Effect of 2,4,6-Trinitrobenzenesulfonic Acid and Pyridoxal 5'-Phosphate on Pantoate Dehydrogenase from *Pseudomonas fluorescens* UK-1

PEKKA MÄNTSÄLÄ

Department of Biochemistry, University of Turku, SF-20500 Turku 50, Finland

Soil pseudomonads grown with D-pantothenate or D-pantoate contain an inducible pantoate dehydrogenase (EC 1.1.1.106).[1,2] The enzyme has been partially purified from *Pseudomonas* P-2[1] and to homogeneity from *P. fluorescens* UK-1.[2,3] Pantoate dehydrogenase has a molecular weight of 83 000 and is dissociable to four identical subunits with subunit weight of 24 000.[2] The enzyme contains essential cysteine and arginine residues.[3] The present investigation demonstrates the existence of an essential lysine residue(s) in pantoate dehydrogenase from *P. fluorescens* UK-1.

Inactivation of pantoate dehydrogenase by 2,4,6-trinitrobenzenesulfonic acid and pyridoxal 5'-phosphate. 2,4,6-Trinitrobenzenesulfonic acid is known to react with protein lysine residues, forming yellow trinitrobenzene derivatives. When pantoate dehydrogenase was incubated in the presence of 1.4 mM 2,4,6-trinitrobenzenesulfonic acid there was a rapid decrease in enzyme activity (Fig. 1 A). If reaction with 2,4,6-trinitrobenzenesulfonic acid were to occur at a lysine residue that is essential for catalytic activity, substrate(s) would be expected to protect against inactivation. As seen, D-pantoate (20 mM) provided better protection against inactivation than NAD (1 mM) (Fig. 1 A). After 10 min incubation pantoate decreased inactivation from 65 to 45 % and NAD from 65 to 60 %. As reported earlier,[3] pantoate dehydrogenase was shown to be sensitive to inactivation by sulfhydryl reagents as well. In order to exclude the possibility that inactivation by 2,4,6-trinitrobenzenesulfonic acid is due to the modification of sulfhydryl groups by 2,4,6-trinitrobenzenesulfonic acid, pantoate dehydrogenase was first treated with 100 μM *p*-chloromercuribenzoic acid and then with 1.4 mM 2,4,6-trinitrobenzenesulfonic acid. Addition of 200 mM 2-mercaptoethanol did not restore any pantoate dehydrogenase activity. Addition of 200 mM 2-mercaptoethanol almost completely restored activity of pantoate dehydrogenase treated only with 100 μM *p*-chloromercuribenzoic acid. The same kind of inactivation of pantoate dehydrogenase by pyridoxal 5'-phosphate and protection by D-pantoate but not by NAD was also detected (Fig. 1 B). Formation of Schiff base during inactivation can be determined by reduction of the enzyme-pyridoxal 5'-phosphate complex with $NaBH_4$. After reduction of pantoate dehydrogenase by $NaBH_4$, the absorption spectrum showed a maximum at 328 nm, which is characteristic for ε-aminophosphopyridoxyllysine.

Number of the modified lysine residues. Although the inactivation of pantoate dehydrogenase by 2,4,6-trinitrobenzenesulfonic acid was pseudo-first order for more than 80 % of the reaction, in the presence of 20 mM D-pantoate inactivation was pseudo-first order for about 40 % of the reaction. As shown in Fig. 2, extrapolation of the number of residues modified by 2,4,6-trinitrobenzenesulfonic acid yields the value of 1.15 mol lysine residues/mol of subunit. Pantoate decreased the number of modified residues by about 0.7 mol/mol of subunit. Although 2,4,6-trinitrobenzenesulfonic acid and pyridoxal 5'-phosphate rapidly inactivated pantoate dehydrogenase, only rather high concentrations of D-pantoate provided some protection. These results suggest that a lysyl residue (or residues) may play

