A NON-REDOX ROLE OF THE COENZYME IN THE CATALYSIS OF ISOCITRATE DEHYDROGENASE

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1. Introduction

NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42) catalyzes the oxidative decarboxylation of threo-D-isocitrate in two steps:

(1) Dehydrogenation of isocitrate into oxalosuccinate.

(2) Decarboxylation of oxalosuccinate into 2-oxoglutarate.

Our purpose was to determine whether the coenzyme was involved only in the redox step or if it also interfered in other steps of the catalytic cycle.

It is known [1] that mitochondrial isocitrate dehydrogenase catalyzes hydrogen exchange between [^{3}H]2-oxoglutarate and water. This reaction is likely to be the first step in the carboxylation of 2-oxoglutarate. NADPH is required for this reaction without playing a redox role. Rippa et al. indeed have shown that NADPH could be replaced by 1,4,5,6 tetrahydro NADP which is devoid of redox power [2].

It has also been shown that isocitrate dehydrogenase exhibited an autocatalytic behaviour due to activation by NADPH produced during the enzymatic oxidative decarboxylation of isocitrate (results submitted for publication).

In this report, we show that NADPH can be replaced by 1,4,5,6 tetrahydro NADP in the catalytic activation reaction.

2. Materials and methods

2.1. *Materials*

Cytoplasmic isocitrate dehydrogenase from beef liver was prepared as previously described [3]. Isocitrate dehydrogenase from pig heart (2 U/mg) was purchased from Boehringer and purified on a Sephadex A 50 column. DL isocitrate, NADP and NADPH were purchased from Sigma. All solutions were passed through a Chelex 100 column (Bio-Rad) to remove contaminating divalent metal ions.

2.2. *Preparation of 1,4,5,6 tetrahydro NADP*

50 mg of NADPH was dissolved in 3 ml of water. The pH of the solution was adjusted, if necessary, to 8.5 with dilute sodium hydroxyde. The hydrogenation was catalyzed by 10 mg of Pd on charcoal 10% (Fluka). The reaction was allowed to proceed until the *A* 288 nm/*A* 265 nm ratio was 0.9, and then the solution was filtered and lyophilised. The absence of NADPH was checked using glutamate dehydrogenase. All solutions of 1,4,5,6 tetrahydro NADP were freshly prepared each day.

2.3. *Preparation of 2-phosphoadenosine diphosphoribose (ADPRP)*

400 mg NADP were dissolved in 70 ml 0.01 M phosphate buffer pH 7.2. After the addition of 100 mg NAD glycohydrolase from pig brain (Sigma), the cleavage of NADP was followed using glutamate

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dehydrogenase as an enzyme probe. No NADP could be detected after 8 h. ADPRP was purified by elution from a DEAE cellulose column with a gradient from 0 to 0.2 M KCl, pH 7. No traces of ADPR could be detected by electrophoresis, nor traces of $NADP^+$ by electrophoresis or in the presence of cyanide or by enzymatic reduction. Cl^-ions were removed on Biogel P2. About 200 mg ADPRP were recovered.

2.4. *Enzymatic assays*

The enzymatic tests were performed in 0.1 M phosphate buffer, pH 7.5, at room temperature. The reagent concentrations were those indicated in the legends. The reaction was started by adding 10 nM enzyme to the cuvette and was followed at 340 nm using the expanded scale (0–0.100) of a Beckman Acta V spectrophotometer. In experiments, where the pH was varied, 10 mM imidazole (pH 6.4 to 7.5) and 10 mM Tris (pH above 7.5) buffers were used. The enzyme and 1,4,5,6 tetrahydro-NADP were stored in 10 mM phosphate buffer (pH 7).

3. Results

In the absence of divalent metal ions, isocitrate dehydrogenase is subject to a catalytic activation by NADPH produced during the reaction. The rate of the reaction increases from V_0 at zero time to v_{ss} at the steady state. We have previously shown that this behaviour reveals the saturation of an enzyme complex by NADPH wiht a dissociation constant K_{act} of 9 μM. This implies that NADPH dissociation rate from this complex is higher than that of the following step of the catalytic cycle. The value of K_{act} depends competitively on the concentration of NADP:

$$K_{act} = K^{\circ}_{act}\left(1 + \frac{NADP}{K_i}\right)$$ where the intrinsic constant $K^{\circ}_{act} = 0.25\ \mu M$.

