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Effects of Adenosine Triphosphate and Calcium Ions on the Bacterial NADP-Specific Isocitrate Dehydrogenases

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Isocitrate dehydrogenase (ICDH) in Krebs cycle has been considered to be one of the key enzymes in the energy metabolism and in the supply of the primary substances of the cell constituents. Therefore, many investigators have concentrated their attentions upon the regulatory mechanisms of ICDH. It has been established that NAD-specific ICDH in higher organisms, which have both NAD- and NADP-specific ICDH's, is affected by adenine nucleotides (1-3). Recently, NADP-specific ICDH in some lower organisms, which usually have either NADP- or NAD-specific ICDH, was reported to be a regulatory enzyme by adenine nucleotides (4-7). On the other hand, some bacterial NADP-specific ICDH's were not affected by adenine nucleotides (8). These facts suggest that there are heterogeneity among different bacterial NADP-specific ICDH's and different regulatory mechanisms in those enzymes.

In this study, the examinations of the effects of adenine nucleotides and calcium ion on the several bacterial NADP-specific ICDH's were performed. The descriptions of the competitive inhibition with ATP in the *Proteus vulgaris* ICDH and the inhibitions by calcium ion in the *Sarcina lutea* ICDH are presented in this paper. The reagents such as NaCl, LiCl and urea known to cause protein denaturation were found to change the effects of ATP and calcium ion on the enzymes. Some discussion about the interactions between these reagents and the enzyme proteins are also presented.

Materials and Methods

Growth conditions of bacteria. The bacteria used in the experiments

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were grown on the ordinary peptone-broth agar plates in the Roux's bottles at their optimal temperatures: *Proteus vulgaris*, *Escherichia coli* and *Pseudomonas aeruginosa* were grown at 37 C for 18hr; *Acetobacter xylinum* at 30 C for 18hr; and *Sarcina lutea* at 24 C for 72hr.

Preparations of isocitrate dehydrogenase. After washing the cells three times with deionized water, they were suspended in 10 mM Tris-HCl (pH 7.4) and then disrupted by sonic oscillation in a 20 kc Umeda's sonor for 7 min. The supernatant obtained by centrifugation at $11,000 \times g$ for 20 min were used as sonic extracts. To each 20 ml of sonic extracts 1 ml of 1% protamine sulfate was added, and the precipitate was removed immediately by centrifugation at $11,000 \times g$ for 15 min. All procedures were done at 4 C except otherwise stated. The NADP-specific ICDH's were partially purified from protamine-treated extracts by repeated fractionation with ammonium sulfate (*Ps. aeruginosa* and *Acetobacter xylinum*). In the cases of *P. vulgaris*, *E. coli*, *S. lutea* and pig heart muscle, the enzymes were further purified by DEAE-cellulose column chromatography. All the enzymes were specific for NADP⁺ and did not show the NADPH oxidizing activities.

Enzyme assay. ICDH was assayed by determining the initial rate of increase in absorbance at 340 nm with a Hitachi Perkin-Elmer spectrophotometer. The standard reaction mixture contained, in a final volume of 3.0 ml, 33 mM of Tris-HCl buffer (pH 7.4), 0.67 mM of MnCl₂, 0.2 mg of NADP⁺, 0.33 mM of DL-isocitrate and an appropriate amount of enzyme. The reactions were carried out at room temperature (22–25 C). Protein concentration was determined by the method of WARBURG and CHRISTIAN (9).

Chemicals. ATP, ADP, AMP and disodium salt of DL-isocitrate were purchased from Sigma Chemical Co. NADP⁺ was a commercial preparation of Boehringer Mannheim. DEAE-cellulose was obtained from Serva. Other chemicals were all analytical grade.

Results

Effect of ATP on the bacterial NADP-specific ICDH. It has been known that NAD-specific ICDH is an enzyme which is regulated by adenine nucleotides (1–4) and the kinetics of the inhibition by ATP was performed elegantly by ATKINSON *et al.* (10) and SANWAL *et al.* (11, 12). However, NADP-specific ICDH has not been considered to be a regulatory enzyme. Recently, ATP-inhibition on a few bacterial NADP-specific ICDH's were reported by MARR and WEBER (5) and other investigators (6). So, reexaminations were performed on the ATP-inhibitions on the NADP-specific ICDH's from five species of bacteria (Table 1). In these experimental condi-

TABLE 1. Effects of ATP and Ca²⁺ on the isocitrate dehydrogenases from various organisms.

Reaction mixture is described in the text. To the reaction mixtures 670 μM of ATP and CaCl₂ were added independently or simultaneously. Degree of inhibition was expressed as per cent of each the control value.

