REACTION OF RABBIT MUSCLE APO-GLYCERALDEHYDE-3-P-DEHYDROGENASE WITH PYRIDOXAL-5'-PHOSPHATE

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1. Introduction

A distinctive feature of glyceraldehyde-3-P-dehydrogenase (GPD) (EC 1.2.1.12) from skeletal muscle is its ability to crystallize with four molecules of firmly bound NAD^+ [1, 2], the removal of which results in the formation of an apoenzyme with markedly different properties, such as a different conformational state and reaction specificity [3–7].

Chemical modifications carried out comparatively on the holo and the apoenzyme should provide information on the nature of the conformational change and, possibly, help elucidate some features of the coenzyme binding site.

We have previously shown [8] that native GPD from rabbit muscle may be preferentially labelled at Lys 191 and 212 by pyridoxal-5'-phosphate (PLP) with a concomitant loss of catalytic activity.

Here we describe the reaction of PLP with the NAD^+ free enzyme from the same source and the identification within the primary structure [9] of the residues labelled.

2. Materials and methods

Crystalline GPD was prepared from rabbit skeletal muscle as already described [8]. Protein concentration was measured at 280 nm assuming an $\epsilon^{280}_{1\%}$ of 9.6 for the holoenzyme and 8.0 for the apoenzyme [2].

NAD^+ was removed by charcoal treatment as described by Cseke and Boross [10] and samples showing a 280/260 absorbance ratio of 1.95–2.0 were used. Enzymic activity measurements, labelling with PLP, carboxymethylation, tryptic digestion, amino acid analysis and ε-pyridoxyl-lysine (ε-pxy-lys) determination were carried out as described previously for native GPD [8]. Peptide maps were prepared as described by Harris and Perham [11]. Peptide mobility was referred to aspartic acid = −1.0. N-terminal amino acids were identified as the dansyl derivatives [12] by chromatography on polyamide layer sheets [13]. Peptides containing ε-phospho-pxy-lys were purified either by ion-exchange chromatography on a PA 35 column, using a linear gradient of pyridine–acetic acid buffer (0.2–2 M) [14] followed, where necessary, by high voltage paper electrophoresis or by a modification on paper of the *column diagonal procedure* described by Strausbauch et al. [15]. In this case, 10 mg of PLP labelled apoenzyme, after having been carboxymethylated and digested with trypsin, were applied to Whatman 3 MM and submitted to high voltage electrophoresis at pH 6.5 (40 min, 50 V/cm). The fluorescent bands, located under UV light, were cut, eluted with 0.1 N NH_3, and digested for 12 hr with 20 μg of *E. coli* alkaline phosphatase in 0.5% NH_4HCO_3 at 25°.

The material was freeze dried and electrophoresed again under the same conditions as before. The fluorescent band, now with a changed mobility owing to enzymic removal of the phosphate, was cut out and eluted.

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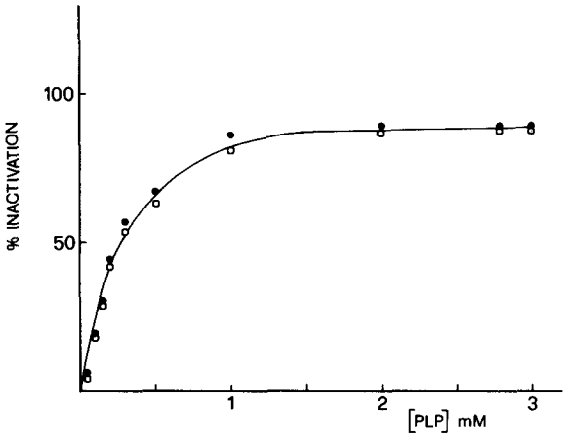

Fig. 1. Effect of increasing concentrations of PLP on the oxidative activity of apo and holo-GPD. Apo or holo-GDP (55.5 μM monomer) were incubated with PLP at the concentrations indicated, in 30 mM Na-pyrophosphate buffer, 5 mM EDTA, pH 8.4. After 5 min at 25°, 0.4 μg of enzyme were assayed for activity. Apoenzyme (•—•—•); holoenzyme (□—□—□).

