Biochimica et Biophysica Acta, 566 (1979) 12—20

BBA 68634

STEADY-STATE KINETICS OF FORMALDEHYDE DEHYDROGENASE AND FORMATE DEHYDROGENASE FROM A METHANOL-UTILIZING YEAST, *CANDIDA BOIDINII*

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(Received June 21st, 1978)

Key words: Formaldehyde dehydrogenase; Formate dehydrogenase; Methanol utilization; (Candida boidinii, Steady-state kinetics)

Summary

Initial velocity studies and product inhibition studies were conducted for the forward and reverse reactions of formaldehyde dehydrogenase (formaldehyde: NAD oxidoreductase, EC 1.2.1.1) isolated from a methanol-utilizing yeast *Candida boidinii*. The data were consistent with an ordered Bi-Bi mechanism for this reaction in which NAD^+ is bound first to the enzyme and NADH released last. Kinetic studies indicated that the nucleoside phosphates ATP, ADP and AMP are competitive inhibitors with respect to NAD and noncompetitive inhibitors with respect to *S*-hydroxymethylglutathione. The inhibitions of the enzyme activity by ATP and ADP are greater at pH 6.0 and 6.5 than at neutral or alkaline pH values.

The kinetic studies of formate dehydrogenase (formate:NAD oxidoreductase, EC 1.2.1.2) from the methanol grown *C. boidinii* suggested also an ordered Bi-Bi mechanism with NAD being the first substrate and NADH the last product. Formate dehydrogenase the last enzyme of the dissimilatory pathway of the methanol metabolism is also inhibited by adenosine phosphates.

Since the intracellular concentrations of NADH and ATP are in the range of the K_i values for formaldehyde dehydrogenase and formate dehydrogenase the activities of these main enzymes of the dissimilatory pathway of methanol metabolism in this yeast may be regulated by these compounds.

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Introduction

Recent studies have shown that in methanol-utilizing yeasts the methanol is successively oxidized in the dissimilatory pathway to carbon dioxide by the inducibly formed enzymes: alcohol oxidase, catalase, formaldehyde dehydrogenase and formate dehydrogenase [1,2]. Since the dissimilatory and assimilatory pathways of the methanol metabolism branch at formaldehyde the possible regulatory mechanism on the two dissimilatory enzymes formaldehyde dehydrogenase and formate dehydrogenase are of great interest.

Formaldehyde dehydrogenase (EC 1.2.1.1) was purified and characterized from several methylotrophic yeasts [3—5] bacteria [6], and from human and animal livers [7—9], human and animal retinas [10] and baker's yeast [11]. Using highly purified formaldehyde dehydrogenase from *Candida boidinii*, *Hansenula polymorpha* and human liver it was demonstrated that the hemimercaptal spontaneously formed between formaldehyde and glutathione is the true substrate of this enzyme and that the reaction product is *S*-formylglutathione [4,5,10].

Formate dehydrogenase (EC 1.2.1.2) was isolated from some methylotrophic yeasts [4,5,12] and bacteria [6,13] and was characterized. Recently, van Dijken et al. [5] reported that the partially purified formate dehydrogenase from *Hansenula polymorpha* has a 40-fold higher affinity for *S*-formylglutathione than for formate. Furthermore, it could be demonstrated that this enzyme hydrolyzes *S*-formylglutathione only in the presence of NAD^+. It is therefore suggested that the hydrolysis of *S*-formylglutathione by the formate dehydrogenase may lead to the formation of enzyme-bound formate which is subsequently oxidized.

The present paper is concerned with steady state kinetics of formaldehyde dehydrogenase and formate dehydrogenase from the methanol-utilizing yeast *Candida boidinii*. This study was undertaken to get a better understanding of the mechanism of control in the metabolism of methanol in this yeast strain.

Materials and Methods

Materials

S-Formylglutathione was synthesized and determined according to van Dijken et al. [5]. All other chemicals used were of the highest purity available from commercial sources.

Enzyme preparation

Formaldehyde dehydrogenase and formate dehydrogenase were purifed from *Candida boidinii* (ATCC 32 195) grown on methanol as described previously [4].

