Steady-State Investigation of the Mechanism of Histidinol Dehydrogenase

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(Received 5 February 1979)

Initial velocities of the histidinol dehydrogenase reaction (EC 1.1.1.23) were measured as a function of the concentrations of the substrates histidinol and NAD^+ and in the presence and absence of the product NADH. The data are consistent with a Bi Uni Uni Bi Ping Pong mechanism. The kinetic constants of this mechanism were determined: K_m for histidinol was found to be $14\mu\text{M}$ and for NAD^+ 0.7mM ; K_i for NAD^+ was 0.4mM .

Histidinol dehydrogenase (EC 1.1.1.23) catalyses the four-electron oxidation of the amino alcohol L-histidinol to the corresponding amino acid L-histidine (Adams, 1954):

The aldehyde histidinal appears to be an intermediate of the reaction, since the enzymes from yeast and *Salmonella typhimurium* will accept histidinal and either oxidize it to histidine in the presence of NAD^+ or reduce it to histidinol in the presence of NADH (Adams, 1955; Loper & Adams, 1965). Although the enzyme will accept the aldehyde as substrate, it is known that the intermediate must be tightly bound to the enzyme during the course of the reaction (Adams, 1954; Loper & Adams, 1965). The present communication reports steady-state kinetic measurements of the histidinol dehydrogenase reaction with the enzyme from *S. typhimurium*.

Materials and Methods

Chemicals

The inorganic chemicals used were of analytical reagent grade. Glycine, succinic acid and Tris were obtained from Merck, Darmstadt, Germany, and DEAE-Sephacrose CL-6B and Sephacryl S-200 were from Pharmacia, Uppsala, Sweden. NAD^+ and NADH were purchased from Boehringer, Mannheim, Germany, and histidinol was from Sigma, St. Louis, MO, U.S.A.

Enzyme isolation

Histidinol dehydrogenase was isolated from *S. typhimurium* strain LT-2 hisO 1242 (Roth *et al.*, 1966) obtained through the courtesy of Dr. Roth, Johns Hopkins University, Baltimore, MD, U.S.A. The organism was grown in a 10-litre laboratory fermentor

with vigorous stirring and an air flow of 10 litres/min at 37°C . The minimal medium of Vogel & Bonner (1956) was supplemented with 0.4% glucose and 0.2% nutrient broth. After 14 h the cells were har-

Fig. 1. Initial-velocity data
 Activities were determined as described in the Materials and Methods section. The reaction mixtures contained 0.15 unit of histidinol dehydrogenase, NAD^+ and various concentrations of histidinol: (a) $5\mu\text{M}$; (b) $10\mu\text{M}$; (c) $15\mu\text{M}$; (d) $30\mu\text{M}$; (e) $45\mu\text{M}$.

vested by centrifugation and stored at -25°C . The purification of the enzyme followed closely the procedure described by Yourno & Ino (1968). Instead of DEAE-Sephadex A50, however, DEAE-Sephacryl S-200 was used. In addition, the enzyme was chromatographed on Sephacryl S-200 eluted with Tris/succinate buffer, pH 6.2, containing 0.1 mM- MnCl_2 and 0.75% NaCl. The purified enzyme showed a specific activity of 12 units/mg of protein and crystallized readily. The highest specific activity reported so far was 11.7 units/mg of protein after several recrystallizations (Yourno & Ino, 1968). Protein was determined by measuring A_{280} and assuming $A_{1\text{cm}}^{1\%} = 4.78$ (Yourno & Ino, 1968).

Kinetic studies

The enzyme activity was measured spectrophotometrically by following the formation of NADH at 340 nm. It is expressed as μmol of histidinol oxidized/min on the basis of 2 equiv. of NAD^+ reduced/ μmol of substrate. Initial rates were measured

by following the reaction continuously in a Zeiss model PMQ III spectrophotometer equipped with a recorder. Temperature was controlled by means of a thermostatically controlled cuvette holder and all assays were conducted at $25 \pm 0.1^{\circ}\text{C}$. One unit of enzyme activity is defined as that amount that converts histidinol to histidine with an initial rate of $1 \mu\text{mol}$ of substrate converted/min at 25°C . The assay mixture of 2 ml contained 0.05 M-glycine/NaOH buffer, pH 9.2, 0.45 mM- MnCl_2 and various concentrations of histidinol and NAD^+ . The enzyme was added to the assay mixture lacking substrate, and the reaction was started 2 min later by the addition of histidinol. The measured velocities were proportional to the enzyme concentration at all concentrations of substrate used. A modification of the photometer allowed the reaction to be followed starting 1.5 s after the addition of histidinol. The initial-rate values shown in the Figures were obtained as means of triplicate measurements. The standard deviations corresponded to 3–6% of the calculated mean rates,

