A ROLE FOR THE PYRIDINE NITROGEN OF REDUCED TRIPHOSPHOPYRIDINENUCLEOTIDE IN AN ENZYMATIC CATALYSIS

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1. Introduction

The 6-phosphogluconate dehydrogenase from *Candida utilis* catalyses the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate, the oxidation of 2-deoxy, 6-phosphogluconate to 3-keto, 2-deoxy, 6-phosphogluconate [1], the decarboxylation of this last product [1] and a tritium exchange reaction between tritiated ribulose 5-phosphate and water [2]. The last two of these four reactions require the presence of 1,4 TPNH, which has not, in these reactions, a redox role [2, 3].

In order to investigate the role of the reduced coenzyme in the tritium exchange reaction, we have studied this reaction substituting for the natural reduced coenzyme two of its analogues devoid of enzymatic redox power. Using tetrahydropyridine TPN, both the pH curve and the pK of the group involved in the binding of the cofactor to the enzyme are shifted toward lower pH values. These findings are interpreted to be due to the different basicity of the pyridine nitrogens of the cofactors.

2. Materials and methods

6-Phosphogluconate dehydrogenase, Type I, was prepared from *Candida utilis* as previously described [4]. One mg of the enzyme used in this work catalysed at pH 8.0 and 20°C the oxidation of 28 μmoles of 6-phosphogluconate per min.

6-Phosphogluconate, TPN and 1,4 TPNH were purchased from Boehringer, Germany. [^{3}H]ribulose 5-phosphate (Ru5P) (66 000 cpm/μmole) and 1,6 TPNH were prepared as previously reported [3]. 1,4,5,6, Tetrahydropyridine, triphosphopyridine-nucleotide (H_4TPN) was prepared by catalytic reduction [5] of TPN, purified by column chromatography on DEAE-cellulose and found to be free of 1,4 TPNH (by assay with pyruvate and lactate dehydrogenase) and TPN (by assay with 6-phosphogluconate and 6-phosphogluconate dehydrogenase).

2.1. *Detritiation assay*

Each ml of the reaction mixture contained: 0.1 μmole of [^{3}H]Ru5P, 0.028 mg (0.79 I.U.) of 6-phosphogluconate dehydrogenase, 0.1 mM β-mercaptoethanol, 1.0 mM EDTA and either 1,4 TPNH or 1,6 TPNH or H_4TPN at five different concentrations. The buffer used in these experiments was 10 mM in each of acetate, imidazole and Tris. After 20 min (when either 1,4 TPNH or 1,6 TPNH were used) or 60 min (when H_4TPN was used) of incubation at 37°C, 0.5 ml of each incubation mixture was passed through a small (0.3 × 2 cm) column of Dowex 1-Cl resin to absorb the Ru5P. The resin was then washed with 1.5 ml of distilled water and 50 μl of the pooled non absorbed material were diluted in the Bray's solution [6] and counted in a Packard liquid scintillation counter. The radioactivity values were then transformed into specific activity, i.e. μmoles of Ru5P detritiated in one min by 1 mg of enzyme at 37°C. Due corrections were made for the small inactivation of the enzyme (at the extreme pH values) and for the very small release of tritium from Ru5P when either 1,4 TPNH or its analogues were omitted from the incubation mixture.

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3. Results and discussion

The enzyme 6-phosphogluconate dehydrogenase catalyses a tritium exchange reaction between [^{3}H]-ribulose 5-phosphate and water [2]. This reaction requires the presence of 1,4 TPNH [2] and we have reported that since the reduced coenzyme can be replaced by 1,6 TPNH, which is devoid of enzymatic redox power, 1,4 TPNH has not, in this reaction, a redox role [3].

We have now found that also 1,4,5,6,tetrahydropyridine TPN (H_4TPN), another analogue of 1,4 TPNH devoid of enzymatic redox power, is active in the tritium exchange reaction and have examined the effects of the pH of the reaction mixture on both the rate of the reaction and on the K_m of the enzyme for 1,4 TPNH, 1,6 TPNH and H_4TPN.

The V_{max} of the tritium exchange reaction in the presence of either 1,4 TPNH or 1,6 TPNH and a pH optimum of 8.0; half maximal rates were obtained at pH 6.3 and at a value higher than 8 (fig. 1A). Substituting 1,4 TPNH with H_4TPN, the pH curve was shifted toward lower pH values; the pH optimum was 7.0 and the half maximal rates were obtained at pH 5.6 and 8.0 (fig. 1B). The maximal rate of detritiation

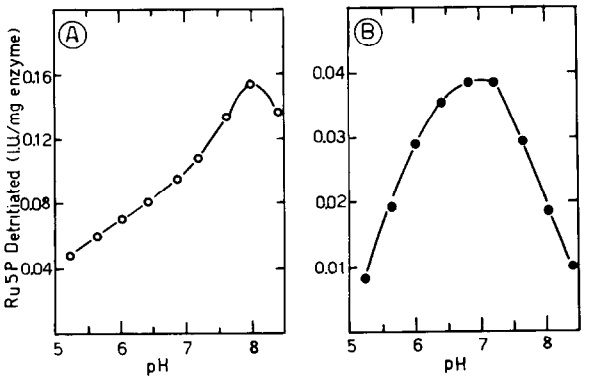

Fig. 1. Effect of the pH of the reaction mixture on the V_{max} of the tritium exchange reaction. The catalytic activity of the enzyme was measured at a fixed Ru5P in the presence pf 5 different concentrations of either 1,4 TPNH of H_4TPN. The data obtained as indicated under Materials and methods were plotted according to Lineweaver and Burk [7] to obtain the value of the V_{max} of the reaction. Ordinate: V_{max} of the tritium exchange reaction in the presence of 1,4 TPNH (fig. 1A) or H_4TPN (fig. 1B) expressed as μmoles of Ru5P detritiated in 1 min by 1 mg of enzyme (specific activity). Using 1,6 TPNH the curve obtained (not reported) was identical to that obtained in the presence of 1,4 TPNH.