Enzyme assays

Enzyme activities were assayed spectrophotometrically at 340 nm using a Zeiss PM QII photometer equipped with a constant temperature bath set at 30°C ± 0.05°C and a recorder. The assay mixture for the forward reaction of the formaldehyde dehydrogenase was 33 mM sodium phosphate buffer (pH

8.0), 2 mM GSH, 1 mM NAD^+, 1 mM fomaldehyde and about 5 mU/ml enzyme. The assay mixture for the reverse reaction contained 33 mM sodium phosphate buffer (pH 8.0), 0.12 mM NADH, 1 mM *S*-formylglutathione and about 20 mU/ml enzyme. The formate dehydrogenase was measured in a mixture of 50 mM potassium phosphate buffer (pH 7.5), 1.5 mM NAD^+, 150 mM sodium formate and about 5 mU/ml enzyme. For studies of CO_2 inhibition various volumes of a saturated solution of $NaHCO_3$ were added to the reaction buffer. The concentration of dissolved CO_2 was calculated to be 8.4 mM [14]. One unit of enzyme activity is defined as the amount of enzyme required to form 1 μmol NADH/min. The nomenclature and equations used for analysis of the data are those of Cleland [15,16].

Concentration of S-hydroxymethylglutathione

The concentration of *S*-hydroxymethylglutathione was calculated by using the dissociation constant determined to be 1.5 ± 0.3 mM of pH 8.0 by Uotila and Koivusalo [10].

Results

(A) Kinetic studies of formaldehyde dehydrogenase

Initial velocities. Initial velocity studies on the forward reaction of formaldehyde dehydrogenase were performed with respect to NAD^+ at fixed concentrations of *S*-hydroxymethylglutathione. As shown in Fig. 1 the intersecting patterns are consistent with a sequential mechanism. The replots for slopes and intercepts were linear. These data are fitted to the following equation:

$$\frac{1}{v} = \frac{K_b}{V}\left(1 + \frac{K_{ia}}{[A]}\right)\frac{1}{[B]} + \frac{1}{V}\left(1 + \frac{K_a}{[A]}\right) \quad (1)$$

where v is the initial velocity, [A] and [B] are substrate concentrations, V is the maximum velocity and the K values are constants [15]. From the secondary plots, the Michaelis constants for NAD^+ (K_a) and *S*-hydroxymethylglutatione (K_b) are 0.043 and 0.070 mM, respectively, and the dissociation constant for $E \cdot NAD^+$ (K_{ia}) is 0.095 mM.

The results of the initial velocity of the reverse reaction with respect to NADH at fixed concentrations of *S*-formylglutathione are illustrated in Fig. 2. Linear intersecting patterns were obtained which gave also linear replots. The Michaelis constants were calculated to be 0.025 mM for NADH and 0.12 mM for *S*-formylglutatione.

Product inhibition. Product inhibition studies indicated that NADH is a competitive inhibitor with respect to NAD^+ in the presence of *S*-hydroxymethylglutathione at levels approaching saturation and a noncompetitive inhibitor with respect to *S*-hydroxymethylglutathione at unsaturated levels of NAD^+. As shown in Fig. 3, *S*-formylglutathione is a noncompetitive inhibitor with respect to both NAD^+ and *S*-hydroxymethylglutathione. These data are consistent with an ordered Bi-Bi mechanism with NAD^+ being the first substrate and NADH being the last product. Furthermore it was also shown that NADPH is a competitive inhibitor with respect to NAD^+. However, the inhibi-

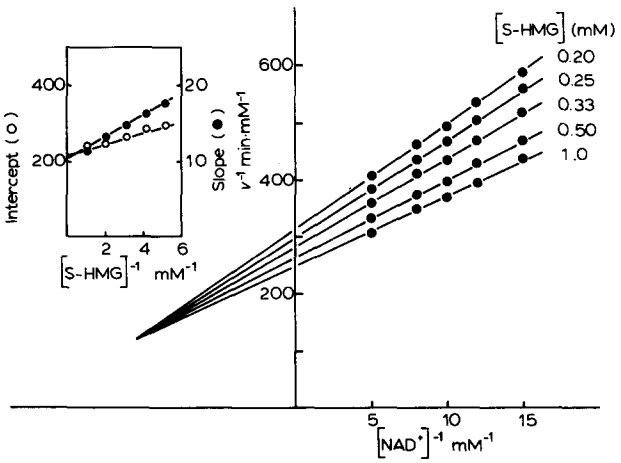

Fig. 1. Double reciprocal plots of the forward reaction of formaldehyde dehydrogenase. Initial velocity versus NAD^+ concentration at various constant levels of *S*-hydroxymethylglutathione (S-HMG). Standard reaction conditions were used described in Materials and Methods. Inset: Replot of the slopes and intercepts versus the reciprocal concentration of *S*-hydroxymethylglutathione.

tion constant was found to be 1.1 mM which is much higher than that of NADH (Table I).

