duplicate samples were hydrolyzed for 24 and 48 hours and analyzed on the amino acid analyzer. Conversion of methionine to methionine sulfone was 85-100% complete. Complete amino acid analyses were carried out on two different enzyme preparations at Syracuse; a third preparation was sent to the AAA Laboratory in Seattle, Washington, for independent analysis.

Separate determinations of sulfhydryl groups were carried out using the

methods of both Boyer (15) and Ellman (16). For the former, 0.15-0.4 mg of enzyme was used. Two determinations were carried out: one in 0.67M acetate pH 4.6 containing 1M sulfate and one in 0.1M phosphate pH 7.0 containing 1M sulfate. The concentration of p-hydroxymercuribenzoate* was maintained between 1 and 60 μM. Known concentrations of glutathione were used as standards and 1 mg samples of bovine serum albumin and ovalbumin served as independent controls. For Ellman's method 0.66 mg and 1.3 mg of enzyme denatured in guanidine hydrochloride were added to 0.1M phosphate pH 8. Controls were included using 0.08 mg of ovalbumin and 1 mg of bovine serum albumin. A molar extinction coefficient for the p-nitrophenol anion of $13.6 \times 10^3$/M/cm at 412 nm was employed (16). Tryptophan was determined by the method of Edelhoch (17). Enzyme was dialyzed for 24 hours against 6M guanidine hydrochloride. Bovine serum albumin, trypsinogen and chymotrypsinogen which were used as control proteins gave tryptophan analyses in good agreement with published values.

Materials. Hydroxylapatite was prepared by the method of Anacker and Stoy (18). Reproducible results were not obtained with commercial hydroxylapatite. Enzyme-grade ammonium sulfate was obtained from Schwarz-Mann; guanidine hydrochloride of extreme purity from Heico, Inc.; Coomassie Blue from Colab; acrylamide and N,N'-methylenebisacrylamide from Eastman Kodak Co.; N,N,N',N'-tetramethylethylenediamine from Matheson, Coleman and Bell, and ammonium persulfate from E-C. Apparatus Corp. The following reagents were obtained from Sigma Chemical Co.: $NADP^+$, glucose 6-phosphate, Na dodecyl sulfate, DTNB, phenazine methosulfate, nitroblue tetrazolium, bovine serum albumin, catalase, glutamic dehydrogenase, fumarase, ovalbumin, trypsinogen, and chymotrypsinogen.

## Results

Subunit molecular weight. Na dodecyl sulfate gels were run with enzyme which had a specific activity of 222 units per mg. Four separate experiments were performed and a total of 12 gels were run. The molecular weight was calcula-

*The following abbreviations are used: pHMB = p-hydroxymercuribenzoate; DTNB = 5,5'-dithiobis (2-nitro-benzoic acid).

TABLE 1. AMINO ACID COMPOSITION OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE

| Amino Acid | μmoles per mg protein[a] | μmoles per 54,800 μg protein | Number of residues per subunit[h] | | |
|---|---|---|---|---|---|
| | | | This analysis[i] | Analysis 2[k] | Analysis 3[ℓ] |
| Asp | 1.3±0.03 | 71.8 | 72 | 70 | 71 |
| Thr | 0.433±0.009[b] | 23.7 | 24 | 24 | 28 |
| Ser | 0.408±0.017[c] | 22.4 | 22 | 22 | 27 |
| Glu | 0.940±0.018 | 51.5 | 52 | 51 | 54 |
| Pro | 0.276±0.056 | 15.1 | 15 | 16 | 13 |
| Gly | 0.638±0.017 | 35.0 | 35 | 33 | 34 |
| Ala | 1.05±0.02 | 57.5 | 58 | 58 | 60 |
| ½ Cys | 0[d] | 0 | 0 | 0 | 0 |
| Val | 0.545±0.015[e] | 29.9 | 30 | 32 | 28 |
| Met | 0.166±0.005[f] | 9.10 | 9 | 9 | 8 |
| Ile | 0.471±0.007[e] | 25.8 | 26 | 27 | 26 |
| Leu | 0.660±0.007[e] | 36.2 | 36 | 36 | 37 |
| Tyr | 0.392±0.007 | 21.5 | 22[j] | 22 | 20 |
| Phe | 0.575±0.016 | 31.5 | 32 | 32 | 27 |
| Lys | 0.669±0.025 | 36.7 | 37 | 36 | 39 |
| His | 0.112±0.005 | 6.14 | 6 | 6 | 6 |
| Arg | 0.319±0.011 | 17.5 | 18 | 19 | 19 |
| Trp | 0.139±0.002[g] | 7.62 | 8 | - | - |

