INACTIVATION OF GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE BY A BIFUNCTIONAL REAGENT

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1. Introduction

The bifunctional reagent p,p′-difluoro-m,m′-dinitrodiphenyl sulphone (F_2DPS) has been used as a cross-linking agent in proteins [1]. However, in most of these studies the reaction was performed without ensuring conditions for a specific reaction of the first functional group of F_2DPS. Recently Cuatrecasas et al. [2] used the low pK of aminotryrosine to enhance the specificity of the reaction and the favour the formation of a monomeric derivative which could then be cross-linked by raising the pH. Glyceraldehyde 3-phosphate dehydrogenase (GPDH, E.C. 1.2.1.12) has been shown to contain one reactive cysteine in the active centre of the enzyme [3,4] and Shaltiel and Soria have reported that under low molar ratio of fluorodinitrobenzene (FDNB) to enzyme, this cysteine is the only one to react, yielding *S*-DNP cysteine [5]. The DNP group could be quantitatively removed by thiolysis [6] with β-mercaptoethanol.

I have used the high reactivity of this cysteinyl residue in GPDH to enhance the specificity of the reaction with F_2DPS in order to form a specific cross-link at the active centre of the enzyme. The apo-enzyme was inactivated within 1 min by two moles of reagent per mole of the tetrameric enzyme. Spectral data suggest that this is due to the rapid formation of *S*-DPS cysteine which is followed by a slower reaction (within 15 min) with anohter residue, possibly lysine. The inactivation could be reversed by thiols as long as the cross-link had not been established.

2. Materials and methods

F_2DPS was purchased from Sigma. *N*-DPS glycine and *S*-DPS, *N*-acetyl cysteine were prepared by the reaction of F_2DPS and either glycine or *N*-acetyl cysteine in 0.1 M $NaHCO_3$ in a molar ratio of 1:2 of F_2DPS to amino acid. After 20 hr unreacted F_2DPS was extracted with ethylacetate. The pH was then lowered by HCl and the yellow derivative was obtained by extraction into ethylacetate followed by extraction into 0.1 M $NaHCO_3$.

Crystalline yeast GPDH was prepared according to the procedure of Krebs [7] and crystalline pig muscle GPDH was prepared according to the method of Amelunxen and Carr [8] for rabbit muscle GPDH. The crystalline GPDH was made free of ammonium sulphate by passing through a Sephadex G-25 column equilibrated with 0.05 M $NaHCO_3$, pH 8.2 or with other buffers when cited. Most of the studies were performed with yeast GPDH (molecular weight and $E^{1\%}_{280}$ were taken as 144,000 and 9.6 respectively). Absorption spectra were measured using a Unicam SP700 spectrophotometer and enzymic activity was determined from the increase in extinction at 340 nm using a Gilford 2000 spectrophotometer. F_2DPS was dissolved in acetone and was added to the enzyme solution in a ratio of 1:100 (v/v). The treated enzyme was assayed according to Krebs et al. [9] except that cysteine was omitted from the reaction mixture. At various intervals samples were also taken and made

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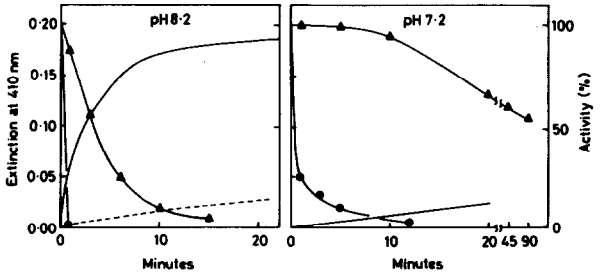

Fig. 1. The effect of F_2DPS on yeast GPDH. Enzyme concentration was 2 mg/ml in 0.05 M $NaHCO_3$ pH 8.2 or 0.05 M Tris-acetate pH 7.2, and the molar ratio of added F_2DPS per enzyme (tetramer) was 4:1. —— extinction at 410 nm; ---- extinction at 410 nm of monocarboxymethylated GPDH that was incubated with F_2DPS under the same conditions. • Activity. ▲ Activity recovered by reduction with β-mercaptoethanol of samples taken at the indicated times.

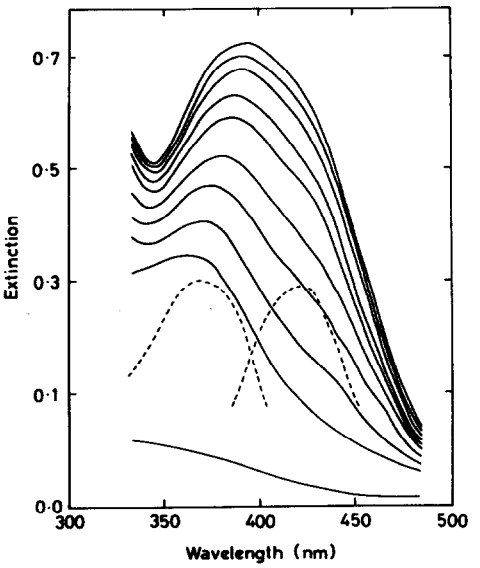

Fig. 2. Visible absorption spectra of yeast GPDH (5 mg/ml in 0.05 M $NaHCO_3$ pH 8.2) during the reaction with F_2DPS (4 moles per tetramer). —— spectra that were taken (from bottom to top), before addition of F_2DPS (lower line) and in the following times after addition of F_2DPS: 2,3,5,8,12,15, 20,23 and 27 min; ---- spectra of *S*-DPS, *N*-acetyl cysteine (left) and of *N*-DPS glycine (right).