Product inhibition studies were also conducted with NAD^+ for the reverse reaction. With NADH as the varied substrate and *S*-formylglutathione non-saturating, competitive inhibition was observed. When *S*-formylglutathione was the varied substrate inhibition by NAD^+ appeared to be noncompetitive. These data are consitent with those of the forward reaction.

Inhibition by ATP, ADP and AMP. Since in *Candida boidinii* formaldehyde dehydrogenase is a key enzyme of the dissimilatory pathway of the methanol metabolism a possible regulatory effect of ATP, ADP and AMP on the enzyme activity was investigated. Kinetic studies indicated that these nucleoside phos-

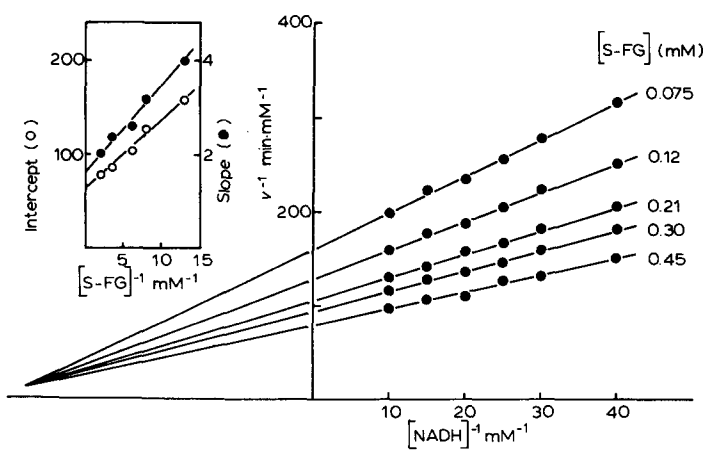

Fig. 2. Double reciprocal plots for the reverse reaction of formaldehyde dehydrogenase. Initial velocity versus NADH concentration at various constant levels of *S*-formylglutathione (S-FG). Inset: Replot of the slopes and intercepts versus the reciprocal concentration of *S*-formylglutathione.

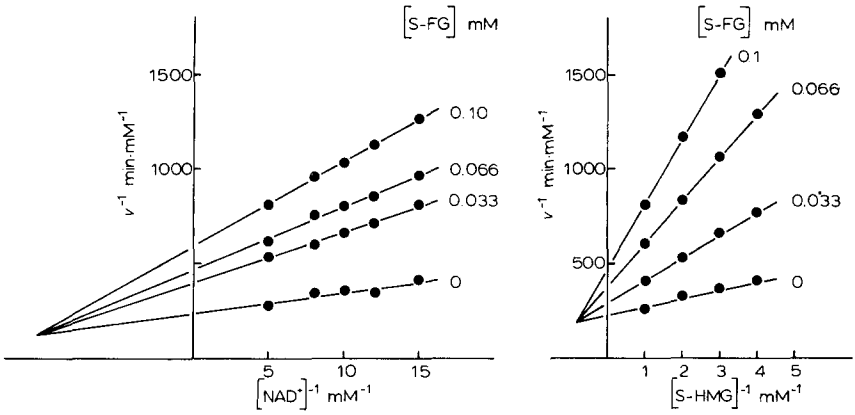

Fig. 3. Double reciprocal plots showing noncompetitive inhibition of formaldehyde dehydrogenase by the product *S*-formylglutathione (S-FG). A, *S*-formylglutathione inhibition at constant *S*-hydroxymethylglutathione concentration of 0.1 mM and varying NAD^+ concentrations. B, *S*-formylglutathione inhibition at constant NAD^+ concentration of 0.1 mM and varying *S*-hydroxymethylglutathione concentrations.

phates are competitive inhibitors with respect to NAD^+ and noncompetitive inhibitors with respect to *S*-hydroxymethylglutathione. As shown in Table II, the K_i value of each nucleoside phosphate is markedly dependent on the pH of the assay system. The inhibition is greater at slightly acidic pH than at neutral or alkaline pH values, the activity of formaldehyde dehydrogenase may be regulated by these nucleoside phosphates.