3. Results

Apo-GPD from rabbit muscle is inactivated by PLP to the same extent as the holoenzyme. Fig. 1 shows the effect of increasing PLP concentrations on the oxidative activity towards the natural substrate glyceraldehyde-3-phosphate (GAP).

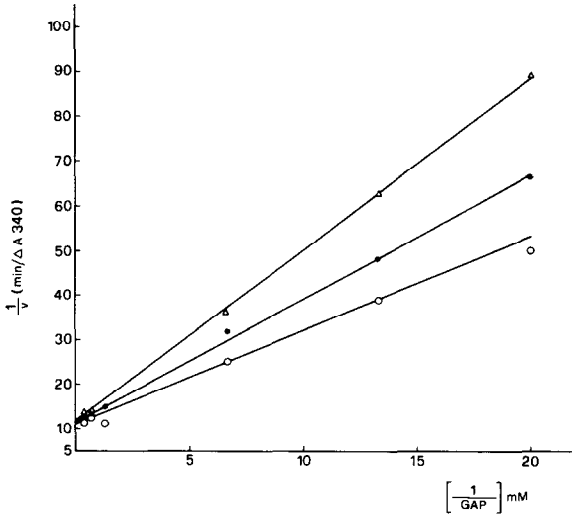

Fig. 2. Inhibition of apo-GPD activity by PLP with respect to varying concentration of GAP. Apoenzyme was incubated with PLP at the indicated concentrations and assayed for activity as described in fig. 1. PLP concentrations: none (○—○—○); 0.15 mM (•—•—•); 0.3 mM (△—△—△).

Table 1
Moles of ε-pxy-lys per monomer in apo- and holo-GPD as a function of time.

Time	% Residual activity		Moles of ε-pxy-lys/monomer	
	Apo-enzyme	Holo-enzyme	Apo-enzyme	Holo-enzyme
0.5 min	70.0	69.0	0.9	1.0
2.5 min	22.0	20.0	3.2	3.1
5.0 min	10.0	12.0	4.0	4.0
15.0 min	9.2	9.5	–	–
30.0 min	8.6	9.0	8.0	4.1
3 hr	–	–	9.9	4.0
24 hr	–	–	9.8	4.2

The dissociation constant K_d for the holo and apoprotein complex is in both cases 2×10^{-4} M. As already reported for the holoenzyme [8], arsenate or phosphate appear to protect in a competitive fashion. The substrate GAP can reverse the inactivation in both the apo and the holoenzyme when arsenate is present in saturating concentrations (fig. 2).

Although no significant kinetic differences are observed, a different reactivity of the lysyl side chains of the holo and apoprotein can be found at the structural level of analysis. Table 1 reports the number of moles of ε-pxy-lys formed per monomer at different times together with the corresponding values of the residual activity. While inactivation is practically complete when 4 moles of ε-pxy-lys are formed in both holo and apoenzyme, the latter can bind a higher amount of PLP, up to a maximum of 10 moles per mole of monomer.

In order to detect differences in the lysyl residues reacting, the holo and the apoprotein were treated with PLP for 5 min under identical conditions, reduced with $NaBH_4$, carboxymethylated and fingerprinted after tryptic digestion (fig. 3). Examination under UV light of the apoenzyme fingerprint did not show one of the two blue fluorescent spots which were present in the holoenzyme (hatched areas in fig. 3). On the basis of the electrophoretic and chromatographic mobilities the missing spot was tentatively concluded to be peptide containing ε-phospho-pxy-lys 191 (spot c) while spot b was identified as peptide containing ε-phospho-pxy-lys 212.

