A NEW POWERFUL INHIBITOR SPECIFIC FOR THE TPN BINDING SITE OF 6-PHOSPHOGLUCONATE DEHYDROGENASE

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1. Introduction

It is known since many years that periodate cleaves the bond between two carbon atoms bearing alcoholic groups. This reaction has been used in the last years also to prepare nucleotide and polynucleotide derivatives to be bound to resins for affinity chromatography [1].

It has been recently reported that, treating TPN with periodate, there is the oxidation to aldehydic groups of the two alcoholic groups present at carbons 2′ and 3′ of the ribose ring bound to the nicotinamide; this periodate oxidized TPN can be bound covalently to a resin to obtain a resin derivative useful for affinity chromatography of dehydrogenases [2].

We have now found that periodate oxidized TPN can be used efficiently as a powerful inhibitor specific for the TPN binding site of 6-phosphogluconate dehydrogenase from *Candida utilis*.

2. Materials and methods

6-phosphogluconate dehydrogenase (6-phospho, D-gluconate:NADP oxidoreductase, decarboxylating, EC 1.1.1.44) crystalline, Type I [3] was prepared from *Candida utilis* as previously described [4]. The crystalline enzyme was dissolved in water and passed through a Sephadex G-25 column, equilibrated with 50 mM phosphate buffer, pH 7.5, containing 1 mM EDTA. One mg of the enzyme used in this work catalyses the oxidation of 40 μmol of 6-phosphogluconate per min at 20°C and pH 8.0.

TPN, TPNH, 6-phosphogluconate (6PG) and DPN were purchased from Boehringer. Ribulose 5-phosphate (Ru5P) was prepared as previously described [5]. Periodate oxidized TPN (PO-TPN) was prepared as described [2] and isolated by alcohol precipitation of the barium salt. The concentration of the PO-TPN was determined by the measure of the absorbance at 260 nm.

The incubation of the enzyme with PO-TPN was carried out at 20°C in 50 mM phosphate buffer, pH 7.5, containing 1 mM EDTA. The enzyme was then diluted in the same buffer and immediately tested for catalytic activity. The enzyme activity was measured at 340 nm for 30 sec, taking readings at 5 sec intervals.

Determination of the dissociation constant of the enzyme–inhibitor complex: the enzyme (1.04 μM) was incubated with 5 different concentrations (from 4.72 to 23.6 μM) of PO-TPN. When the equilibrium was reached, the concentration of the free enzyme was determined by measuring the residual catalytic activity. From this data the concentrations of the enzyme–inhibitor complex and of the free and enzyme bound inhibitor were calculated.

3. Results

3.1. *Kinetics of the enzyme inhibition by periodate oxidized TPN*

The incubation of 6-phosphogluconate dehydrogenase with low concentrations of periodate oxidized TPN (PO-TPN) results in a loss of enzymatic activity. The rate and extent of inhibition are a function of the inhibitor concentration (fig.1). Periodate oxidized DPN, used at higher concentrations (0.6 mM), did not

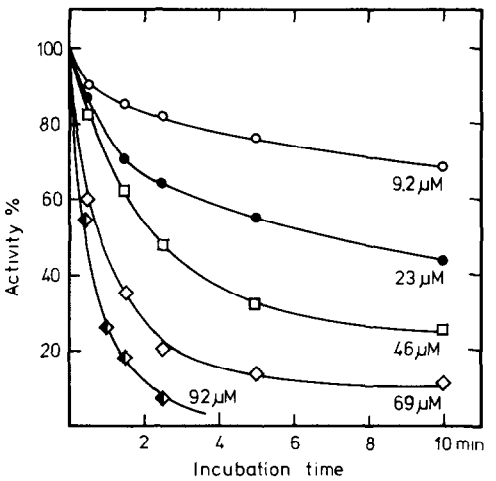

Fig.1. Effect of the concentration of periodate oxidized TPN on the catalytic activity of 6-phosphogluconate dehydrogenase. The enzyme (0.1 mg/ml) was incubated with PO-TPN at the concentrations indicated in the figure. At the time intervals indicated in the abscissa the enzyme was diluted and immediately tested for enzymatic activity.

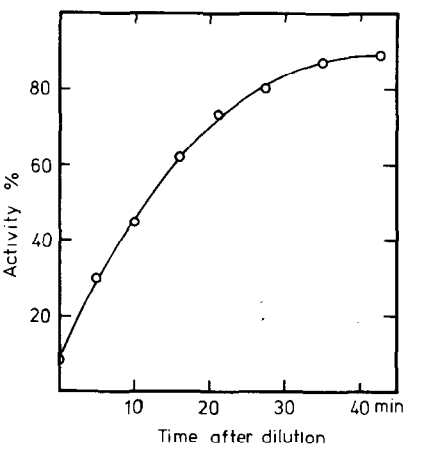

Fig.3. Effect of dilution on the catalytic activity after inhibition. The enzyme (3.3 mg/ml) was incubated with PO-TPN (final concentration 78 μM) in the presence of 1 mM 6-phosphogluconate. After 15 min of incubation the catalytic activity of the enzyme was 8.5% of the original. The enzyme was then diluted 200-fold in 50 mM phosphate buffer, pH 7.5 and tested for enzymatic activity.

