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https://doi.org/10.5109/23653

出版情報：九州大学大学院農学研究院紀要. 22 (1/2), pp.83-88, 1977-10. Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., 22, 83-88 (1977)

NADP --specific Isocitrate Dehydrogenase from *Bombyx mori*, Order of Substrate Addition and Product Release

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(Received *August* 23, 1977)

The kinetics of the reaction of NADP+-specific isocitrate dehydrogenase was examined by using the enzyme purified from the pupae of silkworm, *Bombyx mori*. The binding of the individual substrates, isocitrate and $NADP^+$, depended upon each other, and was competitive with the reaction products, CO_2, α-ketoglutarate and NADPH. The mechanism appears to be of a rapid equilibrium random type. These findings were very similar to those reported for $NADP^+$-specific isocitrate dehydrogenases isolated from several sources.

INTRODUCTION

An NADP+-specific* isocitrate dehydrogenase (NADP-ICDH) has been isolated from the pupae of silkworm, *Bombyx mori*, and its several properties have been shown in the previous paper (Miake *et al.*, 1977). Moreover, it was reported before (Miake *et al.*, 1976) that high activity of NADP-ICDH was detected at the middle of pupal period in this insect. Earlier studies using NADP-ICDH from several sources (Marr and Weber, 1973; Uhr *et al.*, 1974; Wicken *et al.*, 1972) have brought about the concept that these enzymes catalyze the reaction with a random mechanism. It is interesting whether the pupal enzyme exhibits some specific feature of the mechanism. The present paper describes the kinetic analyses of the enzyme.

MATERIALS AND METHODS

Materials

All the reagents used were of special grade from commercial sources. The sodium salt of *threo*-D_s-isocitrate was obtained from Sigma Chemical Co. $NADP^+$ and NADPH were the products of Boehringer. αKG was purchased from Tokyo Chemical Industries.

Preparation of enzyme

The purification of NADP-ICDH from the pupae of *Bombyx mori* was reported separately (Miake *et al.*, 1977).

* Abbreviations: $NADP^+$, nicotinamide-adenine dinucleotide phosphate, oxidized form; NADPH. as above, reduced form ; NADP-ICDH, NADP+-specific isocitrate dehydrogenase ; αKG, a-ketoglutarate

Enzyme assay

The progress of enzymatically catalyzed reactions was monitored by recording the absorbance at 340 nm of the mixed solution using *a* Hitachi 124 double-beam spectrophotometer equipped with a 0.0-0.1 slide-wide recorder. Initial velocities were evaluated from the slopes of the progress curves within the first 3 min of the reaction. The enzyme solution used contained 0.71 μg of protein dissolved in 10 μl of 0.01 M Tris-HCl buffer (pH 7.0) plus 0.1 M KCI. Reactions were initiated by the addition of enzyme solution to a cuvette of 1-cm light path maintained at 30°C, which contained a total volume of 3.0 ml of 0.1 M Tris-HCl buffer (pH 7.8), 0.5 μmole of *threo*-D_s-isocitrate, 1.0 μmole of $MnCl_2$, 0.5 μmole of $NADP^+$ for the forward reaction (oxidative decarboxylation). For the reverse reaction (reductive carboxylation), the reaction mixture was composed of 0.1 M imidazole-HCl buffer (pH 6.6), 60 μmoles of αKG, 60 μmoles of $NaHCO_3$, 30 μmoles of $MnCl_2$, 0.4 μmole of NADPH and enzyme solution in a total volume of 3.0 ml.

Determination of kinetic constants

The slopes and intercepts of the primary plots in the Lineweaver-Burk plots (Lineweaver and Burk, 1934) or the Dixon plots (Dixon, 1953) were obtained by the weighed linear least-squares method outlined by Cleland (1967) with minor modifications. Every dot in the figures was the average of more than 3 experiments.