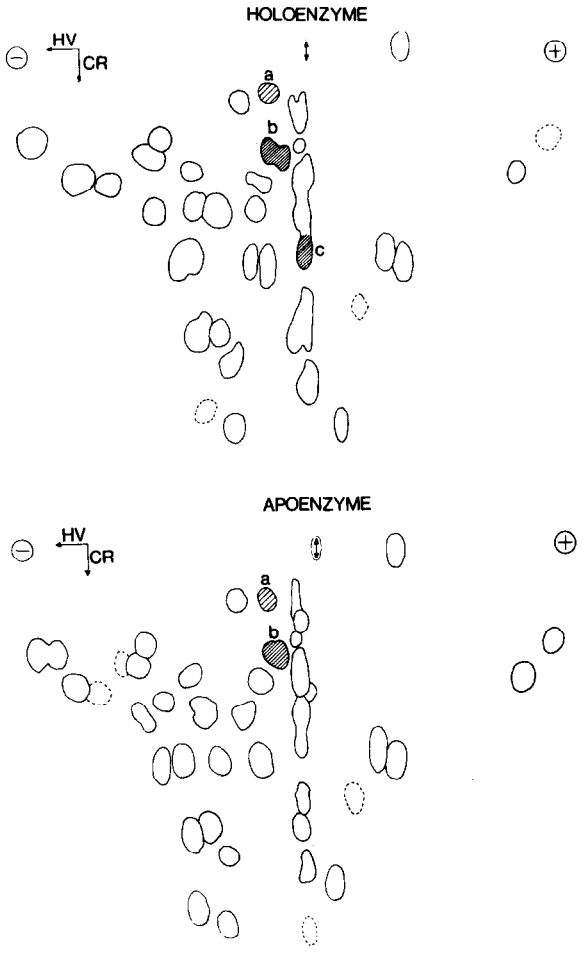

Fig. 3. Peptide maps of a tryptic digest of holo and apo-GPD labelled with PLP. Electrophoresis: pH 6.5, 60 V/cm, 40 min. Chromatography: acetic acid–n-butanol–H_2O–pyridine (6:30:24:20). Blue fluorescent peptides are shown as hatched spots.

To test this, the main fluorescent peptide present in a tryptic digest of the apoenzyme was purified on a preparative scale. Two approaches have been used: i) conventional column chromatography and paper electrophoresis, ii) paper diagonal procedure.

Fig. 4 shows the elution profile from a PA 35 column of a tryptic digest of apo-GPD treated with PLP. While several peaks absorbing at 325 nm can be seen in the chromatogram, indicating that random reaction may occur at several lysine sites, only the major one T_2 was found by paper high voltage electrophoresis to contain strongly blue fluorescent material associated with a single band of mobility +0.13. After further purification at pH 3.5 the peptide proved to be pure and have the following amino acid composition $Gly_{2.8}(Lys_{1.1}$ ϵ-pxy-$lys_{0.8}\,Asp_{1.2}\,Thr_{0.9}\,Ser_{1.1}\,Glu_{1.1}\,Pro_{1.0}\,Ala_{5.7}\,Val_{1.1}\,Ileu_{1.3})$. This is consistent with Lys 212 being labelled by PLP. From peak T_5–T_6 it was possible to purify a peptide with the following amino acid composition $Thr_{0.8}(Lys_{1.0}\,Asp_{1.0}\,Ser_{1.0}\,Pro_{1.0}\,Gly_{2.0}\,Val_{0.9})$ which proves that Lys 191, reacting with PLP in the holoenzyme, is free in the apoenzyme. These results were confirmed in a parallel experiment in which the peptide containing ϵ-pxy-lys 212 was purified by a modification on paper of the column diagonal procedure described by Strausbauch and Fischer [15] for the specific purification of peptides containing covalently bound PLP (see Methods).

After electrophoresis at pH 6.5 of a whole tryptic digest of the protein, the fluorescent PLP peptide, having a mobility of +0.13, was cut, eluted and digested with alkaline phosphatase, so as to alter specifically its mobility, and then rerun under the same conditions as before. The mobility was now +0.31, corresponding to a change of one net charge. The peptide had the following amino acid composition: $Lys_{1.2}$ ϵ-pxy-$lys_{1.0}\,Asp_{1.0}\,Thr_{0.9}\,Ser_{1.0}\,Glu_{1.0}\,Pro_{0.9}\,Gly_{2.8}\,Ala_{5.7}\,Val_{0.9}\,Ileu_{1.2}$. Gly was the N terminus. Final yield of the pure peptide was 18%.

4. Discussion

It has been shown that at the active centre of apo-GPD from several sources [16], there exists a unique Lys residue (Lys 183 in the sequence of the pig enzyme) [9] which can be specifically acetylated via an S–N transfer reaction, with a loss of oxidative activity towards the natural substrate, GA1P [17].

