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Biochem. J. (1966) **99**, 14c

An Extended Amino Acid Sequence around the Reactive Cysteine Residues of Glyceraldehyde 3-Phosphate Dehydrogenase

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(Received 25 February 1966)

As part of a continuing investigation of the amino acid sequence of glyceraldehyde 3-phosphate dehydrogenase [D-glyceraldehyde 3-phosphate-NAD oxidoreductase (phosphorylating), EC 1.2.1.12], a study has been made of the peptides produced by pepsin digestion of the enzyme from ox skeletal muscle. The catalytic role of the reactive cysteine residues in the activity of the enzyme isolated from a number of organisms has been the subject of much recent attention (Harris, Meriwether & Park, 1963; Perham & Harris, 1963, and unpublished work; Allison & Harris, 1965). Also, each enzyme has been shown to consist of four identical polypeptide chains (Harris & Perham, 1965). Other experiments have shown that each polypeptide chain in the enzyme from both pig and rabbit skeletal muscle contains a uniquely reactive lysine residue as indicated by its ability to become acetylated during the enzyme reaction with *p*-nitrophenyl acetate (Harris & Polgar, 1965) and acetyl phosphate (Mathew, Agnello & Park, 1965). It is the purpose of the present paper to report an extension of the amino acid sequence around the reactive cysteine residues of the ox enzyme that enables the relative positions of the reactive cysteine and lysine residues in the primary structure of the pig enzyme to be deduced.

Glyceraldehyde 3-phosphate dehydrogenase was isolated from ox skeletal muscle by the method of Elödi & Szörényi (1956) and oxidized with performic acid (Hirs, 1956). The oxidized protein (120 mg.) was dissolved in 12 ml. of 5% (v/v) formic acid and digested with 0.5% pepsin for 1 hr. at 37°. The resulting peptides were recovered by freeze-drying and applied to a column (22 cm. × 2 cm.) of DEAE-

Sephadex A-25 equilibrated with 0.05N-acetic acid adjusted to pH 8.2 with 5N-NH₃. The column was developed at a flow rate of 36 ml./hr. and 6 ml. fractions were collected. After 204 ml. of effluent had been collected, a linear gradient was started, consisting of 200 ml. of starting buffer into which flowed 200 ml. of 0.5N-acetic acid adjusted to pH 5.3 with 5N-NH₃. Peptides in the column effluent were detected by means of the Folin-Ciocalteu reaction (Lowry, Rosebrough, Farr & Randall, 1951) and were freeze-dried. The peptides were further fractionated by high-voltage paper electrophoresis in pyridine-acetic acid buffers as described by Ryle, Sanger, Smith & Kitai (1955). Three peptides are of particular interest to us here. These are peptides P11c, which emerged in fractions 64–67 and was obtained pure on ionophoresis at pH 6.5, P12a, which emerged in fractions 68–73 and was obtained pure on successive ionophoresis at pH 6.5 and pH 3.5, and P14d2, which emerged in fractions 80–84 and was also obtained pure on successive ionophoresis at pH 6.5 and pH 3.5. Peptide yields were 11, 10 and 7% respectively.

Peptide P11c. Lysine was shown to be the N-terminal amino acid by means of the 'dansyl' method of Gray & Hartley (1963a). On digestion with trypsin four peptide fragments (P11cT1, P11cT2, P11cT3 and P11cT4) were obtained, which were separated by paper electrophoresis at pH 6.5. Peptide P11cT1 had the following amino acid composition: Lys (1), Asp (2), Ser (2), Thr (2), Pro (1), Ala (3), Val (1), Ile (1), Leu (2), CySO₃H* (2). The N-terminal residue was found to be

* Abbreviations: CySO₃H, cysteic acid; MetSO₂, methionine sulphone.

isoleucine by the 'dansyl' method. This information, together with the observed electrophoretic mobility of the peptide, is sufficient to identify it as the peptide containing the reactive cysteine residues of the enzyme and which has the amino acid sequence Ile-Val-Ser-Asp(NH₂)-Ala-Ser-CySO₃H-Thr-Thr-Asp(NH₂)-CySO₃H-Leu-Ala-Pro-Leu-Ala-Lys (R. N. Perham & J. I. Harris, unpublished work), identical with that found for the pig enzyme (Perham & Harris, 1963). Peptide P11cT2 had the following amino acid composition: Lys (2), Asp (2), Ser (2), Thr (2), Pro (1), Ala (3), Val (1), Ile (1), Leu (2), CySO₃H (2). The *N*-terminal residue was found to be lysine by the 'dansyl' method, and peptide P11cT2 gave a peptide identical with peptide P11cT1, together with free lysine, on further digestion with trypsin. Peptide P11cT2 must therefore be Lys-Ile-Val-Ser-Asp(NH₂)-Ala-Ser-CySO₃H-Thr-Thr-Asp(NH₂)-CySO₃H-Leu-Ala-Pro-Leu-Ala-Lys. Peptide P11cT3 had the following amino acid composition: Asp (1), Val (1), Ile (1), Phe (1), His (2). The *N*-terminal residue was found to be valine by the 'dansyl' method. Carboxypeptidase released phenylalanine and traces of histidine from the peptide. The amino acid sequence of the peptide was further examined by the combination 'dansyl-Edman degradation' method of Gray & Hartley (1963b) and shown, by using four successive steps of the degradation, to be Val-Ile-His-Asp-His-Phe. Peptide P11cT4 was free lysine.