[a]Mean value and standard deviation for duplicate analyses after 24, 48 and 72 hr hydrolyses, except as indicated. When these values are calculated as μg of protein, the total recovery = 101.7%. [b]Assuming 95% recovery after 24 hr hydrolysis. [c]Assuming 90% recovery after 24 hr hydrolysis. [d]From performic acid oxidation. [e]Based on 72 hr hydrolysis. [f]Based on 24 hr hydrolysis. In performic acid oxidation methionine sulfone gave 85-100% of this value. [g]Duplicate analyses by method of Edelhoch (17). [h]Nearest integral number, based on subunit molecular weight = 54,800. [i]Performed in Syracuse on enzyme of specific activity = 210 units per mg. [j]Spectrophotometric method (17) gave 19 residues. [k]Performed in Seattle on enzyme of specific activity = 190 units per mg. [ℓ]Performed in Syracuse on enzyme of specific activity = 200 units per mg.

ted to be 55,000 (mean = 54,800; standard deviation = ±1380; range = 51,600 - 56,800).

Amino acid composition. Enzymes purified from 3 different commercial prepa-

rations were subjected to amino acid analysis. The details from one determination are given in Table 1, along with a summary of the other two analyses. The most striking feature is the complete absence of cysteine in this enzyme. In the performic acid oxidations no cysteic acid or methionine could be detected; methionine sulfone was recovered to the extent of 85-100% of the theoretical value in the three sets of duplicate analyses. No sulfhydryl groups could be detected in the enzyme by either Boyer's procedure (15) or Ellman's method (16) whereas the expected values for control proteins were obtained, (Table 2).

TABLE 2. DETERMINATION OF SULFHYDRYL GROUPS IN GLUCOSE 6-PHOSPHATE DEHYDROGENASE BY DTNB AND p-HMB METHODS

| Protein | Moles of sulfhydryl group per mole of protein | | | |
|---|---|---|---|---|
| | Values Determined | | Values Reported[c] | |
| | p-HMB | DTNB[b] | p-HMB | DTNB |
| G6PD[a] | 0.0 | 0.0 | -- | -- |
| Ovalbumin | 1.9 | 4.3 ± 0.8 | 2.7-4.1 | 3.8 |
| Bovine serum albumin | 0.67 | 0.58 ± 0.04 | 0.42-0.52 | 0.32-0.74 |

[a] L. mesenteroides glucose 6-phosphate dehydrogenase.

[b] Average of three determinations.

[c] From Diez et al. (26).

## Discussion

Previously we reported that glucose 6-phosphate dehydrogenase from L. mesenteroides has a molecular weight of 103,700 (3). The present results from Na dodecyl sulfate gels give a subunit molecular weight of 55,000. This value is consistent with a minimum molecular weight calculated from the histi-

dine content obtained from amino acid analysis and indicates that the enzyme consists of two subunits.

Unlike most glucose 6-phosphate dehydrogenases, or indeed most other dehydrogenases, glucose 6-phosphate dehydrogenase from L. mesenteroides resists inactivation under a variety of conditions. This has led to its widespread use in various commercial clinical test kits. It is possible that the unusual stability of this dehydrogenase derives from the total absence of cysteine in its amino acid composition. To our knowledge, no other dehydrogenase has been reported to be devoid of cysteine. It is well established that cysteine residues play vital roles at the active site of numerous dehydrogenases and there is evidence that sulfhydryl groups are essential for catalytic activity of glucose 6-phosphate dehydrogenases from bovine adrenal gland (19) and human erythrocytes (20, 21). The function of the active site cysteine in glyceraldehyde 3-phosphate dehydrogenase is to bind the substrate as a thiohemiacetal (22). The function of essential cysteines in other dehydrogenases is not known, but a role in orienting the coenzyme molecule has been suggested (23) and, in the case of lactic dehydrogenase, is consistent with crystallographic studies (24). The absence of cysteine residues in L. mesenteroides glucose 6-phosphate dehydrogenase establishes the fact that the mechanism of NAD(P)-linked dehydrogenase activity does not require participation of cysteine. Either some dehydrogenases can function without a cysteine at their active site, or the role played by "essential" cysteines can be assumed by another amino acid. The possibility that the absence of cysteine might be a general feature of dehydrogenases of L. mesenteroides, presumably a primitive microorganism, is negated by Garland's demonstration that D(-)-lactate dehydrogenase from this microorganism contains 28 cysteine residues per molecule of enzyme (25).

The isoelectric point of L. mesenteroides glucose 6-phosphate dehydrogenase is 4.6 (3), which is consistent with the large excess of acidic over basic amino acids. We reported that the ultraviolet absorption spectrum of this enzyme displays considerable fine structure (3) and speculated that this would

prove to be a reflection of a low ratio of tryptophan to phenylalanine. This prediction is borne out by the data in Table 1 and by the similarity between the ultraviolet absorption spectra of the enzyme and a mixture of phenylalanine, tryptophan and tyrosine in the same ratio as that found in the enzyme.*

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