INTRODUCTION OF AN INTRAMOLECULAR CROSSLINK AT THE ACTIVE SITE OF GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE

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Received June 1, 1971

Summary. Reaction of rabbit muscle apo-glyceraldehyde 3 -phosphate dehydrogenase with one mole of 1, 5-difluoro, 2, 4-dinitrobenzene per mole of enzyme protomer brings about total loss of enzymatic activity and concomitant introduction of covalent intramolecular crosslinks at the active site of the enzyme. Following peptic digestion, a crosslinked couple of peptides was purified and found to have the structure:

Ala-Ser-Cys-Thr-Thr-Asn
|
DNP
|
Gln-Lys-Thr-Val-Asp-Gly-Pro-Ser-Gly-Lys-Leu

The crosslinked cysteine and lysine residues, though some 32 amino acid residues apart in the primary sequence, may approach each other to a distance of 5-6 Å in the three dimensional structure of the enzyme.

Glyceraldehyde 3-phosphate dehydrogenase (GAPD*) from rabbit muscle is a tetramer with a molecular weight of 145.000 (1, 2). Each of the enzyme protomers contains four sulfhydryl groups, one of which (cys 149) appears to be at its active site (3-5). We have recently shown that, in the absence of NAD^+, this cysteine residue can be selectively dinitrophenylated with concomitant loss of catalytic activity (6, 7). In the present study we used the preferential reactivity of this cysteine towards FDNB as an anchoring point for the introduction of intramolecular crosslinks at the active site of the enzyme. The crosslinking reagent was F_2DNB (8), which is very similar to FDNB in its size, shape and reactivity.

* Abbreviations: B.A.W., 1-butanol-acetic acid-water (4:1:4, v/v); DNP, 2, 4-dinitrophenyl; FDNB, 1-fluoro, 2, 4-dinitrobenzene; F_2DNB, 1, 5-difluoro, 2, 4-dinitrobenzene; GAPD, D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12).

MATERIALS AND METHODS

GAPD (from Sigma) was assayed by the method of Velick (9). Apo-GAPD (the NAD^+-free enzyme) was prepared according to Havsteen (10). The preparations used had a $A_{280\ nm} : A_{260\ nm}$ ratio of 1.85 - 1.95 . Protein concentrations were determined spectrophotometrically at 280 nm, using absorbance coefficients of $1.00 cm^2 mg^{-1}$ for GAPD and $0.83 cm^2 mg^{-1}$ for apo-GAPD (11). The molecular weight of the enzyme was taken as 140000 (1, 2).

F_2DNB was purchased from Aldrich. The amino acid composition of peptides was determined with an amino acid analyzer (12) after hydrolysis in 5.7 N HCl (22hr, 110^0) in evacuated sealed tubes, N-terminal amino acids were determined by the Dansyl method (13) and C-terminals by the use of carboxypeptidases A and B (14).

RESULTS

Inactivation of Apo-GAPD with F_2DNB. Reaction of apo-GAPD with one mole of F_2DNB per mole of enzyme protomer(pH 7.8, 22^0) brings about a quantitative inactivation of the enzyme within 30 minutes (Fig.1). Unlike the inactivation with FDNB which can be fully reversed by thiolysis (6), the loss of activity caused by F_2DNB is only partially reversed (ca. 60%, Fig. 1). One of the possible causes for incomplete reactivation could be the involvement of ε-amino groups of lysines in the crosslink since, under these conditions, N-DNP derivatives of lysine do not undergo thiolysis (15).

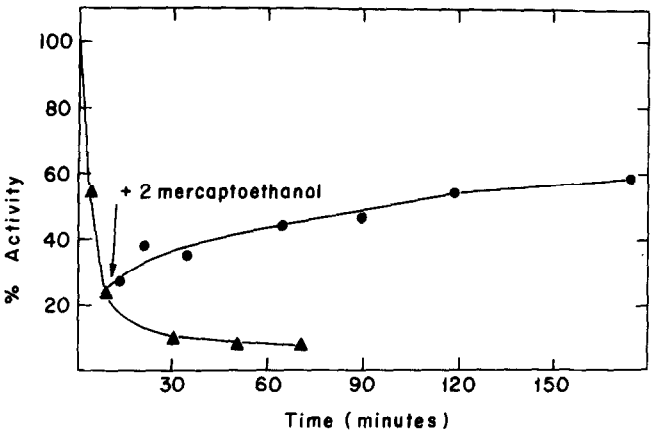

Fig. 1: Inactivation of apo-GAPD with F_2DNB and subsequent reactivation by thiolysis. The reaction mixture contained apo-GAPD (1.55 mg/ml) and F_2DNB (one mole/mole of enzyme protomer), pH 7.8 . The reaction was allowed to proceed at 22^0 and followed with time (—▲—). Ten minutes after the beginning of the reaction a sample was removed and thiolyzed with 2-mercaptoethanol (—●—) (500 moles per mole of enzyme protomer, pH 8.0, 22^0).

