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EVIDENCE FOR THE IMPORTANCE OF CYSTEINE AND ARGININE RESIDUES IN *PSEUDOMONAS FLUORESCENS* UK-1 PANTOATE DEHYDROGENASE

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Summary

Homogeneous pantoate dehydrogenase (D-pantoate:NAD^+ 4-oxidoreductase, EC 1.1.1.106) was shown to be sensitive to inactivation by *p*-chloromercuribenzoate (100 μM), 5,5′-dithiobis(2-nitrobenzoic acid) (1 mM), iodoacetic acid (1 mM) and phenylglyoxal (5.3 mM). Potassium D-pantoate and NAD protected against inactivation by *p*-chloromercuribenzoate, 5,5′-dithiobis-(2-nitrobenzoic acid) and iodoacetic acid. NAD and D-pantoate also provided substantial protection against inactivation by phenylglyoxal. Titration of the sulphydryl groups by 5,5′-dithiobis(2-nitrobenzoic acid) and incorporation of [^{14}C]carboxymethyl revealed that there are two cysteine residues which are modified and one of those is essential for activity. In the presence of NAD and D-pantoate, incorporation of [^{14}C]phenylglyoxal was decreased by 0.42 mol/mol of subunit.

Introduction

Pantoate dehydrogenase (D-pantoate:NAD^+ 4-oxidoreductase, EC 1.1.1.106) has been partially purified from *Pseudomonas* P-2 [1] and *P. fluorescens* UK-1 [2]. The enzyme is rather unstable without 2-mercaptoethanol. NAD stabilizes it to a certain extent. At pH values below 5.5 and above 10.0 the purified enzyme is unstable, even in the presence of NAD [1].

In this paper we describe the purification of pantoate dehydrogenase to homogeneity and the modification of cysteine and arginine residues of the enzyme by group specific reagents.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanosulphonic acid.

Experimental

Materials. N^6-(6-Aminohexyl)5′AMP-Sepharose 4B was purchased from Pharmacia, Sweden. *p*-Mercuribenzoate and phenylglyoxal monohydrate were obtained from Aldrich, Belgium. 5,5′-Dithiobis(2-nitrobenzoic acid) and *S*-carboxymethyl-L-cysteine were from Sigma, St. Louis, MO, U.S.A. Iodo-[1-^{14}C]acetic acid (2.8 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks, U.K. [*carbonyl*-^{14}C]Acetophenone was obtained from New England Nuclear Corp., Boston, MA, U.S.A. [^{14}C]Phenylglyoxal was prepared by the method of Riley and Gray [3]. The specific activity was 0.11 mCi/mmol.

Purification of pantoate dehydrogenase. Crude cell extract was prepared from *P. fluorescens* UK-1 (2.5 g wet wt.) and pantoate dehydrogenase precipitated with $(NH_4)_2SO_4$ as previously described [2]. The precipitate was dissolved in 30 ml of 10 mM potassium phosphate buffer, pH 7.0, and dialyzed overnight against 4 l of the same buffer. The dialyzate was applied to a 5′AMP-Sepharose 4B column (1.2 × 9.0 cm) equilibrated with 10 mM potassium phosphate, pH 7.0. The column was first washed with 0.1 M potassium phosphate, pH 7.5 and then with NAD (0—0.9 mM) plus potassium D-pantoate (0—3.0 mM) gradient in 0.1 M potassium phosphate, pH 7.5. The most active fractions were pooled and dialyzed against 20 mM potassium phosphate, pH 7.5, and stored at −70° C.

Gel electrophoresis. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was performed on a 10% (w/v) gel [4,5].

Inactivation experiments. Pantoate dehydrogenase was inactivated at room temperature in reaction mixtures (0.2 ml) containing 25 mM potassium borate, Hepes or 0.1 M $NaHCO_3$ (pH 7.2—7.6) and 22 μg enzyme protein. The time course of inactivation was measured by removing samples of 0.02 ml at the intervals indicated and diluting 50-fold into the enzyme assay mixture.

Pantoate dehydrogenase was assayed as described earlier [1]. Protein was determined according to the method of Lowry et al. [6].

Measurement of sulphydryl groups. The number of sulphydryl groups of pantoate dehydrogenase was determined by titrating with 5,5′-dithiobis-(2-nitrobenzoic acid) [7]. Exposed SH groups were measured at room temperature in a 1 ml reaction mixture containing 0.1 M sodium phosphate, pH 7.0, 1.5% glycerol, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) and 0.24 mg enzyme protein. The titration of SH groups was measured at 412 nm. When the reaction was complete all of the SH groups were titrated by addition of 0.1% SDS. A freshly prepared cysteine solution was used as a standard.