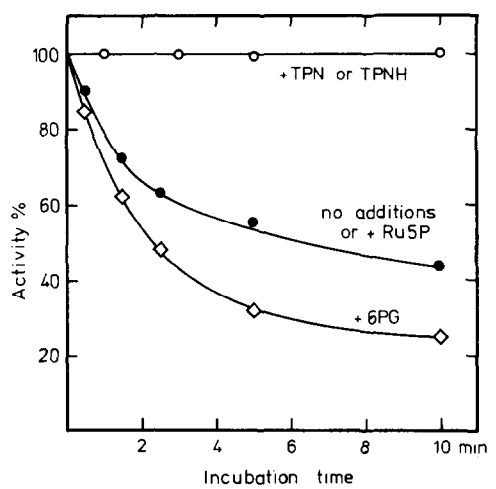

Fig.2. Effect of the presence of the coenzyme and of the substrates on the inhibition by periodate oxidized TPN. The enzyme (0.1 mg/ml) was incubated with 23 μM PO-TPN in the presence of either 1 mM TPN, or 0.12 mM TPNH, or 1 mM ribulose 5-phosphate or 1 mM 6-phosphogluconate.

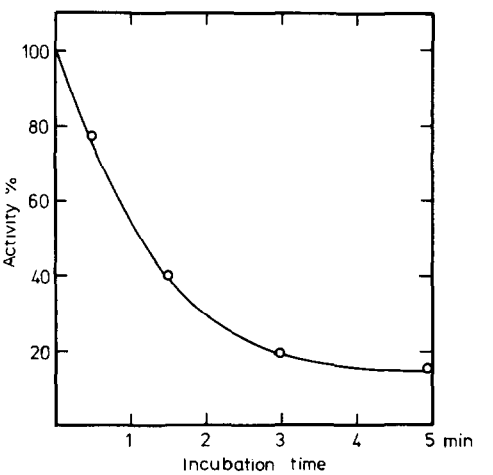

Fig.4. Kinetics of enzyme inactivation by stoicheiometric addition of inhibitor. The enzyme (4.25 mg/ml, corresponding to 85 nmol/ml) was treated with PO-TPN (final concentration 86 nmol/ml); at the time intervals indicated in the abscissa the enzyme was diluted and immediately assayed for catalytic activity.

produce any appreciable inhibition. The dissociation constant of the enzyme–inhibitor complex is 3 ± 0.4 μM.

The presence in the incubation mixture of either TPN or TPNH fully protects the enzyme against inhibition (fig.2). While ribulose 5-phosphate has no effect on the inhibition rate, the 6-phosphogluconate increases the rate of inhibition (fig.2).

The enzyme inhibited by incubation with PO-TPN, fully recovers, upon dilution, its original enzymatic activity (fig.3).

3.2. *Stoicheiometry of the inhibition*

The enzyme treated with an equimolecular amount of inhibitor (1 mole of PO-TPN for each sub-unit mole) looses the 85% of its activity, according a second order kinetics (fig.4). The second order rate constant is 1.4×10^2 M^{-1} sec^{-1}.

The titration of the enzyme with the inhibitor is reported in fig.5. Extrapolating the line obtained, it appears that a complete loss of activity requires the binding of only one mole of inhibitor to each sub-unit mole.

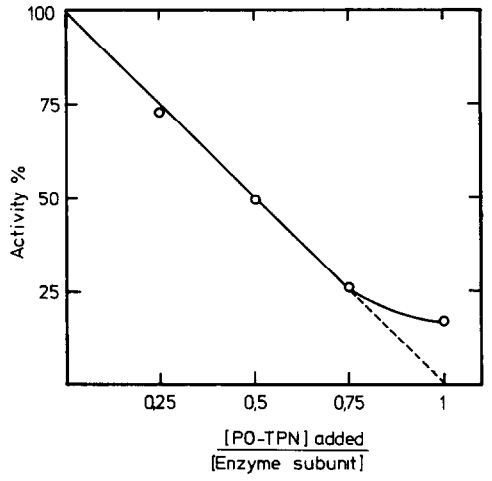

Fig.5. Titration of the enzyme with the inhibitor. Four 0.5 ml aliquots of enzyme (3.8 mg/ml) were treated with 4 different amounts of inhibitor. After 30 min of incubation the enzyme was diluted and tested for catalytic activity.

4. Discussion

The incubation of 6-phosphogluconate dehydrogenase with periodate oxidized TPN results in a reversible loss of the catalytic activity of the enzyme. From our experiments it appears that the inhibitor binds specifically at the TPN binding site of the enzyme; indeed: a) the natural coenzyme, both in its oxidized or reduced form, fully protects the enzyme against inhibition; b) the substrate 6-phosphogluconate specifically enhances the rate of inhibition; c) the addition of only one mole of inhibitor to each sub-unit mole results in the almost complete inhibition; d) periodate oxidized DPN does not cause inhibition of the enzyme.

The inhibition can be reversed by dilution, indicating that, upon inhibition, there is not an irreversible damage of the protein, and that there is an equilibrium between free and protein-bound inhibitor.

We do not know yet neither the precise mode of binding of the inhibitor to the enzyme, nor the nature of the amino acid residue involved in the binding; these problems are now under active investigation.

Preliminary experiments carried out in this laboratory indicate that other periodate oxidized mono- and dinucleotides have an inhibitory effect on other enzymes that require mono and dinucleotides as substrate, cofactor or effector.

Acknowledgements

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