1,4,5,6 tetrahydro NADP can replace NADPH in the activation step (fig.1). At zero time the same steady state was reached at saturation as well with 1,4,5,6 tetrahydro NADP as with NADPH. Measurements of the initial rate of the reaction were used

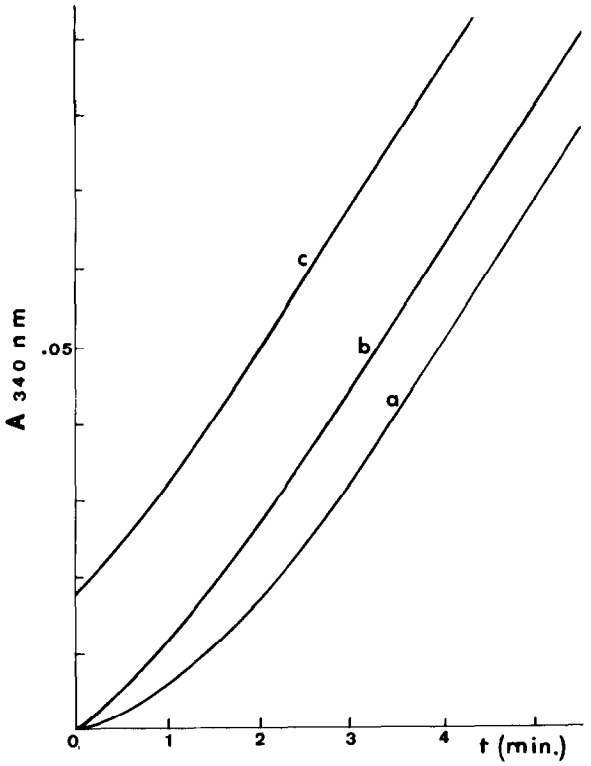


Fig.1. Catalytic activation of isocitrate dehydrogenase. The assay was performed under the following conditions: 0.1 M phosphate buffer pH 7.5, 50 μM $NADP^+$, 8.3 mM DL-isocitrate. The reaction was started by the addition of 10 nM enzyme. (a) No addition of 1,4,5,6 tetrahydro NADP nor NADPH. (b) 1,4,5,6 tetrahydro NADP = 49 μM. (c) NADPH = 3 μM.

to obtain at pH 7.50 the activation constant: 90 μM ± 20 μM of 1,4,5,6 tetrahydro NADP (fig.2). Independently, by competition between NADPH and 1,4,5,6 tetrahydro NADP, we determined by extrapolation the value of 70 μM for the activation constant of 1,4,5,6 tetrahydro NADP, which is in good agreement with the preceding determination (fig.3). It is interesting to note that the affinity of 1,4,5,6 tetrahydro NADP is about ten times lower than that of NADPH whereas its efficiency towards activation is the same as NADPH. These data compare favourably with those concerning the hydrogen exchange reaction [2].

No activation was observed with ADP, ADPRP or NADH, even at high concentrations (1 to 2 mM).

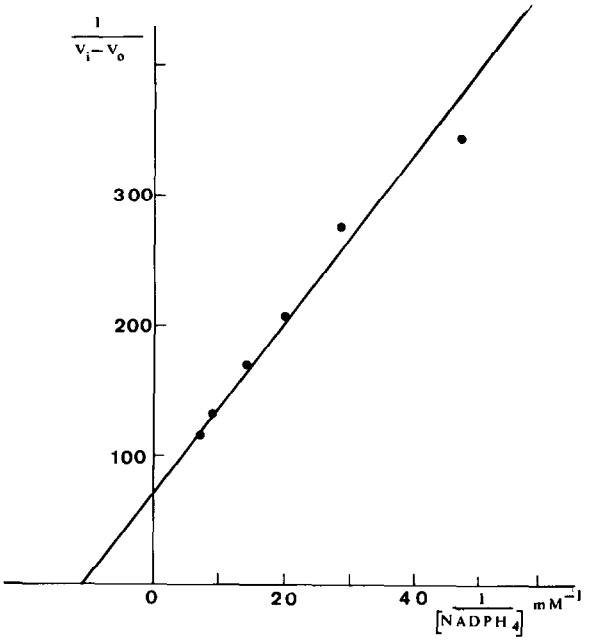


Fig.2. Study of the catalytic activation by 1,4,5,6 tetrahydro NADP. v_i = initial rate of the reaction at different concentrations of 1,4,5,6 tetrahydro NADP. v_0 = initial rate in the absence of 1,4,5,6 tetrahydro NADP. The conditions of the assays are the same as in fig.1.