It was of interest, therefore, to test whether the same Lys residue would be labelled by PLP in the present study. Our failure to detect any significant labelling of Lys 183 in the apoenzyme which, incidentally, is confirmed by the isolation in good yields of the tryptic peptide following this lysine in the sequence, suggests that the reactivity of Lys 183 may well be a consequence of the S–N transfer reaction and may indeed vary with the reagent used to probe it.

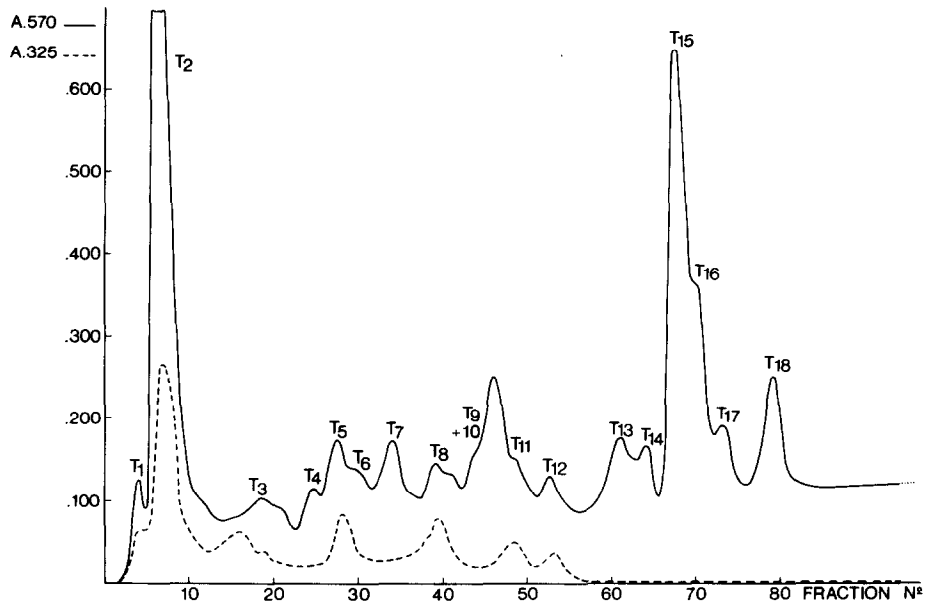

Fig. 4. Elution profile of tryptic peptides from apo-GPD treated with PLP. 30 mg of protein digest were dissolved in 1.2 ml of 0.12 N HCl and chromatographed at 50° on a column (0.9 X 18 cm) PA 35 with a linear gradient obtained by 250 ml of 0.2 N pyridine-acetate buffer pH 3.1 in the mixer and 250 ml of 2 N pyridine-acetate buffer pH 5 in the reservoir. Flow rate 30 ml/hr. Fractions of 2.7 ml were collected. (——) Absorbance at 570 nm, after alkaline hydrolysis and reaction with ninhydrin; (----) absorbance at 325 nm.

While the holoenzyme is capable of binding a maximum of 4 moles of PLP per monomer, even after prolonged times of incubation, the apoenzyme, under the same conditions, can bind up to 10 moles of PLP. It is conceivable that, in the absence of NAD^+, the introduction of the negative charges of the phosphate group may facilitate unfolding of the apoenzyme and, thus, further random reaction at other lysine sites. However, as early as 5 min, at which time the inactivation is practically complete, only Lys 212 is labelled to a significant extent, while both Lys 212 and 191 are labelled in the holoenzyme. It seems not unreasonable to explain the absence of reaction at Lys 191 with the different conformation of the apoenzyme and to predict that the conformational change will affect at least this part of the sequence. Since Lys 212 is the only residue which is significantly labelled in both the holo and the apoenzyme we suggest that the inactivation by PLP is brought about by reaction at this site, and, in view of the protection afforded by substrates, that this residue is in the active centre of the enzyme. It is of interest that the sequence around Lys 212 shows considerable homology, detected also by computer analysis [18], with that around Lys 97 of glutamate dehydrogenase which is also specifically labelled by PLP [19].

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