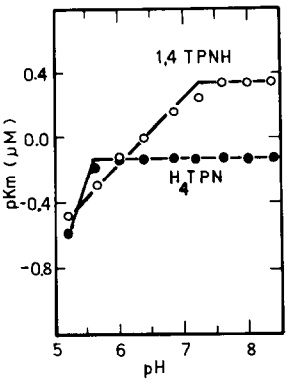

Fig. 2. Effect of the pH of the reaction mixture on the K_m of the enzyme for either 1,4 TPNH or H_4TPN. The K_m of the enzyme for these compounds were obtained from the Lineweaver and Burk plot described in the legend of fig. 1. Ordinate: pK_m of the enzyme for 1,4 TPNH (○—○—○) and for H_4TPN (●—●—●). Using 1,6 TPNH a line (not reported) superimposable to that reported for 1,4 TPNH was obtained.

was found to be one fourth of that obtained in the presence of 1,4 TPNH.

Also the K_m of the enzyme for the three compounds tested is a function of the pH of the reaction mixture. In a plot of the pK_m of the enzyme for either 1,4 TPNH or 1,6 TPNH, as a function of the pH, a line with an inflection point at pH 7.2 was obtained (fig. 2, ○—○—○) suggesting that a group with a pK of 7.2 was involved in the binding of the coenzyme or its analogue to the enzyme. We are unable to offer an explanation of the unusual slope of 0.4 observed in this graph; how-

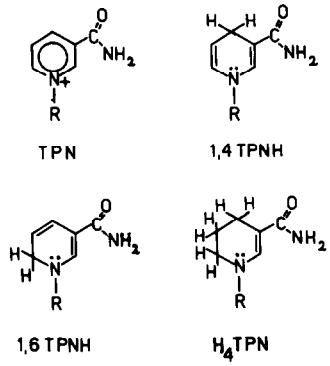

Fig. 3. Formulas of the nicotinamide moiety of TPN, 1,4 TPNH, 1,6 TPNH and H_4TPN. R indicated the residue of the TPN molecule.

ever the same slope was obtained for the pK_m of the enzyme for TPN [8, 9].

Plotting instead the pK_m of the enzyme for H_4TPN as a function of the pH, the line obtained (fig. 2, ●—●—●) has an inflection point at pH 5.6, suggesting that in this case the group involved in the binding of the cofactor had a pK of 5.6. At high pH values, the K_m of the enzyme for H_4TPN is four times higher than that for 1,4 TPNH (1.3×10^{-6} M against 0.46×10^{-6} M).

Summarizing these experimental results, three main observations can be made: a) 1,6 TPNH and H_4TPN are both able to substitute efficiently for 1,4 TPNH in the tritium exchange reaction; b) substituting 1,4 TPNH with H_4TPN there is a shift toward acidity of the pK of two groups involved in the tritium exchange reaction and of the group involved in the binding of the cofactor to the enzyme; c) while 1,4 TPNH and 1,6 TPNH have the same K_m for the enzyme and the same effect on V_{max}, H_4TPN has a higher K_m and gives a lower V_{max}.

These three different phenomena can be explained considering the essential features of the formulas of the pyridine ring of TPN and of the three derivatives used (fig. 3).

Recalling that TPN is inactive in this reaction [2] while the three other compounds are active, it appears that only the presence of an uncharged nitrogen in the pyridine ring of the cofactor is crucial for the tritium exchange reaction, while the number or the position of the hydrogens, the conformation of the ring and the enzymatic redox power are unimportant.

The change of the pK of the groups involved in the reaction and in the binding of the cofactor could be attributed to the different basicity of the nitrogen of the pyridine rings of 1,4 TPNH and H_4TPN. The pyridine rings of 1,4 TPNH and 1,6 TPNH have the same resonance properties and thus their nitrogen should have the same (or almost the same) pK value; instead the pyridine ring of H_4TPN has a lower number of conjugated double bonds, hence a lower resonance and thus the nitrogen of the ring, having a higher tendency to accept a proton, is more basic than that of 1,4 TPNH. Now substituting in the tritium exchange reaction the 1,4 TPNH with H_4TPN, the presence at the active site of the enzyme of a more basic nitrogen could cause a more easy deprotonization of the groups directly involved in both the tritium exchange reaction and in the binding of the cofactor to the enzyme, making them more acidic, as experimentally found.

Considering that the pK of the group involved in the binding of the cofactor is either 7.8 [8] or 7.2 or 5.6 when either TPN or 1,4 TPNH or H_4TPN are respectively involved, it appears that increasing the basicity of the pyridine nitrogen there is a shift toward acidity of the pK of the group involved in the binding.

The decrease of the V_{max} and the increase of the K_m for the cofactor when H_4TPN is used instead of 1,4 TPNH or 1,6 TPNH, could be ascribed to a steric hindrance. While the last two compounds have almost the same steric hindrance, the presence of two additional hydrogens and possibly a modification of the conformation of the pyridine ring could cause, in the active site of the enzyme, a small conformational change which, although allowing the reaction to proceed, results in a higher K_m and a lower V_{max} when H_4TPN is used.

In conclusion, from our results it appears that the nitrogen of the pyridine ring of the reduced coenzyme has a role in both the tritium exchange reaction and in the binding of the coenzyme to the enzyme. It is tempting to postulate an acid base role for this nitrogen, but this hypothesis requires further investigations.

Acknowledgements

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