The pH dependance of activation constant is for NADPH small and monotonous, whereas for 1,4,5,6 tetrahydro NADP two inflexion points at pH 8.2 and 8.4 were detected. Commercial mitochondrial isocitrate dehydrogenase showed the same activation by NADPH and 1,4,5,6 tetrahydro NADP (activation constants 8 μM and 70 μM respectively) and gave pH profiles of K_{act} for NADPH and tetrahydro NADP similar to those obtained with the cytoplasmic enzyme (fig.4).

4. Discussion

The 1,4,5,6 tetrahydro NADP and NADPH can act as activator for both cytoplasmic and mitochondrial isocitrate dehydrogenases. The coenzyme must therefore play a non-redox role in the same step of the overall forward reaction.

The question now arising is: at which step of the catalytic cycle, and in which enzymatic complex is NADPH involved in accelerating the reaction? We already know that NADPH is a competitive inhibitor toward NADP, at the dehydro-

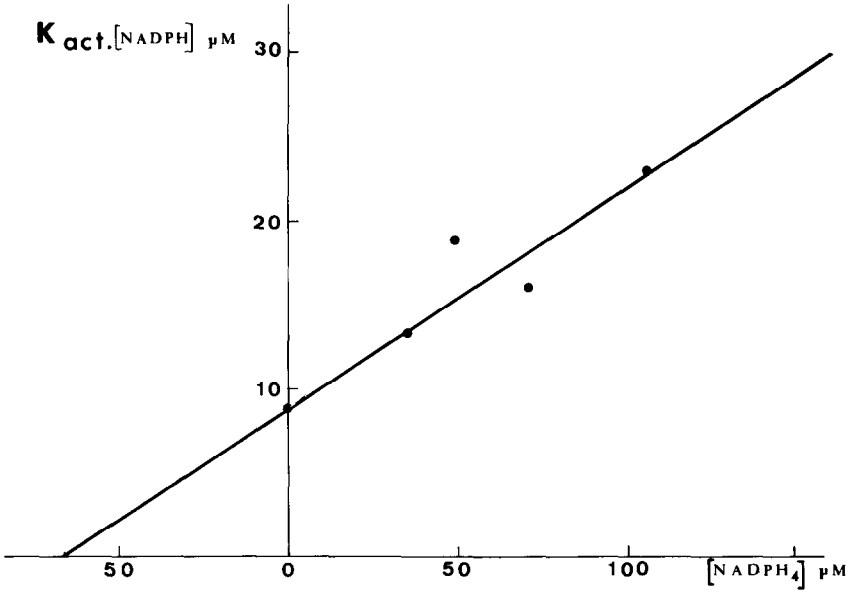


Fig.3. Competition between NADPH and 1,4,5,6 tetrahydro NADP in the activation. The apparent activation constant for NADPH, K_{act}, was determined at different concentrations of 1,4,5,6 tetrahydro NADP. The conditions of the assays are the same as in fig.1.