(B) Kinetic studies of formate dehydrogenase

Initial velocities. Initial velocity studies on the forward reaction of formate dehydrogenase were performed at pH 7.5 with respect to NAD^+ at fixed concentrations of formate. As shown in Fig. 4, intersecting lines in the double

TABLE I

INHIBITION OF FORMALDEHYDE DEHYDROGENASE

Reaction	Inhibitor	Substrate varied	Pattern	K_i (mM)	
				Slope	Intercept
S-Hydroxymethylglutathione oxidation	NADH	NAD^+	c *	0.020	—
	NADH	*S*-Hydroxymethylglutathione	nc **	0.032	0.054
S-Hydroxymethylglutathione oxidation	*S*-Formylglutathione	NAD^+	nc	0.075	0.040
	S-Formylglutathione	*S*-Hydroxymethylglutathione	nc	0.020	0.080
S-Hydroxymethylglutathione oxidation	NADPH	NAD^+	c	1.10	—
S-Formylglutathione reduction	NAD^+	NADH	c	0.050	—
	NAD^+	*S*-Formylglutathione	nc	0.032	0.038
S-Formylglutathione reduction	*S*-Hydroxymethylglutathione	NADH	nc	0.072	0.083
	S-Hydroxymethylglutathione	*S*-Formylglutathione	nc	0.054	0.076

* c: competitive.

** nc: noncompetitive.

TABLE II

INHIBITION OF FORMATE DEHYDROGENASE BY ATP, ADP AND AMP AT DIFFERENT pH VALUES

NAD^+ was the variable substrate (0.05 to 0.20 mM) at fixed concentrations of formate (10, 12.5 and 20 mM).

Inhibitor	K_i at pH (mM)				
	6.0	6.5	7.0	7.5	8.0
ATP	0.070	0.18	0.40	1.8	2.0
ADP	0.15	0.12	0.30	0.75	1.8
AMP	0.40	0.25	0.35	1.3	1.8

reciprocal plots indicate a sequential mechanism for the enzyme reaction and the data are fitted also to Eqn. 1. From the secondary plots, the Michaelis constants for NAD^+ (K_a) and formate (K_b) were calculated to be 0.10 and 16 mM. The dissociation constant for E · NAD^+ (K_{ia}) is 0.25 mM. The reverse reaction of formate dehydrogenase could be demonstrated. However, it was difficult to perform kinetic analysis, since the reaction rate was very low [4].

Product inhibition. Product inhibition studies were conducted with NADH and CO_2. NADH is a competitive inhibitor with respect to NAD^+ in the presence of a saturation level of formate (50 mM) and a noncompetitive inhibitor with respect to formate. However, no inhibition was observed at the saturation level of NAD^+ (1.0 mM) and with various levels of formate and NADH. Inhibition studies with CO_2 were conducted with NAD^+ as the variable substrate and constant formate concentrations (10—30 mM). It was found that under these conditions CO_2 is a noncompetitive inhibitor and the K_i(slope) and K_i(inter.) values were constant over the range of formate concentrations (Table III). With

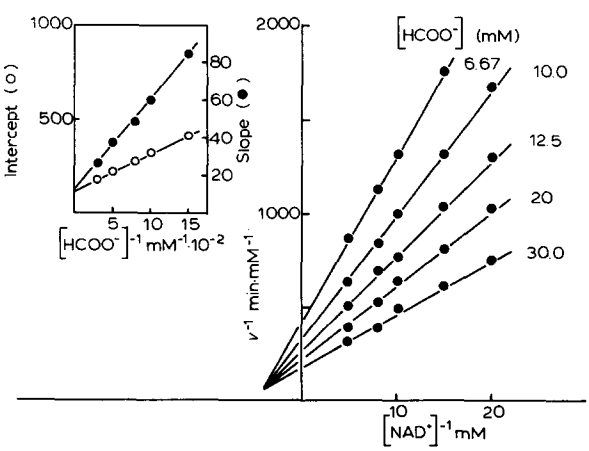

Fig. 4. Double reciprocal plots for formate dehydrogenase. Initial velocity versus NAD^+ concentration at various constant levels of formate. Inset: Replot of the slopes and intercepts versus the reciprocal concentration of formate.