RESULTS

Effect of substrate and cofactor concentration

The enzyme exhibited normal Michaelis-Menten kinetics as manifested by straight lines in the Lineweaver-Burk plots (Figs. 1, 2). All the lines intersect-

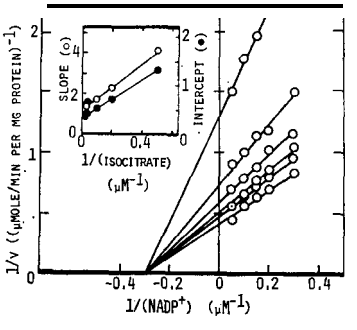

Fig. 1. Double-reciprocal plots of initial velocity versus $NADP^+$ concentration at various levels of isocitrate. The standard assay conditions of the forward reaction were used at the $NADP^+$ concentration varied as shown. The concentration of isocitrate was fixed at 2, 5, 10, 100 or 200 μM (from top to bottom plots in the main graph). Initial velocities were determined from the initial slopes of the reaction progress curves. Inset: replots of slopes and intercepts.

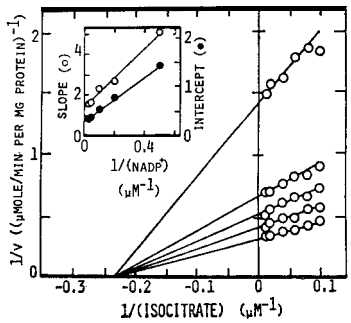

Fig. 2. Double-reciprocal plots of initial velocity versus isocitrate concentration at various levels of $NADP^+$. The standard assay conditions of the forward reaction were used at the isocitrate concentration varied as shown. The concentration of $NADP^+$ was fixed at 2, 5, 10 or 50 μM (from top to bottom plots in the main graph). Initial velocities were determined from the initial slopes of the reaction progress curves. Inset: replots of slopes and intercepts.

ed at a point on the abscissa, thus eliminating a "Ping Pong" mechanism (Cleland, 1963). The apparent Michaelis constants, K_a and K_b (for $NADP^+$ and isocitrate, respectively), could be evaluated as $K_a = 3.83 \pm 0.19$ μM and $K_b = 4.34$ k0.22 μM. The dissociation constant (K_{ia}) was 2.61 FO. 13 μM for $NADP^+$. While, the dissociation constant (K_{ib}) was 0.30 ± 0.20 μM for isocitrate and the maximum velocity (V_f) for the forward reaction was 2.89 ± 0.14 μM/min per mg of protein. These constants gave the following numbers by multiplication :

$$K_{ib} \cdot K_a = 3.30 \times 3.83 = 12.64 \ (\mu M^2)$$
$$K_{ia} \cdot K_b = 2.61 \times 4.49 = 11.76 \ (\mu M^2).$$

Where a and b represent $NADP^+$ and isocitrate, respectively. Each substrate in the forward reaction apparently influenced the binding of the other, although a compulsory sequence was ruled out (Vestling, 1962).

Apparent Michaelis constant for NADPH, αKG and CO_2

Initial velocity patterns in the reverse reaction for the enzyme with NADPH, αKG and CO, were examined. Every double-reciprocal plot was linear at the excess of the other substances. The apparent Michaelis constants were 28.2 ± 1.41 μM for NADPH, 1.2 ± 0.1 mM for αKG and 12.4 ± 0.6 mM for CO_2, respectively. The maximum velocity (V_r) for the reverse reaction was 1.491 0.18 μM/min per mg of protein.

Product inhibition

In order to determine the order of binding of the substrates and release of products, inhibitory effects of various substances were studied. When NADPH was used as a product inhibitor in the forward reaction, linear double-reciprocal plots (and the replots of the slopes, as well as the Dixon plots) indicated that NADPH was competitive *versus* both $NADP^+$ (K_{ir}^a = 36.4 $\pm$ 6.1 μM) and isocitrate ($K_{ir}^b = 25.5 \pm 8.0$ μM) (Figs. 3, 4). When αKG was used as a product

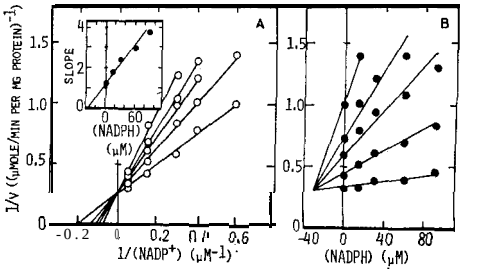

Fig. 3. A : competitive product inhibition obtained at varying concentrations of $NADP^+$ by changing concentrations of NADPH as an inhibitor. NADPH: 90, 60, 30, 15 and 0 μM from top to bottom plots (main graph). Inset: secondary plots of slopes against the concentration of NADPH. B: inhibition of NADP-ICDH by NADPH with respect to $NADP^+$ (Dixon plots).