Structural studies indicate that this is indeed the case.

Labeling of the Apoenzyme. Apo-GAPD (912 mg, 6.5 μmoles) was reacted with 33 μmoles of F_2DNB (1.27 moles/mole of enzyme protomer) in a buffer composed of sodium pyrophosphate (0.03 M) and EDTA (10^{-3}M), pH 7.4. The reaction was allowed to proceed for 30 minutes at 22^0 and then the reaction mixture was dialyzed, first (1 hour) against 3 liters of the buffer solution mentioned above but adjusted to pH 9.0, and then (48 hours) against 6 liters of 0.02N HCl.

Labeling of the apoenzyme with F_2DNB did not change the aggregation state of the protein. Apo-GAPD and its labeled derivative (before dialysis against HCl) were found to have identical s_{20} values (s_{20} = 7.3 S, measured at 22^0 in sodium pyrophosphate (0.03 M), pH 7.4 .

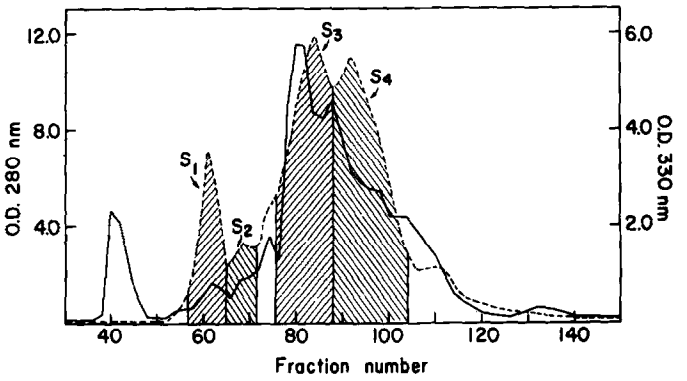

Fig. 2: Elution pattern of the peptic digest of apo-GAPD which had been labeled with F_2DNB. The column (Sephadex G-25, fine; 180 x 1.5 cm) was equilibrated at 22^0 with 2% acetic acid. Fractions of 3.2 ml were collected and their absorbancy at 280 nm (——) and at 330 nm (----) were monitored.

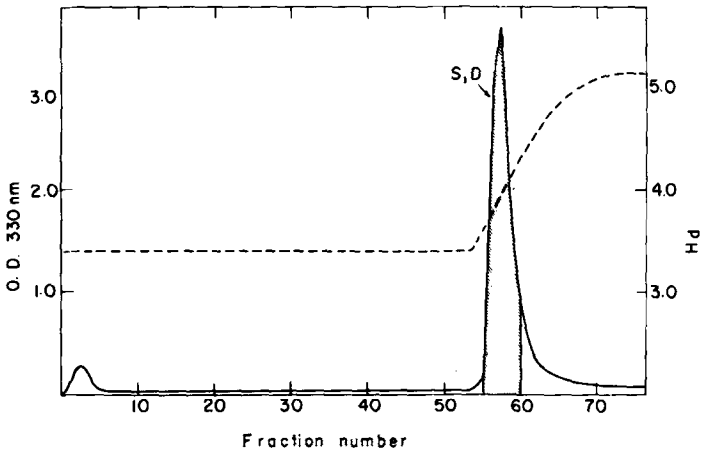

Fig. 3: Purification on Dowex 50-x8 of the fraction S_1 . The column (35 x 0.9 cm) was water-jacketed and eluted at 50^0, first with 0.2 M pyridine acetate, pH 3.3 and then with 2 M pyridine acetate pH 5.0 . Fractions of 2.5 ml were collected and their absorbance at 330 nm (——) as well as their pH (----) were monitored.

Isolation of a Crosslinked Peptide. The labeled protein (in 0.02N HCl) was digested by the addition of 91 mg pepsin dissolved in 3 ml of water. The proteolytic digestion was allowed to proceed for 22 hours at 30°. The reaction mixture was then lyophylized, dissolved in 6 ml of 2% acetic acid and subjected to gel filtration on Sephadex G-25 (fine). Fraction S_1 (Fig. 2) was applied on a Dowex 50-X8 column from which it emerged as a single yellow peak (S_1D, Fig. 3). This fraction (yield 12%) appeared to be a S-N crosslinked peptide since it was found to have an absorption spectrum (Fig. 4) identical to that reported for a model compound in which the sulfhydryl group