1. Sequential mechanism

2. Ping Pong mechanisms

Scheme 1. Possible mechanisms for the histidinol dehydrogenase reaction

Key: A, L-histidinol; B, NAD^+ ; E and F, stable enzyme forms; P, NADH; Q, L-histidine.

whereby the higher values tended to be associated with the smaller rates at low substrate concentrations.

The concentrations of the NAD^+ and the histidinol solutions were determined enzymically by using histidinol dehydrogenase. A sample of each substrate solution was converted to products in the presence of an excess of the second substrate.

Results and Discussion

Initial rates of the histidinol dehydrogenase reaction were determined as a function of the NAD^+ concentration in the presence of various fixed concentrations of histidinol. Linear double-reciprocal plots were obtained (Fig. 1).

In a mechanistic sense the oxidation of histidinol by 2 equiv. of NAD^+ may be regarded as a reaction with three substrates, with NAD^+ binding twice during the reaction sequence. The participation of water is not considered, since its concentration remains constant. The rate equations of a number of theoretically possible mechanisms were derived. For mechanisms assuming steady-state conditions, the method of King & Altman (1956) was used to derive the rate equations, and the kinetic constants were defined as described by Cleland (1963). In the double-reciprocal form the velocity equations of all possible sequential mechanisms that involve a quaternary complex of enzyme, histidinol and two NAD^+ molecules will show a non-linear dependence on the concentration of NAD^+ . This is true for the Ordered Ter Ter (Scheme 1), partially Random Ter Ter and the completely Random Ter Ter mechanisms. For Random mechanisms under steady-state conditions the double-reciprocal form of the velocity equations will also exhibit a non-linear dependence on the concentration of histidinol, even though it binds only once during the reaction sequence. The velocity equations of the Ping Pong mechanisms in the double-reciprocal form, however, will show a linear dependence on the concentration of both substrates histidinol and NAD^+ . The experimental results shown in Fig. 1 indicate that the reaction of histidinol dehydrogenase may follow a Ping Pong mechanism. Of the three possible Ping Pong mechanisms (Scheme 1) the Hexa Uni Ping Pong case can be eliminated. This mechanism gives a pattern of parallel lines in the Lineweaver-Burk plot, when the initial velocity is studied at variable concentrations of one substrate in the presence of different fixed concentrations of the other. This, however, appears not to be the case for the histidinol dehydrogenase reaction, because the slopes of the double-reciprocal plots do depend on the histidinol concentration, as is shown in Fig. 1. The Bi Uni Uni Bi Ping Pong and the Bi Bi Uni Uni Ping Pong mechanisms both result in a pattern of intersecting straight lines in the absence of products.

If the histidinol dehydrogenase reaction is studied in the presence of NADH, product inhibition is observed. From Fig. 2 it is also apparent that the velocity equation in its double-reciprocal form does not yield a straight line in the presence of NADH if the concentration of NAD^+ is varied. This dependence is exactly what is expected for the Ping Pong mechanisms discussed. NADH establishes a reversible link between the two points where NAD^+ binds in the reaction sequence, resulting in a non-linear dependence on the reciprocal NAD^+ concentration (Cleland, 1970). The linear dependence on the NAD^+ concentration of the velocity equation in the double-reciprocal form in the absence of NADH and the non-linear behaviour in its presence strongly indicate that the histidinol dehydrogenase reaction follows the Bi Uni Uni Bi Ping Pong or the Bi Bi Uni Uni Ping Pong mechanism.