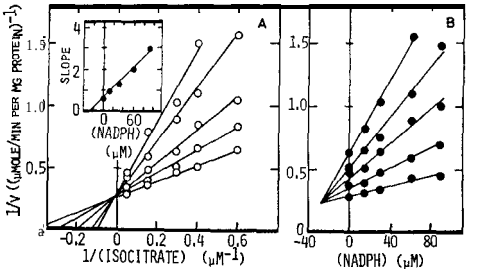

Fig. 4. A : competitive product inhibition obtained at varying concentrations of isocitrate by changing concentrations of NADPH as an inhibitor. NADPH: 90, 60, 30, 15 and 0 μM from top to bottom plots (main graph). Inset: secondary plots of slopes against the concentration of NADPH. B: inhibition of NADP-ICDH by NADPH with respect to isocitrate (Dixon plots).

inhibitor in the forward reaction, it gave also a Iinear competitive inhibition *versus* either $NADP^+$ (K_{iq}^a = 1.7±0.8 mM) or isocitrate (K_{iq}^b=0.25±0.14 mM), resembling those for NADPH (not shown). Product inhibtion by CO_2 was also competitive ***versus*** $NADP^+$ (K_{ip}^a=51.6±11.9 mM) and isocitrate (K_{ip}^b=33.5±15.6 mM), showing similar patterns to those for NADPH (not shown). Where K_{ir}^a and others indicate inhibition constants, in which product a inhibits substrate r and so on. Small letters a, b, p, q and r represent $NADP^+$, isocitrate, CO_2, αKG and NADPH, respectively. In view of the patterns in product inhibition (Figs. 3, 4) and initial velocity (Figs. 1, 2), it was concluded that the forward reaction of NADP-ICDH from the pupa of ***Bombyx mori*** is of a rapid equilibrium random mechanism.

DISCUSSION

The results presented above support a rapid equilibrium random reaction in the forward direction. This type of mechanism has previously been discussed by Cleland (1963), based upon the findings that all of the reaction prod-

ucts are competitive with the substrates. This was also the case with the present enzyme. The dissociation constant for αKG is larger than that for NADPH, suggesting that the products are released in the order of CO_2, αKG and NADPH. This finding is in agreement with the earlier reports (Marr and Weber, 1973 ; Uhr *et al.,* 1974 ; Wicken *et al.,* 1972). On the other hand, Sanwal and Cook (1966) and Sanwal *et al.* (1965) showed that, in the presence of adenosine 5′-monophosphate, the *Neurospora* NAD+-specific isocitrate dehydrogenase converted its reaction mechanism from a random to an ordered addition of substrates, pointing out a change of double-reciprocal plots for NAD+ from linear to nonlinear. This may hold for the present enzyme, since it has been shown that the activity of the enzyme is affected by several metabolites (Miake *et al.,* 1977). The present data about the product inhibition may not explain clearly the order of binding of the substrates ($NADP^+$ and isocitrate), since this type of inhibition, in general, may partly be effected by the accelerated reverse reaction due to the addition of products. The features shown in the present study are consistent with those for the enzymes from other sources (Marr and Weber, 1973; Uhr *et al.,* 1974; Wicken *et al.,* 1972) and no significance has been found for an insect enzyme.

In summary, kinetic analysis of the forward reaction showed that the NADP+-specific isocitrate dehydrogenase from the silkworm reacts in a rapid equilibrium random mechanism, sequentially dissociating CO_2, αKG and NADPH.

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