A ROLE FOR THE PYRIDINE NITROGEN OF REDUCED TRIPHOSPHOPYRIDINE-NUCLEOTIDE IN THE MECHANISM OF ACTION OF ISOCITRATE DEHYDROGENASE

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1. Introduction

The isocitrate dehydrogenase (L_s-isocitrate: NADP oxidoreductase, decarboxylating, EC 1.1.1.42) catalyses, in the presence of TPN, the oxidative decarboxylation of isocitrate to α-ketoglutarate and CO_2. The enzyme catalyses also a tritium exchange reaction between tritiated α-ketoglutarate and water; this reaction requires the presence of 1,4 TPNH [1], but the role of the reduced coenzyme in this reaction is unknown.

Alsot the tritium exchange reaction, catalysed by 6-phosphogluconate dehydrogenase, between tritiated ribulose 5-phosphate and water, requires the presence of 1,4 TPNH [2]. In this case there are indications that the nitrogen of the nicotinamide ring of the reduced coenzyme influences the ionization of amino acid residues involved in both the tritium exchange reaction and in the binding of the coenzyme to the enzyme [3, 4].

In the present paper we report some experiments which indicate that also in the case of isocitrate dehydrogenase the pyridine nitrogen of the reduced coenzyme seems to play a role in the tritium exchange reaction.

2. Materials and methods

Isocitrate dehydrogenase from pig heart was purchased from Boehringer.The specific activity of the enzyme at pH 7.5 was 1.41 μmoles of isocitrate oxidized per min per mg of protein. Isocitrate, α-ketoglutarate, TPN, TPNH, and glutamate dehydrogenase were Boehringer products. Tritiated α-ketoglutarate was prepared as previously described [1] and had a specific radioactivity of 230 cpm/nmole. α-Ketoglutarate concentration was determined spectrophotometrically using glutamate dehydrogenase and TPNH. 1, 4, 5, 6 tetrahydropyridine triphosphopyridinenucleotide (H_4TPN) was prepared as previously described [4].

Detritiation assay. Each ml of the detritiation mixture contained: 3.3 μmoles of tritiated α-ketoglutarate (230 cpm/nmole), 0.25 mg (0.35 I.U.) of isocitrate dehydrogenase, 1 mM EDTA, 10 mM $MgCl_2$ and either 1,4 TPNH or H_4TPN at five different concentrations. The buffer used in these experiments was 10 mM in each acetate, imidazole and Tris. After 20 min (when 1, 4 TPNH was used) or 60 min (when H_4TPN was used) of incubation at 37°C, 0.5 ml of the reaction mixture were passed through a small (0.3 × 2 cm) column of Doxex 1-Cl resin to absorbe the α-ketoglutarate. The resin was then washed with 1.5 ml of quartz distilled water and 50 μl of the non absorbed material were diluted in the Bray solution [5] and counted in a Packard liquid scintillation counter. The radioactivity values were corrected for the small inactivation of the enzyme (at the extreme pH values) and for the very small release of tritium from α-ketoglutarate when either 1, 4 TPNH of H_4TPN were omitted from the incubation mixture. The corrected radioactivity values where then transformed in specific activity, i.e. μmoles of α-ketoglutarate detritiated in one min by one mg of protein at 37°C.

The V_{max} of the tritium exchange reaction and the K_M of the enzyme for either 1, 4 TPNH or H_4TPN were calculated by plotting the data, obtained as indicated above, according to Lineweaver and Burk [6].

3. Results and discussion

The incubation of tritium labelled α-ketoglutarate with isocitrate dehydrogenase and Mg^{2+} ions in the presence of 1, 4 TPNH results in the release of the tritium in the medium. In the absence of the reduced co-

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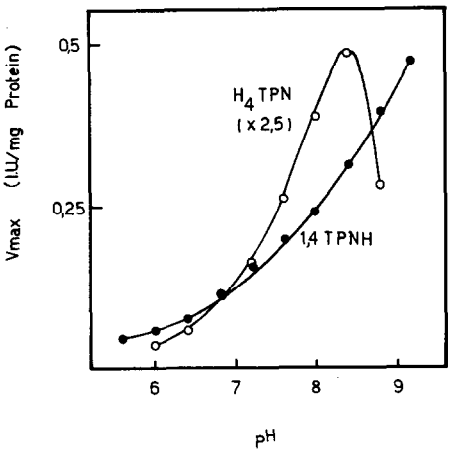

Fig. 1. Effect of the pH of the reaction mixture on the V_{max} of the tritium exchange reaction. The catalytic activity of the enzyme was measured at a fixed α-ketoglutarate concentration in the presence of 5 different concentrations of either 1,4 TPNH or H_4TPN. Abscissa: pH of the reaction mixture. Ordinate: V_{max} of the reaction expressed as specific activity, i.e. μmoles of α-ketoglutarate detritiated in one min by one mg of protein. (0–0–0) in the presence of 1, 4 TPNH; (0–0–0) in the presence of H_4TPN. The values obtained with H_4TPN are multiplied by 2.5.

enzyme, this reaction does not occur [1].