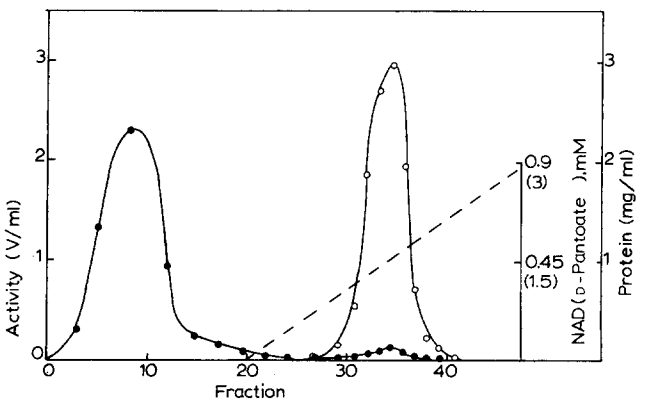
Incorporation of [^{14}C]carboxymethyl and [^{14}C]phenylglyoxal. Pantoate dehydrogenase (0.45 mg) was modified by reaction with 0.36 mM iodo[1-^{14}C]-acetic acid (4830 cpm per nmol) in 20 mM potassium Hepes, pH 7.2, or 25 mM potassium borate, pH 7.6, in a final volume of 0.38 ml. After 1 h incubation at room temperature the reaction was stopped by gel filtration through a 1 × 20 cm Sephadex G-25 column. The column was eluted with 20 mM potassium Hepes, pH 7.2, and the protein fractions were pooled, dialyzed and counted for radioactivity. A portion of the dialyzate was hydrolyzed in 6 M HCl at 110° C in an evacuated tube for 24 h and chromatographed on Whatman No. 1 paper

using *n*-butanol/acetic acid/water (4 : 1 : 5). Incorporation of [^{14}C]phenylglyoxal into pantoate dehydrogenase was determined in the reaction mixtures (1.0 ml) containing 0.45 mg protein/0.8 mM phenylglyoxal (specific radioactivity 0.006 mCi/mmol)/0.1 M $NaHCO_3$, pH 7.6. Samples (0.03 ml) were assayed for pantoate dehydrogenase activity and after inactivation the enzyme was treated as described [8,9].

Results

Purification of pantoate dehydrogenase from P. fluorescens UK-1

It was reported earlier [2] that pantoate dehydrogenase was not completely separated from dimethylmalate dehydrogenase by using gel filtrations and hydroxyapatite chromatography. However, by using 5'AMP-Sepharose 4B these two dehydrogenases were eluted in two separate fractions. As seen in Fig. 1, only a small amount of protein was retained to the 5'AMP-Sepharose column when crude extract precipitated by $(NH_4)_2SO_4$ (0—38% saturation) was applied to the column. Most protein passed through with the washing buffer. A small



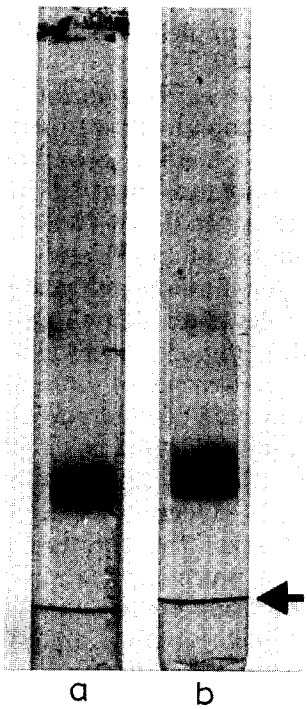


Fig. 1. Purification of pantoate dehydrogenase from *Pseudomonas fluorescens* UK-1 on a 5'AMP-Sepharose 4B column (1.2 X 9.0 cm). Fractions of 3 ml were collected. The column was eluted with 0.1 M potassium phosphate, pH 7.5, and then with a linear NAD (0—0.9 mM) and D-pantoate (0—3.0 mM) gradient. (●———●), absorbance at 280 nm; (○———○), pantoate dehydrogenase activity.