TABLE III

INHIBITION OF THE FORWARD REACTION OF FORMATE DEHYDROGENASE

Inhibitor	Substrate varied	Pattern	K_i (mM)	
			Slope	Intercept
NADH	NAD^+	c *	0.030	—
NADH	Formate	nc **	0.030	0.038
NADPH	NAD^+	c	1.20	—
CO_2	NAD^+	nc	1.56	1.40
CO_2	Formate	nc	1.68	1.70

* c: competitive.
** nc: noncompetitive.

TABLE IV

INHIBITION OF FORMALDEHYDE DEHYDROGENASE BY ATP, ADP AND AMP AT DIFFERENT pH VALUES

NAD^+ was the variable substrate (0.03 to 0.12 mM) at fixed concentrations of *S*-hydroxymethylglutathione (0.067, 0.1 and 0.2 mM).

Inhibitor	K_i at pH (mM)			
	6.0	6.5	7.0	7.5
ATP	0.80	0.85	1.65	3.1
ADP	0.12	0.25	0.40	0.75
AMP	6.0	2.8	4.0	1.6

formate as the variable substrate and NAD^+ concentrations in the range of 0.066 to 0.2 mM noncompetitive inhibition was observed (Table III). The initial velocity studies and product inhibition patterns are consistent with an ordered Bi-Bi mechanism with NAD^+ being the first substrate and NADH the last product.

Inhibition of ATP, ADP and AMP. It was also found that formate dehydrogenase the last enzyme of the dissimilatory pathway of the methanol metabolism in this yeast is inhibited by adenosine phosphates. Kinetic studies indicated that these nucleoside phosphates are competitive inhibitors with respect to NAD^+ and noncompetitive inhibitors with respect to formate. As shown in Table IV, the inhibition by each nucleoside phosphate is also pH dependent. The inhibitions by ATP and ADP are greater at acidic pH than at neutral.

Discussion

The results of the initial velocity and product inhibition studies described in this paper demonstrate that the reaction mechanism of formaldehyde dehydrogenase from *Candida boidinii* is an ordered Bi-Bi one [15,16] in which NAD^+

(A) is bound before *S*-hydroxymethylglutathione (B), and *S*-formylglutathione (P) is released first and then NADH (Q).

(Reaction 1)

Since P is a noncompetitive inhibitor with respect to both A and B in the forward reaction and B is a noncompetitive inhibitor with respect to both P and Q in the reverse reaction, the two other sequential mechanisms for two substrates, the Theorell-Chance and rapid equilibrium random mechanism may be eliminated for this enzyme reaction. Although, unfortunately, no dead-end inhibitor has been found for this enzyme reaction, only the ordered Bi-Bi mechanism of those listed by Cleland [15—17] is compatible with the inhibition patterns.

On a similar basis, the reaction mechanism of formate dehydrogenase from *Candida boidinii* seems also an ordered Bi-Bi mechanism in which NAD^+ (A) is bound first and NADH (Q) is released last. In this case B is for formate and P for CO_2 in reaction 1. For the bacterial formate dehydrogenase, Rodionov et al. [13] have postulated a rapid equilibrium random mechanism in which NADH is a competitive inhibitor with respect to formate. The inhibition pattern is different from that of the yeast enzyme.

The intracellular concentration of NADH and ATP are in the range of the K_i values for formaldehyde dehydrogenase and formate dehydrogenase [18]. Thus the activities of these enzymes may be regulated by the intracellular levels of NADH and ATP during growth of *Candida boidinii* on methanol. Since the K_i values for NADPH are more than 100 times higher than the intracellular concentration of this compound, it seems improbable that under physiological conditions the activities of these enzymes are affected by NADPH.

Acknowledgements

This investigation was supported by grants from the Bundesministerium für Forschung und Technologie der Bundesrepublik Deutschland. We thank Mr. H. Schütte for the purifications of the formaldehyde dehydrogenase and formate dehydrogenase. We gratefully acknowledge the skilled assistance of Mrs. R. Städing.

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