We have now seen that in this reaction the 1, 4 TPNH can be efficiently substituted by 1, 4, 5, 6 tetrahydropyridine triphosphopyridinenucleotide (H_4TPN). Since this last compound is devoid of enzymatic redox power, it appears that the natural reduced coenzyme has not a redox role in the tritium exchange reaction.

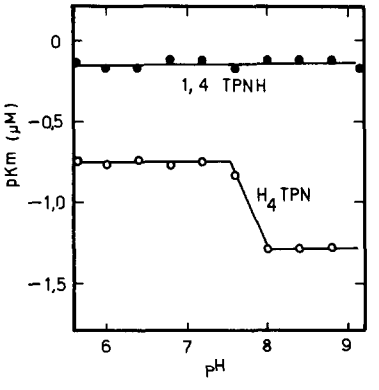

Fig. 2. Effect of the pH of the reaction mixture on the K_M of the isocitrate dehydrogenase for 1, 4 TPNH and for H_4TPN. Abscissa: pH of the reaction mixture; ordinate: pK_M of the enzyme for 1, 4 TPNH(•–•–•) and H_4TPN (o–o–o).

The effect of the pH on the V_{max} of the tritium exchange reaction indicated (fig. 1) that substituting the 1, 4 TPNH with H_4TPN there is a shift toward acidity of the pH curve.

The effect of the pH on the K_M of the enzyme for either 1, 4 TPNH or H_4TPN is reported in fig. 2. It appears that in the pH range examined the pH of the medium does not affect the K_M of the enzyme for 1, 4 TPNH, while affects that for H_4TPN. Indeed the line representing the pK_M of the enzyme for H_4TPN has two inflection points.

These experimental data indicate that substituting in the tritium exchange reaction the natural reduced coenzyme with H_4TPN, the tritium exchange reaction still proceeds, but the pH values of the groups involved in the tritium exchange reaction are decreased, while the pK of the group involved in the binding of the cofactor to the enzyme is modified.

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, could be ascribed to a steric hindrance. The presence in the nicotinamide ring of two additional hydrogens and possibly a modification of the conformation of the nicotinamide ring, could cause in the active site of the enzyme a small conformational change, which, although allows the reaction to proceed, results in a higher K_M and a lower V_{max} when H_4TPN is used.

These same results were obtained studying the tritium exchange reaction catalysed by 6-phosphogluconate dehydrogenase [4]. Recalling that TPN is inactive in these tritium exchange reactions, it appears that in order to be effective in these reactions, the cofactor is required to have the nitrogen of the pyridine ring in the uncharged basic form.

The pK values of the nitrogen of the pyridine ring of these TPN derivatives is still unknown, but organic chemistry suggests that the pyridine nitrogen of the H_4TPN is more basic than that of 1, 4 TPNH.

Introducing now in the active site of the enzyme a more basic nitrogen, the groups directly involved in the tritium exchange reaction (and in the binding of the cofactor to the enzyme) could become more acidic, as experimentally found.

It thus appears that the nitrogen of the pyridine ring of the coenzyme has a so far unsuspected role in the enzymatic catalysis, i.e. influences the ionizations of the amino acid residues involved both in the tritium exchange reaction and in the binding of the coenzyme to

both isocitrate dehydrogenase and 6-phodphogluconate dehydrogenase.

It is known that almost all the pyridine nucleotide dependent dehydrogenases have a higher affinity for the reduced than for the oxidized from of the coenzyme [7]; this difference has been attributed [8] to steric reasons, not paying perhaps due attention to the possibility that the major event occuring during the reduction of the coenzyme could be the change of the pyridine nitrogen from a positively charged to an uncharged basis form. It is tempting now to postulate that this difference in the affinity could be due to the change in the basicity of the nitrogen of the pyridine ring of the coenzyme.

Acknowledgements

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References

[1] Rose, Z.B. (1960) J. Biol. Chem. 235, 928–933.
[2] Lienhard, G.E. and Rose, I.A. (1964) Biochemistry 3, 190–195.
[3] Rippa, M., Signorini, M. and Dallocchio, F. (1972) Biochem. Biophys. Res. Commun 48, 761–768.
[4] Rippa, M., Signorini, M. and Dallocchio, F. (1973) FEBS Letters 36, 148.
[5] Bray, G.A. (1960) Anal. Biochem. 1, 79.
[6] Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 4703.
[7] Colowick, S.P., van Eys, J. and Park, H.J. (1966) (Florkin, M. and Stotz, E.H. eds.) in: Comprehensive Biochemistry, Elsevier, Amsterdam. Vol. 14, p. 1.
[8] Velick, S.F. (1961) (Mc Elroy, W.D. and Glass, H.B. eds.), in: Light and Life, Johns Hopkins Press, Baltimore. p. 108.