0.05 M with respect to β-mercaptoethanol. The activity of these samples was assayed after 1 hr of incubation at room temperature, pH 8.2, or after 24 hr of incubation at 4°, pH 7.2. This was found necessary since at pH 7.2 and room temperature the treated enzyme solution became turbid on addition of mercaptoethanol. Yeast GPDH carboxymethylated in the cysteine residue of the active centre was prepared as previously described [4].

3. Results

The addition of one mole of F_2DPS per mole of subunit of yeast GPDH (which contains very little NAD [10]) resulted in an immediate and complete inactivation of the enzyme at pH 8.2 and in a somewhat slower inactivation at pH 7.2 (fig. 1). This inactivation could be slightly inhibited by the presence of phosphate (0.15 M) or of NAD (0.0015 M); and in the latter case the inactivation at pH 8.2 was complete after 15 min. Similarly the pig muscle GPDH which contains bound NAD (the ratio of 280 nm/260.nm absorbance was 1.3) was inactivated at pH 8.2 only after 15 min whereas the charcoal treated apoenzyme (the ratio of 280 nm/260 nm absorbance was 1.95) was inactivated within 2 min. The rapid inactivation of yeast GPDH was followed by an increase in the extinction at 410 nm which reached a plateau after 15 min of reaction. Addition of another mole of reagent per mole of subunit at this point did not change the rate of increase in extinction. Such an increase in extinction was not observed with the *S*-carboxymethylated enzyme, or when the reaction with the native enzyme was performed at pH 7.2 (fig. 1). This suggests that the inactivation is due to a fast reaction of F_2DPS with the reactive cysteine residue which is then followed by reaction of the second functional group of F_2DPS with another residue, due to the high local concentration of reagent in the active centre of the enzyme. This is corroborated by the visible absorption spectrum of the treated enzyme (fig. 2). It is

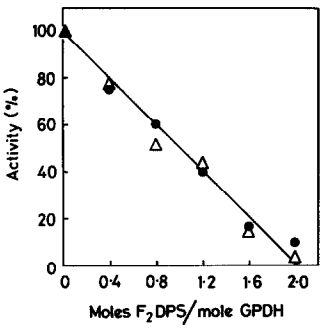

Fig. 3. The stoichiometry of the reaction of F_2DPS with GPDH (tetramer): F_2DPS at different concentrations in acetone was added (10 µl/ml) to either yeast or pig muscle GPDH (0.3 mg/ml in 0.05 M $NaHCO_3$ pH 8.2). Assay was performed 5 min or 1 hr after mixing with the yeast or pig enzymes respectively. • pig muscle GPDH, △ yeast GPDH.

shown that during the reaction the peak of maximum absorption is shifted from 370 nm, which is typical for *S*-DPS, *N*-acetyl-cysteine, to 394 nm, which is in an intermediate position between the maximum absorption of an amino derivative of DPS (420 nm) and that of *S*-DPS cysteine. This suggestion is also consistent with the reversibility of inactivation by mercaptoethanol due to thiolysis of the *S*-nitrophenyl bond [6]. At pH 8.2 the extent of reactivation by mercaptoethanol is inversely proportional to the increase of extinction at 410 nm, whereas at pH 7.2 the inactivation is reversible for a longer period, presumably due to the very slow reaction with lysine residues at this pH.

Fig. 3 demonstrates that only 2 moles of F_2DPS per mole of tetrameric enzyme are required in order to inactivate either yeast or pig muscle GPDH. This is in contrast to the inactivation with the monofunctional FDNB where 4 moles of reagent were incorporated per mole of enzyme [5].

4. Discussion

F_2DPS is less reactive than FDNB and has usually been reacted with proteins at pH 9.5 to 10.5 [1]. The rapid and stoichiometric reaction of F_2DPS with GPDH must be due to reaction with the reactive cysteine since *S*-carboxymethylated enzyme did not react under almost identical conditions. The data presented indicate that the reaction of cross-linking takes place in two stages:

(1) Fast reaction with the reactive cysteine.

(2) A slower reaction, presumably with a neighbouring lysine residue. The first stage could be reversed by thiols and the second stage takes place very slowly at pH 7.2, where ϵ-NH_2 groups are fully ionized.

The isolation of a cross-linked peptide from DPS-GPDH seems difficult due to the lability of the *S*-nitrophenyl bond under conditions of tryptic digestion in the presence of relatively high concentration of α- and ϵ-amino groups. However when the DPS-GPDH was reduced with mercaptoethanol in 8 M urea, followed by tryptic digestion, 70% of the yellow colour was recovered in one fraction of tryptic peptides, indicating that the second functional group of F_2DPS indeed reacted with another residue to form a non-thiolysable derivative, presumably with an ϵ-amino group of lysine. The location of the DPS-lysyl residue is now in progress and it is hoped that specific cross-linking by F_2DPS as well as other bifunctional reagents will provide some information on vicinal groups at the active centre of the enzyme. The yellow yeast DPS-GPDH could be crystallized under the same conditions as those used for the native enzyme and this might help also in crystallographic studies.

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