

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

Miake, Fumio

Laboratory of Sericultural Chemistry, Faculty of Agriculture, Kyushu University

Torikata, Takao

Laboratory of Sericultural Chemistry, Faculty of Agriculture, Kyushu University

Koga, Katsumi

Laboratory of Sericultural Chemistry, Faculty of Agriculture, Kyushu University

Hayashi, Katsuya

Laboratory of Sericultural Chemistry, Faculty of Agriculture, Kyushu University

<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：

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

**Fumio Miake, Takao Torikata, Katsumi Koga
and Katsuya Hayashi**

Laboratory of Sericultural Chemistry, Faculty of Agriculture,
Kyushu University 46-02, Fukuoka 812

(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, CO₂, 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*-D₃-isocitrate was obtained from Sigma Chemical Co. NADP⁺ and NADPH were the products of Boehringer. α KG 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 ; α KG, α -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 KCl. 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*-D₃-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-

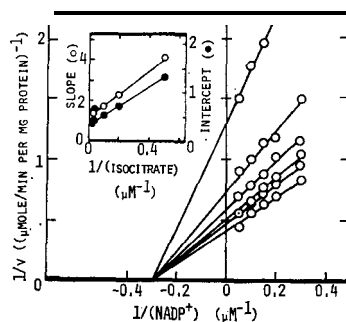


Fig. 1. Double-reciprocal plots of initial velocity versus NADP^+ concentration at various levels of isocitrate. The standard assay conditions of the forward reaction were used at the NADP^+ concentration varied as shown. The concentration of isocitrate was fixed at 2, 5, 10, 100 or 200 μM (from top 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.

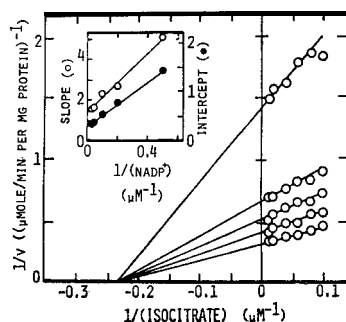


Fig. 2. Double-reciprocal plots of initial velocity versus isocitrate concentration at various levels of NADP^+ . The standard assay conditions of the forward reaction were used at the isocitrate concentration varied as shown. The concentration of NADP^+ was fixed at 2, 5, 10 or $50 \mu\text{M}$ (from 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 $K_a = 3.83 \pm 0.19 \mu\text{M}$ and $K_b = 4.34 \pm 0.22 \mu\text{M}$. The dissociation constant (K_{ia}) was $2.61 \pm 0.13 \mu\text{M}$ for NADP^+ . While, the dissociation constant (K_{ib}) was $0.30 \pm 0.20 \mu\text{M}$ for isocitrate and the maximum velocity (V_r) for the forward reaction was $2.89 \pm 0.14 \mu\text{M}/\text{min}$ per mg of protein. These constants gave the following numbers by multiplication :

$$K_{ib} \cdot K_a = 3.30 \times 3.83 = 12.64 (\mu\text{M}^2)$$

$$K_{ia} \cdot K_b = 2.61 \times 4.49 = 11.76 (\mu\text{M}^2).$$

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_2 , were examined. Every double-reciprocal plot was linear at the excess of the other substances. The apparent Michaelis constants were $28.2 \pm 1.41 \mu\text{M}$ for NADPH, $1.2 \pm 0.1 \text{ mM}$ for αKG and $12.4 \pm 0.6 \text{ mM}$ for CO_2 , respectively. The maximum velocity (V_r) for the reverse reaction was $1.491 \pm 0.18 \mu\text{M}/\text{min}$ 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^+ ($K_{ir}^a = 36.4 \pm 6.1 \mu\text{M}$) and isocitrate ($K_{ir}^b = 25.5 \pm 8.0 \mu\text{M}$) (Figs. 3, 4). When αKG was used as a product

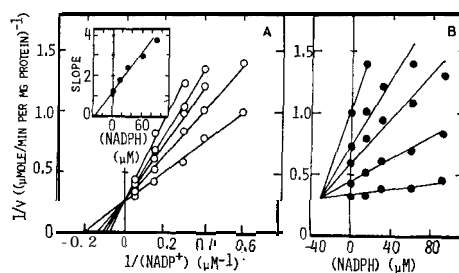


Fig. 3. A : competitive product inhibition obtained at varying concentrations of NADP^+ by changing concentrations of NADPH as an inhibitor. NADPH: 90, 60, 30, 15 and $0 \mu\text{M}$ from top to bottom plots (main graph). Inset: secondary plots of slopes against the concentration of NADPH. B: inhibition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