In order to describe the histidinol dehydrogenase reaction by the Bi Bi Uni Uni Ping Pong mechanism, it is necessary to assume that there exists no free

Fig. 2. Initial-velocity data in the presence and absence of NADH

Activities were determined as described in the Materials and Methods section. The reaction mixtures contained 0.13 unit of histidinol dehydrogenase, NAD^+ and $15 \mu\text{M}$ -histidinol. (a) In the absence of NADH; (b) in the presence of $90 \mu\text{M}$ -NADH.

enzyme. Instead the enzyme would have to exist as a complex with NADH as depicted in Scheme 1. Sequences starting with a complex of enzyme and NAD⁺ or histidinol or histidine are also possible. On the basis of the results of Loper & Adams (1965), who showed that the enzyme purified from *S. typhimurium* strain LT-7 does not contain more than 0.06 mol.equiv. of coenzyme, employing an approximate mol.wt of 75000, the Bi Bi Uni Uni Ping Pong mechanism may be safely ruled out. Only the Bi Uni Uni Bi mechanism gives a meaningful

sequence for the association and dissociation steps of substrates and products in the reaction under consideration.

The sequence in which histidinol and the first NAD⁺ molecule bind to the enzyme in the Bi Uni Uni Bi Ping Pong mechanism has not been established, i.e. it is not clear whether histidinol or an NAD⁺ molecule binds first. In the absence of products, both possibilities lead to the same velocity equation. The complete velocity equation of the Bi Uni Uni Bi Ping Pong mechanism as shown in Scheme 1 is given in eqn. (1) in terms of rate constants:

$$\frac{v}{[E]_t} = \frac{k_{+1}k_{+2}k_{+3}k_{+4}k_{+5}k_{+6}[A][B]^2 - k_{-1}k_{-2}k_{-3}k_{-4}k_{-5}k_{-6}[P]^2[Q]}{k_{-1}k_{+4}k_{+5}k_{+6}(k_{+3}+k_{-2})[B] + k_{+2}k_{+3}k_{+4}k_{+5}k_{+6}[B]^2 + k_{+1}k_{+6}(k_{+3}k_{+4}k_{+5} + k_{+4}k_{+5}k_{-2} + k_{+2}k_{+3}k_{+5} + k_{+2}k_{+3}k_{-4})[A][B] + k_{+1}k_{+2}k_{+4}(k_{+5}k_{+6} + k_{+3}k_{+6} + k_{+3}k_{+5})[A][B]^2 + k_{-1}k_{-2}k_{-3}k_{+6}(k_{+5}+k_{-4})[P] + k_{-1}k_{-2}k_{-3}k_{-4}k_{-5}[P]^2 + k_{+1}k_{+6}k_{-2}k_{-3}(k_{+5}+k_{-4})[A][P] + k_{+1}k_{-2}k_{-3}k_{-4}k_{-5}[A][P]^2 + k_{+1}k_{+2}(k_{+5}k_{+6}k_{-3} + k_{+6}k_{-3}k_{-4} + k_{+3}k_{-4}k_{-5})[A][B][P] + k_{+1}k_{+2}k_{+3}k_{+4}k_{-5}[A][B]^2[P] + k_{+1}k_{+2}k_{-3}k_{-4}k_{-5}[A][B][P]^2 + k_{-1}k_{-6}k_{+4}k_{+5}(k_{+3}+k_{-2})[B][Q] + k_{+2}k_{+3}k_{+4}k_{+5}k_{-6}[B]^2[Q] + k_{-1}k_{-6}(k_{+3}k_{-4}k_{-5} + k_{-2}k_{-4}k_{-5} + k_{-2}k_{-3}k_{+5} + k_{-2}k_{-3}k_{-4})[P][Q] + k_{-3}k_{-5}k_{-6}(k_{-2}k_{-4} + k_{-1}k_{-4} + k_{-1}k_{-2})[P]^2[Q] + k_{-5}k_{-6}(k_{+2}k_{+3}k_{-4} + k_{+3}k_{+4}k_{-1} + k_{+4}k_{-1}k_{-2})[B][P][Q] + k_{+2}k_{-3}k_{-4}k_{-5}k_{-6}[B][P]^2[Q] + k_{+2}k_{+3}k_{+4}k_{-5}k_{-6}[B]^2[P][Q]} \quad (1)$$

Fig. 3. Determination of K_m^A and K_m^B

(a) Replot of the ordinate intercepts of Fig. 1 as a function of the reciprocal histidinol concentration. The graph cuts the baseline at a point giving $-1/K_m$ for histidinol. (b) Replot of the ordinate intercepts as a function of the reciprocal NAD⁺ concentration. The data were obtained from a Lineweaver-Burk plot in which the initial velocity was studied as a function of the histidinol concentration at different fixed concentrations of NAD⁺. The graph cuts the baseline at a point giving $-1/K_m$ for NAD⁺.