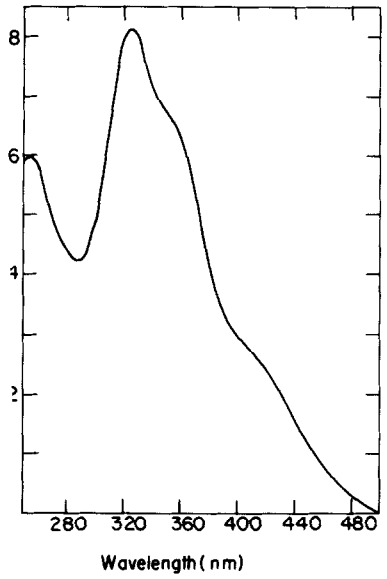

Fig. 4: Absorption spectrum of the fraction S_1D obtained from the Dowex column (Fig. 3). The sample was lyophylized to remove the pyridine acetate, dissolved in 2% acetic acid (pH 2.6) and the spectrum was taken.

Purification of fraction S_1D

Step N°	Method of Purification	Schematic Representation
1	Electrophoresis pH 3.5 ; 60 V/cm, 120 min.	
2	Electrophoresis pH 1.9 ; 30 V/cm , 75 min.	
3	Chromatography B.A.W. (4:1:4 v/v ; upper phase)	

Fig. 5: Further purifications of the S_1D fraction (obtained from the Dowex column) by paper electrophoresis and chromatography. Black bars are yellow and dotted bars are ninhydrin-positive bands.

of cysteine was crosslinked (with F_2DNB) to the ε-amino group of lysine (16). Further purification of the S_1D fraction by a series of preparative paper electrophoresis and chromatography yielded a homogeneous yellow peptide (Fig. 5).

Structure of the Labeled Peptide. After acid hydrolysis (6 N HCl, 110°, 22 hours) the purified yellow peptide was found to have the following amino acid composition: Asp, 2.3 (2) ; Thr, 2.6 (3) ; Ser, 1.7 (2) ; Glu, 1.0 (1) ; Pro, 1.3 (1) ; Gly, 1.8 (2) ; Ala, 1.6 (2) ; Val, 1.2 (1) ; Leu, 0.8 (1) ; Lys, 1.4 (1). The N-terminal amino acids of the crosslinked peptide were found to be Ala and Gln by the Dansyl method and the C-terminals (determined with carboxypeptidases A and B) were Asn and Leu followed by Thr and Lys.

On the basis of the above results and the sequence data about the enzyme (17, 18) we assigned to the crosslinked peptide the sequence depicted in Fig. 6. The crosslinked cysteine and lysine residues correspond to positions 149 and 183 (respectively) in the sequence of GAPD from pig muscle (17) whose structure is very similar to the rabbit muscle enzyme.

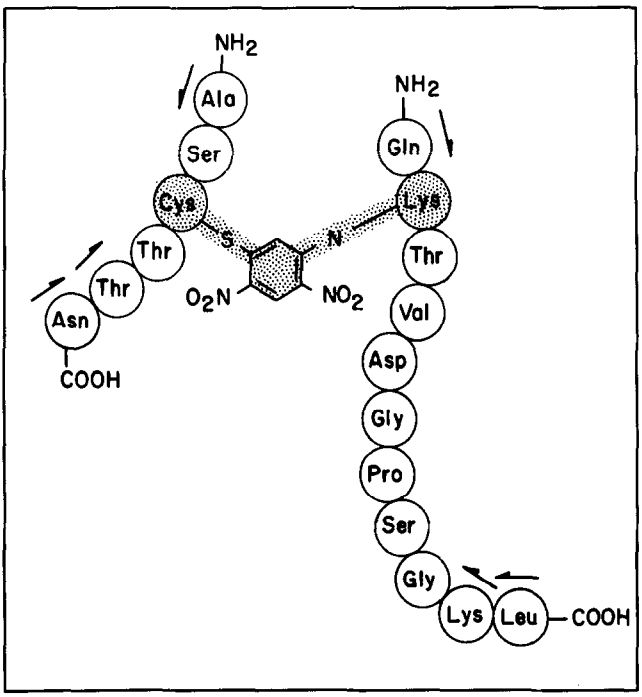

Fig. 6: Proposed structure of the crosslinked couple of peptides. The symbol ⟶ indicates a determination by the Dansyl procedure and ⟵, a determination with carboxypeptidase A or B.

DISCUSSION

A large body of evidence indicates that the sulfhydryl group of cys 149 is part of the active site of GAPD (18, 19). This functional group is super-reactive towards a variety of alkylating agents and is also uniquely acetylated during the enzyme-catalyzed hydrolysis of p-nitrophenyl acetate (3) or acetyl phosphate (4, 5). The elegant work of Harris, Merriwether and Park (3) has shown that a lysine residue (at position 183) is also at the active site, since the S-acetyl group (on cys 149) of the acetylated enzyme can be made to migrate to the ϵ-amino group of lys 183 upon raising the pH of the medium (4, 5, 20).