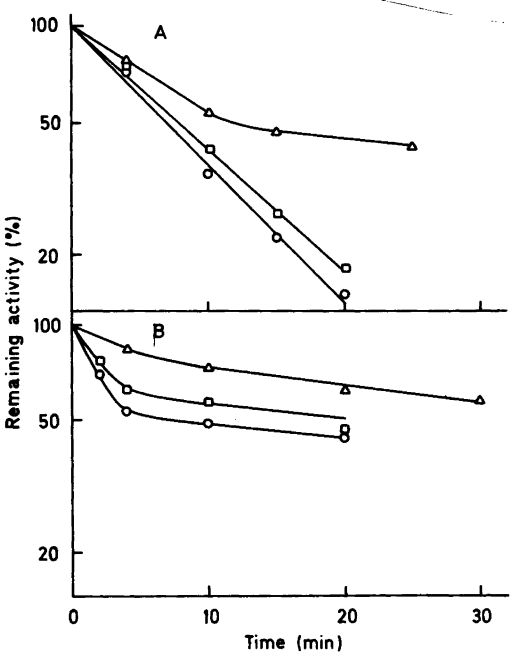

Fig. 1. Inactivation of pantoate dehydrogenase by 2,4,6-trinitrobenzenesulfonic acid and pyridoxal 5'-phosphate. A. (○) 1.4 mM 2,4,6-trinitrobenzenesulfonic acid; (△) 1.4 mM 2,4,6-trinitrobenzenesulfonic acid plus 20 mM D-pantoate; (□) 1.4 mM 2,4,6-trinitrobenzenesulfonic acid plus 1 mM NAD. B. (○) 5 mM pyridoxal 5'-phosphate; (△) 5 mM pyridoxal 5'-phosphate plus 20 mM D-pantoate; (□) 5 mM pyridoxal 5'-phosphate plus 2 mM NAD.

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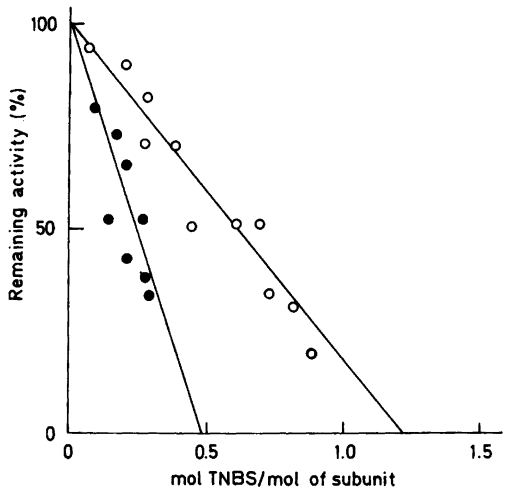

Fig. 2. Amount of lysine residues modified during inactivation by 2,4,6-trinitrobenzenesulfonic acid. The reaction mixture was incubated in the presence of 20 mM D-pantoate (●) or in the absence of D-pantoate (○).

some role in binding pantoate to the enzyme. However, it does not play any direct role in the catalytic process but, more probably, in the orientation of the reagent.

Experimental. Materials. 2,4,6-Trinitrobenzenesulfonic acid and pyridoxal 5'-phosphate were from Sigma, St. Louis, Mo., U.S.A. N^6-(6-Aminohexyl) 5'AMP-Sepharose 4B was from Pharmacia, Sweden. Pantoate dehydrogenase was purified by using a N^6-(6-aminohexyl) 5'AMP-Sepharose 4B affinity column as described previously.[3]

Inactivation experiments. Pantoate dehydrogenase (22 μg) was incubated in a 0.2 ml reaction mixture with 1.4 mM 2,4,6-trinitrobenzenesulfonic acid or 5 mM pyridoxal 5'-phosphate at room temperature. The reaction was conducted in a 25 mM HEPES buffer, pH 7.6. Inactivation was followed by removing samples of 0.03 ml at the intervals indicated and diluting 100-fold into the assay mixture. Pantoate dehydrogenase was assayed as described.[1]

Method of trinitrophenylation. The number of groups modified by 2,4,6-trinitrobenzenesulfonic acid (based on the subunit molecular weight of 24 000 [2]) was determined as described by Coffee *et al.*[4] and Goldin and Frieden.[5] Pantoate dehydrogenase (0.8 mg/ml) was reacted with 0.35 mM 2,4,6-trinitrobenzenesulfonic acid in 25 mM potassium HEPES, pH 7.6. Aliquots were withdrawn and assayed for pantoate dehydrogenase.

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