Peptide P14d2. Lysine was shown to be the *N*-terminal amino acid by the 'dansyl' method. On digestion with trypsin, four peptides (P14d2T1, P14d2T2, P14d2T3 and P14d2T4) were obtained, which were separated by paper electrophoresis at pH 6.5. Peptide P14d2T1 was identical in all respects with peptide P11cT1. Peptide P14d2T2 contained valine, isoleucine, histidine, aspartic acid,

phenylalanine, glycine and glutamic acid. The *N*-terminal residue was found to be valine by the 'dansyl' method. On digestion with chymotrypsin two peptides (P14d2T2C1 and P14d2T2C2) were produced, which were separated by paper electrophoresis at pH 6.5. Peptide P14d2T2C2 was identical in all respects with peptide P11cT3. Peptide P14d2T2C1 had the following amino acid composition: Val (1), Ile (1), Glu (1), Gly (2). The amino acid sequence was shown, by using four successive steps of the 'dansyl-Edman degradation' method, to be Gly-Ile-Val-Glu-Gly. Peptide P14d2T2 must therefore be Val-Ile-His-Asp-His-Phe-Gly-Ile-Val-Glu-Gly. Peptide P14d2T4 was free lysine.

Peptide P12a. This peptide had the following amino acid composition: Gly (2), Ile (1), Val (1), Glu (1), Leu (1), Thr (1), MetSO₂ (1). The *N*-terminal residue was found to be glycine by the 'dansyl' method. Carboxypeptidase released leucine, threonine and methionine sulphone. The amino acid sequence was shown, by using six successive steps of the 'dansyl-Edman degradation' method, to be Gly-Ile-Val-Glu-Gly-Leu-MetSO₂-Thr.

These results for peptides P11c, P14d2 and P12a are summarized in Fig. 1. From them the amino acid sequence around the reactive cysteine residue of ox glyceraldehyde 3-phosphate dehydrogenase may now be written: Lys-Ile-Val-Ser-Asp(NH₂)-Ala-Ser-CySH-Thr-Thr-Asp(NH₂)-CySH-Leu-Ala-Pro-Leu-Ala-Lys-Val-Ile-His-Asp-His-Phe-Gly-Ile-Val-Glu-Gly-Leu-Met-Thr.

The amino acid sequence from residues 19-32 is identical with the *N*-terminal sequence of the peptide (S2To1) produced by tryptic digestion of pig glyceraldehyde 3-phosphate dehydrogenase (R. N. Perham & J. I. Harris, unpublished work) and which contains the lysine residue uniquely

Fig. 1. Amino acid sequences of peptides isolated from ox glyceraldehyde 3-phosphate dehydrogenase.

reactive in the native enzyme (Harris & Polgar, 1965). The amino acid sequences of the pig and ox enzymes are known to be very similar indeed (Perham, 1964) and hence, on the reasonable assumption that a sequence of 14 residues does not occur twice in a chain of about 330 residues, an amino acid sequence containing the reactive cysteine and lysine residues of pig glyceraldehyde 3-phosphate dehydrogenase may be deduced to be: Lys-Ile-Val-Ser-Asp(NH₂)-Ala-Ser-CySH-Thr-Thr-Asp(NH₂)-CySH-Leu-Ala-Pro-Leu-Ala-Lys-Val-Ile-His-Asp-His-Phe-Gly-Ile-Val-Glu-Gly-Leu-Met-Thr-(Thr,Val,His)-Ala-Ile-Thr-Ala-Thr-Glu(NH₂)-Lys-Thr-Val-Asp-Gly-Pro-Ser-Gly-Lys-Leu. Further experiments on the pig enzyme support this conclusion (J. I. Harris & R. N. Perham, unpublished work). The specific acetylation of the ϵ -amino group of lysine-42 during reaction of the NAD-free pig enzyme with *p*-nitrophenyl acetate appears to occur by intramolecular migration of acetyl groups from cysteine-12, where they are initially bound in thio ester linkage, indicating a close proximity of these two amino acid residues in the three-dimensional structure of the NAD-free enzyme (Harris & Polgar, 1965). This proximity could occur either by folding of the individual polypeptide chain or by suitable orientation of the four polypeptide chains in the active enzyme so that the reactive cysteine and lysine residues of different monomers are then juxtaposed.

The amino acid sequences around the reactive cysteine residues in glyceraldehyde 3-phosphate

dehydrogenase from ox and pig have been shown to be identical for at least 32 residues. It seems likely that this identity will extend even further and stands in marked contrast with the amino acid replacements found in the alcohol dehydrogenases (Harris, 1964).

I am grateful to Dr J. I. Harris for his continued interest, to Professor F. G. Young for encouragement and to Dr C. J. R. Thorne for performing the amino acid analyses. My thanks are also due to Mrs L. Oliver for valuable technical assistance.

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Biochem. J. (1966) **99**, 16c

A Hydroxy Fatty Acid Dehydrogenase in *Euglena gracilis*

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(Received 28 February 1966)

A requirement for NADPH and molecular oxygen in the enzymic desaturation of fatty acyl-CoA or fatty acyl-ACP* esters in a number of organisms (Bloomfield & Bloch, 1960; Marsh & James, 1962; Davidoff, 1964; Nagai & Bloch, 1965) has led to the proposition that such desaturations might proceed by way of an oxygenase

type of reaction, involving hydroxy acid intermediates. Testing this hypothesis, Light, Lennarz & Bloch (1962) failed to obtain conversion of 9- or 10-hydroxystearic acid into oleic acid either in extracts or in growing cells of the yeast *Saccharomyces cerevisiae*. Marsh & James (1962) obtained some evidence for a transformation of 9- or 10-hydroxystearic acid into oleate by rat-liver homogenates, but the conversion of the hydroxy acids

* Abbreviation: ACP, acyl carrier protein.