Enzyme source	Inhibition (%)		
	ATP	Ca ²⁺	ATP+Ca ²⁺
<i>P. vulgaris</i>	30		
<i>S. lutea</i>	0	50	80
<i>E. coli</i>	0	0	0
<i>Ps. aeruginosa</i>	0	0	20
<i>A. xylinum</i>	10	0	15
Pig heart	0	0	0

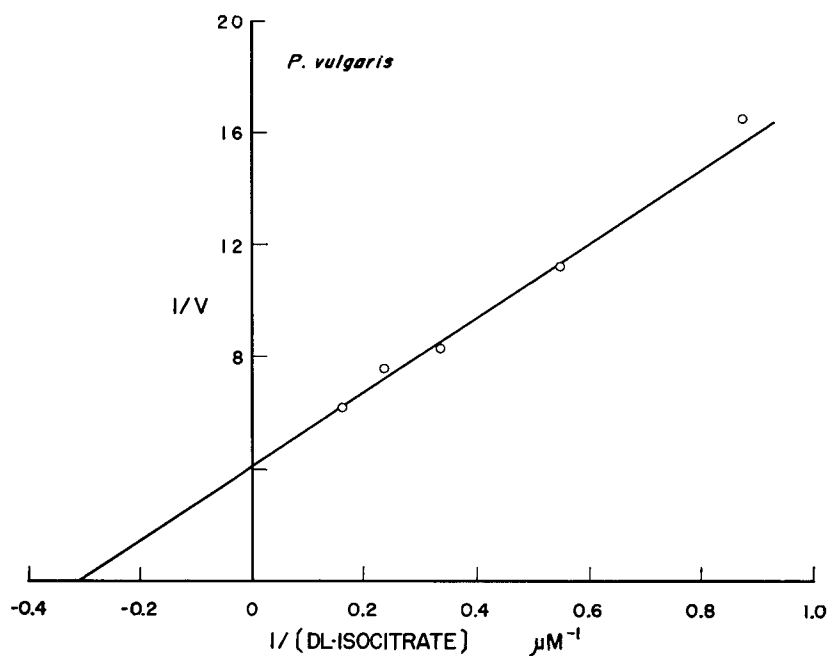


Fig. 1. Km value for isocitrate of the *P. vulgaris* ICDH. Reaction mixture was the same as described in the text, except that the indicated amount of isocitrate was added.

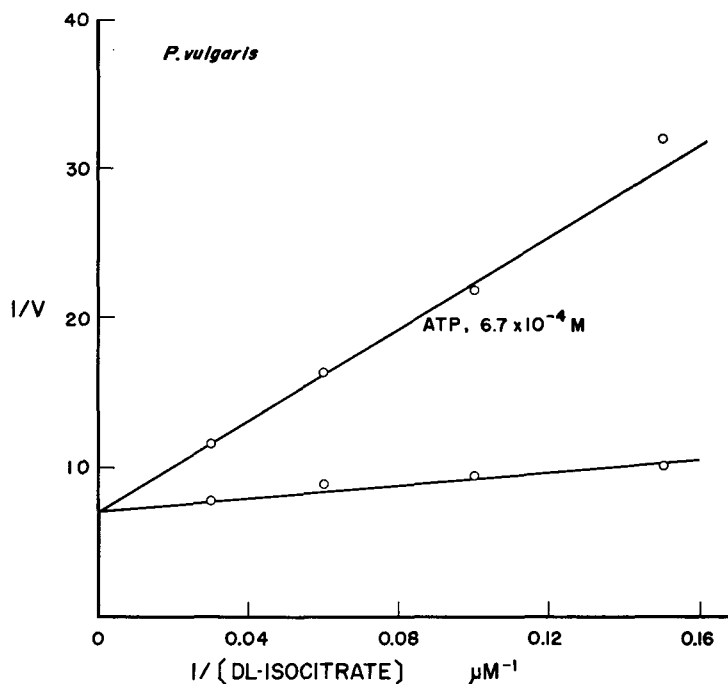


Fig. 2. Inhibition by ATP of the *P. vulgaris* ICDH. Reaction mixture was the same as in Fig. 1, except that the indicated amount of ATP and isocitrate were added.

tions only the *P. vulgaris* enzyme was inhibited significantly by ATP.

Km value for isocitrate of the *P. vulgaris* enzyme was 3.13 μM, the type of the inhibition by ATP was competitive for isocitrate, and Ki value for ATP was 223 μM (Figs. 1 and 2). That any degradation of ATP did not occur during the enzyme assay was confirmed by the determination using paper chromatography.