Eqn. (2) shows the rate equation in the absence of products and in a kinetic constant form:

$$v = \frac{V_{\max} [A][B]}{K_1^B K_m^A + K_m^A [B] + K_m^B [A] + [A][B]} \quad (2)$$

where

$$V_{\max} = \frac{\text{num}_1}{\text{coeff}_{AB2}} = \frac{k_{+3} k_{+5} k_{+6} [E]_t}{(k_{+5} k_{+6} + k_{+3} k_{+6} + k_{+3} k_{+5})}$$

$$K_m^A = \frac{\text{coeff}_{B2}}{\text{coeff}_{AB2}} = \frac{k_{+3} k_{+5} k_{+6}}{k_{+1} (k_{+5} k_{+6} + k_{+3} k_{+6} + k_{+3} k_{+5})}$$

$$K_m^B = \frac{\text{coeff}_{AB}}{\text{coeff}_{AB2}} = \frac{k_{+6} (k_{+3} k_{+4} k_{+5} + k_{+4} k_{+5} k_{-2} + k_{+2} k_{+3} k_{+5} + k_{+2} k_{+3} k_{-4})}{k_{+2} k_{+4} (k_{+5} k_{+6} + k_{+3} k_{+6} + k_{+3} k_{+5})}$$

$$K_1^B = \frac{\text{coeff}_B}{\text{coeff}_{B2}} = \frac{k_{-1} (k_{+3} + k_{-2})}{k_{+2} k_{+3}}$$

The defined kinetic constants are also given. Eqn. (3) is the velocity equation in a coefficient form, demonstrating the non-linear dependence with respect to the NAD^+ concentration in the presence of NADH.

$$\frac{V_{\max}}{v} = \left(1 + \frac{\text{coeff}'_{B2}}{[A]} + \text{coeff}'_{AB2P}[P] + \left(\text{coeff}'_{AB} + \frac{\text{coeff}'_B}{[A]} + \text{coeff}'_{ABP}[P] + \text{coeff}'_{ABP2}[P]^2 \right) \frac{1}{[B]} + \left(\frac{\text{coeff}'_P}{[A]} [P] + \frac{\text{coeff}'_{P2}}{[A]} [P]^2 + \text{coeff}'_{AP}[P] + \text{coeff}'_{AP2}[P]^2 \right) \frac{1}{[B]^2} \right) \quad (3)$$

where

$$V_{\max} = \frac{\text{num}_1}{\text{coeff}_{AB2}}$$

$$\text{coeff}'_x = \frac{\text{coeff}_x}{\text{coeff}_{AB2}} \text{ e.g. } \text{coeff}'_{B2} = \frac{\text{coeff}_{B2}}{\text{coeff}_{AB2}}$$

The kinetic constants defined in eqn. (2) can be determined from secondary replots of the slopes and intercepts of the primary Lineweaver-Burk plots, which are obtained by studying the initial velocity as a function of the NAD^+ concentration in the presence of different fixed concentrations of

Fig. 4. Determination of K_1^B

Replot of the slopes as a function of the reciprocal concentration of NAD^+ . The data were obtained from a Lineweaver-Burk plot in which the initial velocity was studied as a function of the histidinol concentration at different fixed concentrations of NAD^+ . The graph cuts the baseline at a point giving $-1/K_1$ for NAD^+ .

histidinol and vice versa. The three replots of intercepts and slope for the determination of the kinetic constants are shown in Figs. 3 and 4. The Michaelis constants are $14 \mu\text{M}$ and 0.7mM for histidinol and NAD^+ respectively. The inhibition constant for NAD^+ is 0.4mM .

I thank Professor Dr. F. Lingens for his continuous interest in this work and for financial support. I am also grateful to Mrs. I. Beck for skillful technical assistance.

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