The results presented here provide direct evidence that cys 149 and lys 183 may approach each other to a distance of 5-6 Å, since they participate in a crosslink of F_2DNB in which the two fluorine atoms are 5-6 Å apart.

We have previously shown that FDNB can be selectively and exclusively directed to cys 149 (6, 7), and it was shown that this reaction is prevented by the presence of NAD^+. The crosslinking reagent that was used in the present study (F_2DNB) is very similar to FDNB in size, shape and reactivity. Therefore one could assume that the bifunctional reagent would first become anchored to cys 149 and then explore the surrounding of this locus and become attached with its other reactive carbon atom to other nucleophiles in the vicinity. The size of F_2DNB is small relative to NAD^+ and it is therefore possible that this reagent, once anchored to the reactive cysteine residue, may freely rotate within the site and form several types of crosslinks. The possibility also exists that the second C-F bond of F_2DNB may undergo hydrolysis before or after the reagent becomes anchored to the site. In view of all these possibilities and in view of the fact that S⟶N migrations may take place, it is necessary to accurately identify each of the crosslinks and monosubstitutions formed.

In the present study we have isolated one of the crosslinked peptides, purified it, established its structure and demonstrated that it contains a crosslink between cys 149 and lys 183. It should be emphasized, however, that among the other peptides that are formed there appears to be another type of crosslink (according to spectral evidence) connecting two sulfhydryl groups.

The crosslinks introduced by F_2DNB are formed intramolecularly, since there is no change in the sedimentation coefficient following the reaction of the apoenzyme with the bifunctional reagent. Nevertheless, it is not clear yet whether the cysteine and lysine residues which become crosslinked originate both from the same protomer or from two adjacent protomers within the same molecule. Dis-

sociation of the F_2DNB-labeled enzyme under conditions in which no crosslink is detached, may enable us to establish whether the crosslink is formed within a protomer or between two protomers. This question is of special relevance in the case of GAPD: on the one hand, each of the enzyme protomers (α) contains one super-reactive sulfhydryl group and binds one NAD^+ (18, 19). On the other hand, crystallographic data on the lobster muscle enzyme (21) are compatible with a structure containing two distinct interacting pairs of protomers ($\alpha_2\alpha'_2$ rather than α_4). This is supported by physicochemical studies with the native and the acyl enzyme (22).

Introduction of intramolecular crosslinks in GAPD may therefore be of great use both in the mapping of the enzyme active site and in determining the spatial relationship between its protomers.

REFERENCES

1. Harris, J.I., and Perham, R.N., J. Mol. Biol., 13, 876 (1965).
2. Harrington, W.F., and Karr, G.M., J. Mol. Biol., 13, 885 (1965).
3. Harris, J.I., Merriwether, B.P., and Park, J.H., Nature, 197, 154 (1963).
4. Park, J.H., Agnello, C.F., and Mathew, E., J. Biol. Chem., 241, 769 (1966).
5. Mathew, E., Meriwether, B.P., and Park, J.H., J. Biol. Chem., 242, 5024 (1967).
6. Shaltiel, S., and Soria, M., Biochemistry, 8, 4411 (1969).
7. Shaltiel, S., and Tauber-Finkelstein, M., FEBS Letters, 8, 345 (1970).
8. Zahn, H., and Stuerle, H., Biochem. Z., 331, 29 (1958).
9. Velick, S.F., Methods in Enzymol., 1, 401 (1955).
10. Havsteen, B.H., Acta Chem. Scand., 19, 1643 (1965).
11. Fox, J.B., and Dandliker, W.B., J. Biol. Chem., 221, 1005 (1956).
12. Spackman, D.H., Stein, W.H., and Moore, S., Anal. Chem., 30, 1190 (1958).
13. Gray, W.R., Methods in Enzymol., 11, 139 (1967).
14. Ambler, R.P. Methods in Enzymol., 11, 436 (1967).
15. Shaltiel, S., Biochem. Biophys. Res. Commun., 29, 178 (1967).
16. Marphey, P.S., Uziel, M., and Little, J., J. Biol. Chem., 240, 3270 (1965).
17. Harris, J.I., and Perham, R.N., Nature, 219, 1025 (1968).
18. Harris, J.I., in "Pyridine Nucleotide Dependent Dehydrogenases" Sund, H., Ed., Springer-Verlag, Berlin, p. 57.
19. Park, J.H., in "Current Aspects of Biochemical Energetics", Kaplan, N.O. and Kennedy, E., Ed., Academic Press, New York, p. 298.
20. Polgàr, L., Biochim. Biophys. Acta., 118, 276 (1966).
21. Watson, H.C., and Banaszak, L.J., Nature, 204, 918 (1964).
22. Malhorta, O.P., and Bernhard, S.A., J. Biol. Chem., 243, 1243 (1968).