Fig. 2. Polyacrylamide gel electrophoresis in the presence of 0.1% SDS. (A) Pantoate dehydrogenase purified by 5'AMP-Sepharose 4B affinity chromatography; (b), Pantoate dehydrogenase purified by the conventional method [2]. The arrow shows the position of the tracking dye.

single protein peak corresponding to pantoate dehydrogenase activity was eluted by using D-pantoate-NAD gradient (0—3.0 mM and 0—0.9 mM, respectively). All dimethylmalate dehydrogenase activity was in the washing buffer (results not shown). Pantoate dehydrogenase was purified 62-fold by using these two purification steps (Table I). By using the conventional method the enzyme was purified 69-fold [2]. Fig. 2 shows polyacrylamide gel electrophoresis patterns of the two preparations in the presence of 0.1% SDS. As seen, the enzyme purified in the conventional method (b) contains a small impurity corresponding to the dimethylmalate dehydrogenase.

Inactivation of pantoate dehydrogenase by SH inhibitors and by alkylating reagents

p-Chloromercuribenzoate (100 μM) inactivated pantoate dehydrogenase about 85% within 15 min (Fig. 3A). 2-Mercaptoethanol reactivated the inactivated enzyme up to 70% within 10 min. Over the time period necessary to inactivate pantoate dehydrogenase the control did not undergo any loss in activity. Substrates were tested to determine whether they would protect native pantoate dehydrogenase from inactivation by *p*-chloromercuribenzoate. As shown in Fig. 3A, potassium D-pantoate (20 mM) and NAD (1 mM) provided substantial protection against inactivation. When added simultaneously they almost completely prevented inactivation. Iodoacetic acid (1 mM) decreased pantoate dehydrogenase activity by 70% within 50 min at pH 7.2 Fig. 3B). Pantoate (20 mM) decreased inactivation from 70 to 30%. Inactivation of pantoate dehydrogenase by SH inhibitors was pseudo-first order for almost 80% of the reaction. In every case investigated pantoate provided better protection against inactivation than NAD. As shown in Table II pantoate increased the half-inactivation times of pantoate dehydrogenase by 64% in the case of 5,5′-dithiobis(2-nitrobenzoic acid) and by 107% in the case of iodoacetamide salicylate. NAD increased the half-inactivation times by 24% and 32%, respectively. These results, as well as those shown in Fig. 3, suggest that a cysteine residue (or residues) may be involved in the binding of pantoate to pantoate dehydrogenase. However, it is not excluded that the effect is due to prevention of structural distortions through the presence of the substrates.

Titration of sulphydryl residues and incorporation of [^{14}C]carboxymethyl

In order to further investigate the role of cysteine (or cysteines) involved in the activity of pantoate dehydrogenase, 5,5′-dithiobis(2-nitrobenzoic acid) and

TABLE I

PURIFICATION OF PANTOATE DEHYDROGENASE FROM *P. FLUORESCENS* UK-1

1 unit of activity corresponds to the amount of enzyme catalyzing the utilization of 1 μmol NAD per min at 25°C.

Step	Protein (mg)	Total activity (units)	Spec. activity (units/mg)
1. Crude extract	552.0	191.3	0.35
2. $(NH_4)_2SO_4$ precipitate 0—0.38 saturation	60.1	99.7	1.66
3. 5′-AMP-Sepharose 4B	1.1	23.1	21.0

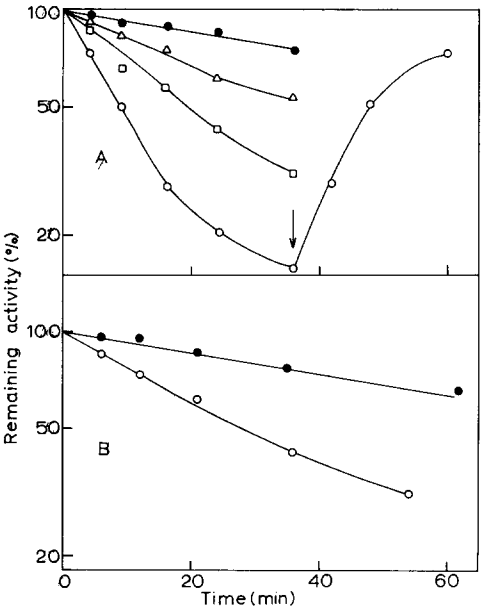


Fig. 3. Inactivation of pantoate dehydrogenase by *p*-chloromercuribenzoate and iodoacetic acid in the presence of substrates. Inactivation was carried out in reaction mixtures (0.2 ml) containing 25 mM potassium Hepes, pH 7.2, 22 μg pantoate dehydrogenase and *p*-chloromercuribenzoate or iodoacetic acid and substrates as indicated. A. 100 μM *p*-chloromercuribenzoate, (○———○) no additions; (□———□) plus 1 mM NAD; (△———△) plus 20 mM D-pantoate; (●———●) plus 1 mM NAD and 20 mM D-pantoate. B. 1 mM iodoacetic acid, (○———○) no additions; (●———●) plus 20 mM D-pantoate. The arrow shows the addition of 0.2 M 2-mercaptoethanol.