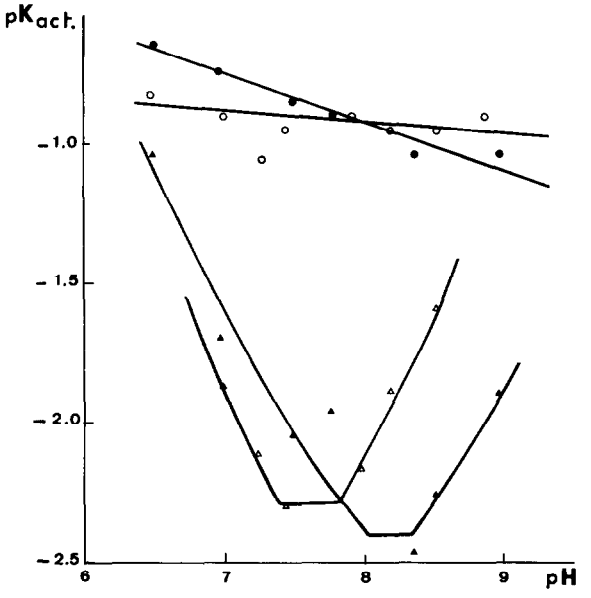
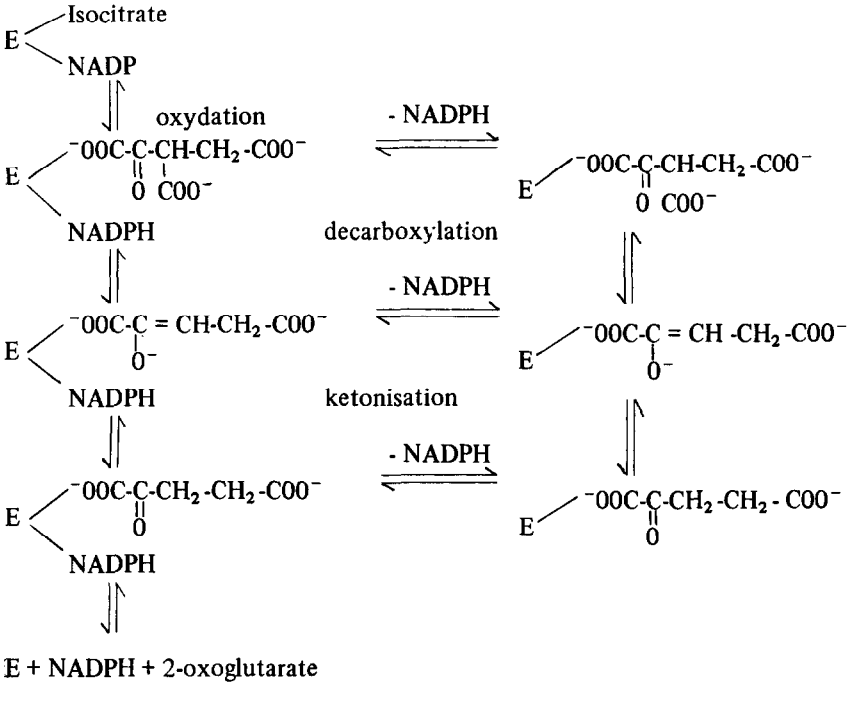


Fig.4. pH profile of the activation constants for NADPH and 1,4,5,6 tetrahydro NADP in isocitrate dehydrogenase catalysed oxidation of isocitric acid. Open symbols: cytoplasmic enzyme. Filled symbols: mitochondrial enzyme. Circle: NADPH. Triangle: 1,4,5,6 tetrahydro NADP.

genating site, with K_i = 35 μM, a much higher value than K_{act}. If, according to Rippa [2], NADPH is necessary in the first step (enolisation) of the reverse reaction, the microreversibility rule tells it must also be involved in the ketonisation step. The activation would then be explained by the existence of a complex similar to that catalyzing the hydrogen exchange of 2-oxoglutarate and water. NADPH and 1,4,5,6 tetrahydro NADP might accelerate the ketone ⇌ enol reaction. The present state of our knowledge of the mechanism of isocitrate dehydrogenase does not allow us to determine whether NADPH is also necessary for the decarboxylation step:



Indeed the direct study of the role of NADPH in the enzymatic decarboxylation is not easy for isocitrate dehydrogenase because of the spontaneous decarboxylation of oxalosuccinate at neutral pH. However, in the case of malic enzyme [4] and 6-phosphogluconate dehydrogenase [5–7], it has been shown that:

(1) The β hydrogen of pyruvate and ribulose 5 phosphate respectively will exchange only in the presence of NADPH and of the related enzyme.

(2) Decarboxylation of the dehydrogenated intermediate catalyzed by 6-phosphogluconate dehydrogenase takes place only in the presence of NADPH or 1,4,5,6 tetrahydro NADP and 6-phosphogluconate dehydrogenase.

Is the dependance of non-redox step on the presence of reduced coenzyme general for all NAD(P) dependent decarboxylating dehydrogenase?

References

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