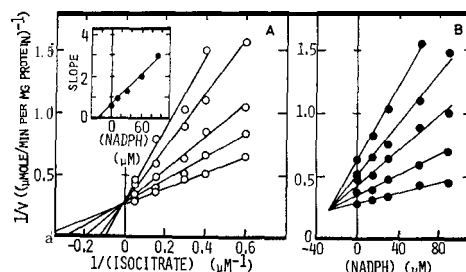


Fig. 4. A : competitive product inhibition obtained at varying concentrations of isocitrate by changing concentrations of NADPH as an inhibitor. NADPH: 90, 60, 30, 15 and $0 \mu\text{M}$ from top to bottom plots (main graph). Inset: secondary plots of slopes against the concentration of NADPH. B: inhibition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

inhibitor in the forward reaction, it gave also a linear competitive inhibition *versus* either NADP^+ ($K_{i_q}^a = 1.7 \pm 0.8 \text{ mM}$) or isocitrate ($K_{i_q}^b = 0.25 \pm 0.14 \text{ mM}$), resembling those for NADPH (not shown). Product inhibition by CO_2 was also competitive *versus* NADP^+ ($K_{i_p}^a = 51.6 \pm 11.9 \text{ mM}$) and isocitrate ($K_{i_p}^b = 33.5 \pm 15.6 \text{ mM}$), showing similar patterns to those for NADPH (not shown). Where $K_{i_r}^a$ 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 α KG is larger than that for NADPH, suggesting that the products are released in the order of CO_2 , α KG 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 , α KG and NADPH.

REFERENCES

- Cleland, W. W. 1963 The kinetics of enzyme-catalyzed reactions with two or more substrates or products. I. Nomenclature and rate equations. *Bioclchim. Biophys. Acta*, 67: 104-137
- Cleland, W. W. 1967 The statistical analysis of enzyme kinetic data. *Adv. Enzymol.*, 29: 1-32
- Dixon, M. 1953 The determination of enzyme inhibitor constants. *Biochem. J.*, 55 : 170-171
- Lineweaver, H. and D. Burk 1934 The determination of enzyme dissociation constants. *J. Am. Chem. Soc.*, 56: 658-666
- Marr, J. J. and M. M. Weber 1973 Kinetic studies of a NADP⁺-specific isocitrate dehydrogenase from *Salmonella typhimurium*. Purification and reaction mechanism. *Arch. Biochem. Biophys.*, 158: 782-791
- Miake, F., T. Torikata, S. Hanada, E. Soeda, K. Koga and K. Hayashi 1976 Developmental changes in activities of several enzymes involved in glycolysis and TCA cycle during the pupal period of *Bombyx mori*. *J. Fac. Agr., Kyushu Univ.*, 20: 1433-149
- Miake, F., T. Torikata, K. Koga and K. Hayashi 1977 Isolation and characterization of NADP⁺-specific isocitrate dehydrogenase from the pupa of *Bombyx mori*. *J. Biochem.*, 82 : 449-454
- Sanwal, B. D. and R. A. Cook 1966 Effect of adenylic acid on the regulatory nicotinamide-adenine dinucleotide-specific isocitrate dehydrogenase. *Biochemistry*, 5 : 886-894
- Sanwal, B. D., C. S. Stachow and R. A. Cook 1965 A kinetic model for the mechanism of allosteric activation of nicotinamide-adenine dinucleotide-specific isocitrate dehydrogenase. *Biochemistry*, 4 : 410-421
- Uhr, M. L., V. W. Thompson and W. W. Cleland 1974 The kinetics of pig heart triphosphopyridine nucleotide-isocitrate dehydrogenase. I. Initial velocity, substrate and

- product inhibition, and isotope exchange studies. *J. Biol. Chem.*, **249**: 2920-2927
- Vestling, C. S. 1962 Determination of dissociation constants for two-substrate enzyme systems. *Methods in Biochemical Analysis*, **10** : 137-173
- Wicken, J. S., A. E. Chung and J. S. Franzen 1972 Isocitrate dehydrogenase from *Azotobacter vinelandii*. Order of substrate addition and product release. *Biochemistry*, **11** : 4766-4778