iodo[1-^{14}C]acetic acid were used to follow the modification of cysteine residues. The data in Table III show the titration of two exposed and six buried SH groups per native pantoate dehydrogenase. When the titration was carried out in the presence of 25 mM potassium D-pantoate only 1.3 mol of SH groups per mol of pantoate dehydrogenase were titrated with 5,5′-dithiobis(2-nitrobenzoic acid) within the same time period. Almost 2 mol [^{14}C]carboxymethyl

TABLE II

EFFECT OF SUBSTRATES ON INACTIVATION OF PANTOATE DEHYDROGENASE BY 5,5′-DITHIOBIS (2-NITROBENZOIC ACID) AND IODOACETIC ACID

Inactivation experiments were carried out as described in the legend to Fig. 3. τ is the half-inactivation time.

Addition	τ (min)	
	5,5′-Dithiobis (2-nitrobenzoic acid) (1 mM)	Iodoacetic acid (1 mM)
None	4.6	14
D-Pantoate (20 mM)	7.0	29
NAD (1 mM)	5.2	18
D-Pantoate (20 mM) plus NAD (1 mM)	7.3	31

TABLE III

TITRATION OF SH GROUPS WITH 5,5'-DITHIOBIS(2-NITROBENZOIC ACID) AND ALKYLATION WITH IODO[1-^{14}C]ACETIC ACID

Pantoate dehydrogenase (0.24 mg) was titrated with 5,5'-dithiobis(2-nitrobenzoic acid) in the presence and absence of 0.1% SDS and 25 mM D-pantoate. Alkylation of the enzyme (0.45 mg) was carried out in the presence of 0.36 mM iodo[1-^{14}C]acetic acid. Other experimental conditions were as described under Experimental.

Addition	Inactivation (%)	mol SH/mol of enzyme	Incorporation of [^{14}C]carboxymethyl (mol/mol of enzyme)
Experiment I			
None	82	1.93	
D-pantoate (25 mM)	35	1.32	
SDS (0.1%)	—	7.68	
Experiment II			
None	68		1.76
D-pantoate (25 mM)	23		1.18
NAD (1 mM)	—		1.54

per mol of pantoate dehydrogenase were incorporated during 30 min incubation with 0.36 mM iodo[1-^{14}C]acetic acid (Table III). The addition of D-pantoate (25 mM) decreased the incorporation of [^{14}C]carboxymethyl by 0.58 mol/mol of pantoate dehydrogenase. Most radioactivity (68%) was recovered as *S*-carboxymethyl cysteine after gel filtration through a 1.2 × 18 cm column of Sephadex G-25 and after chromatography on Whatman No. 1 paper by using *n*-butanol/acetic acid/water (4 : 1 : 5) of the hydrolyzed ^{14}C-labeled enzyme. The radioactivity corresponded to 1.2 mol of cysteine per mol of pantoate dehydrogenase. In the presence of 20 mM D-pantoate 0.74 mol of *S*-[^{14}C]carboxymethyl cysteine was recovered from the hydrolyzed ^{14}C-labeled enzyme.

Inactivation by phenylglyoxal

When incubated in the borate or $NaHCO_3$ buffers (pH 7.6) in the presence of 5.3 mM phenylglyoxal, pantoate dehydrogenase was inactivated by 70% and 92% within 10 min (Fig. 4). Inactivation was pseudo-first order for the first 70%. Prolonged incubation with phenylglyoxal resulted in complete inactivation of the enzyme. The substrates NAD and D-pantoate protected efficiently, increasing half-inactivation time from 6 min (without substrate) to 40 min and 17 min, respectively. When half-inactivation time was drawn as a function of phenylglyoxal concentration, in the presence and absence of NAD, competitive kinetics were detected (results not shown). Although the apparent K_m for NAD was found to be much lower (0.078 mM) than the K_i for inactivation by phenylglyoxal (0.94 mM), these results suggest that an essential arginine residue is located at or close to the binding site of the nucleotide. The number of arginine residues modified (total number 5.4 residues per subunit [2]) was estimated by incorporation of [^{14}C]phenylglyoxal to the enzyme as described [8]. Extrapolation of the amount of [^{14}C]phenyglyoxal to inactivate completely pantoate dehydrogenase yielded the value 1.40 mol of phenylglyoxal

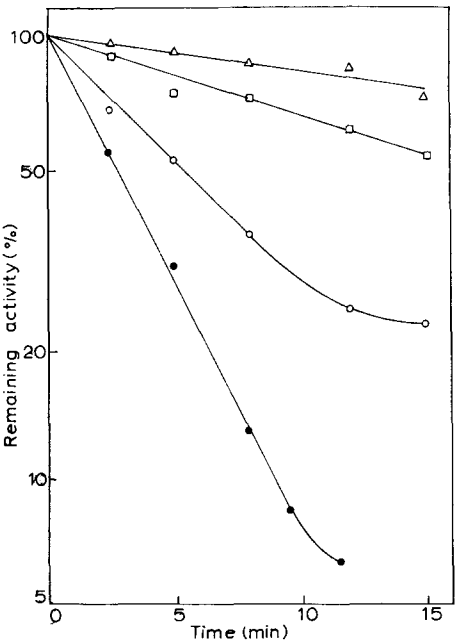


Fig. 4. Inactivation of pantoate dehydrogenase by phenylglyoxal. Experimental conditions were those described in the legend to Fig. 3. (○———○) 5.3 mM phenylglyoxal in 25 mM borate buffer, pH 7.6; (●———●) 5.3 mM phenylglyoxal in 0.1 M $NaHCO_3$, pH 7.6; (△———△) 5.3 mM phenylglyoxal in the borate buffer plus 1 mM NAD; (□———□) 5.3 mM phenylglyoxal in the borate buffer plus 20 mM D-pantoate.

per mol of subunit (40% inactivation gave 0.55 mol of phenylglyoxal per mol of subunit). In the presence of 1 mM NAD and 20 mM D-pantoate, incorporation of 0.26 equivalent of phenylglyoxal accompanied the 27% inactivation of pantoate dehydrogenase. Extrapolation to 100% yields the value 0.98 mol of phenylglyoxal per mol of subunit.

Discussion

Covalent attachment of radioactive iodoacetic acid to the enzyme may allow determination of some modified amino acid residues. In most of the cases examined, alkylation of cysteine residues appears to result. It is important that this modification is not random, but that loss of enzymatic activity is accompanied by covalent binding of one equivalent of reagent per one equivalent of enzyme subunit. Less than 0.5 mol of [^{14}C]carboxymethyl per subunit was incorporated when the enzyme was inactivated by 68% (Table III). The same residue (or residues) were probably alkylated with iodoacetic acid or titrated with 5,5′-dithiobis(2-nitrobenzoic acid), because no reaction with 5,5′-dithiobis(2-nitrobenzoic acid) was detected after complete inactivation of pantoate dehydrogenase by iodoacetic acid. Furthermore, D-pantoate prevented the titration of about the same amount of sulphydryl groups per mol of pantoate dehydrogenase (0.61 mol, 82% inactivation) as it prevented incorporation of [^{14}C]carboxymethyl per mol of pantoate dehydrogenase (0.58 mol, 68% inactivation) (Table III).

Phenylglyoxal reacts most rapidly with arginine residues. Among other side-chains, only those of lysine and cysteine residues are able to react, but at a much lower rate [10]. Modification of cysteine residues were ruled out by incubating pantoate dehydrogenase first in the presence of 100 μM *p*-chloromercuribenzoate and then in the presence of 5.3 mM phenylglyoxal. A treatment with 0.2 M 2-mercaptoethanol restored 70% of the activity of the *p*-chloromercuribenzoate treated enzyme, but less than 5% of the activity of the enzyme treated first with *p*-chloromercuribenzoate and then with phenylglyoxal. Arginine residues have been reported to be essential for alcohol [11], lactate [12], malate [13] and glutamate [14—16] dehydrogenase activities and for many other enzyme activities [17—23]. Normally modification of several arginine residues occurs during inactivation, but only one or two are protected by the coenzyme [14]. Because of a weak inactivation of pantoate dehydrogenase by 0.8 mM [^{14}C]phenylglyoxal (40% inactivation) it was difficult to estimate the exact number of arginine residues modified during complete inactivation. However, inactivation by phenylglyoxal was pseudo-first order at least for the first 70% of the reaction, and extrapolation to 100%, closely correlates to the amount of essential arginine residues.

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