Effect of calcium ions. The activities of both the NAD- and the NADP-specific ICDH's are stimulated intensely by Mg²⁺ or Mn²⁺, but other bivalent metals have less or no stimulatory effects. The effect of Ca²⁺ on the NADP-specific ICDH is shown in Table 1. When the equimolar concentrations of Ca²⁺ and Mn²⁺ were presented in the reaction mixture, the activities of the *S. lutea* ICDH were inhibited significantly, but the enzymes from other bacteria were not at all. The requirements for Mn²⁺ of the enzymes from *S. lutea*, *E. coli* and *P. vulgaris* were at the same level.

As shown in Fig. 3, the requirement for Mn²⁺ of the *S. lutea* ICDH was increased by addition of Ca²⁺ and the inhibition by Ca²⁺ were disap-

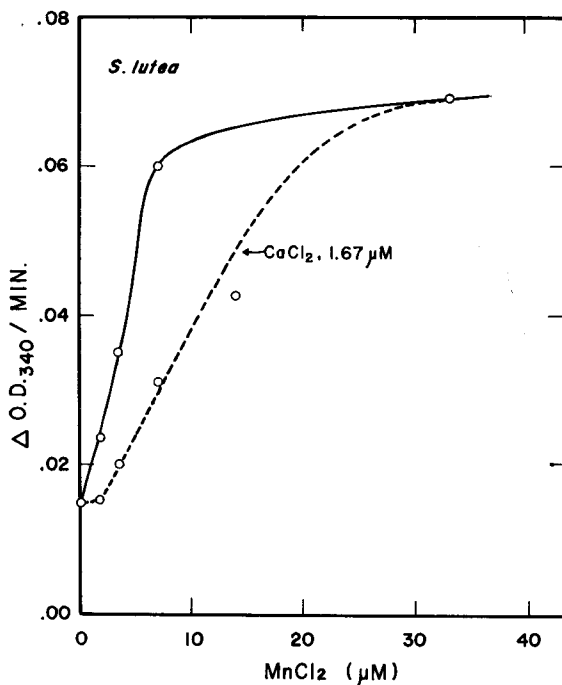


Fig. 3. Requirement for Mn²⁺ of the *S. lutea* ICDH in the presence of Ca²⁺. Reaction mixture was the same as described in the text, except that the indicated amount of MnCl₂ or CaCl₂ was added.

peared when the ratio of Mn²⁺ to Ca²⁺ was more than 20 : 1. Table 2 shows that bivalent metals except Sr²⁺ had no inhibitory effect on the *S. lutea* enzyme. Strontium diminished the enzyme activity to about 30% of the control.

Optimal pH and Km value for isocitrate of the S. lutea ICDH. To clarify the mechanism of Ca²⁺-inhibition of the NADP-specific ICDH of *S. lutea*, some characteristics of this enzyme were investigated. Optimal pH was 7.4. Km value for isocitrate was 3.13 μM that was the same value as that obtained from the *P. vulgaris* enzyme.

Changes of the inhibition degree by Ca²⁺ under different pH. If the competition between Ca²⁺ and Mn²⁺ occur on the same site of the enzyme protein, antagonistic effect of Ca²⁺ to Mn²⁺ would be introduced and analogous responses in Ca²⁺-inhibition and Mn²⁺-activation would be expected by changing the states of enzyme protein under various conditions. So, the changes of the inhibition degree by Ca²⁺ and the activation by enough

amount of Mn^{2+} were estimated under different pH. These results are summarized in Table 3. When the *S. lutea* enzyme were assayed at pH 6.0, dependency on Mn^{2+} was increased and the inhibition by Ca^{2+} was lost. However, when the *E. coli* enzyme was assayed at pH 6.0, the dependency on Mn^{2+} was also lost completely (Table 3). Over the pH ranges of 6.5 to 10 both the degrees of activation by Mn^{2+} and inhibition by Ca^{2+} did not change significantly. When the *S. lutea* enzyme or the *E. coli* enzyme was assayed at neutral pH after the preincubation at pH 6.0 or 5.5 at room temperature (about 25 C) for 30 min, the degree of Ca^{2+} -inhibition and the Mn^{2+} -activation in both the enzymes were reversed to those of the original enzymes.

Effects of NaCl and LiCl. It has been known that the treatment of the enzyme with NaCl or LiCl caused alterations of some char-

TABLE 2. Effects of various bivalent metals on the *S. lutea* NADP-specific isocitrate dehydrogenase.

Reaction mixture was the same as described in the text. To the reaction mixture 670 μ M of the indicated bivalent metal was added. Activities were expressed as per cent of the control.

Metal	Relative activity (%)
None	100
Cd (NO ₃) ₂	109
CoCl ₂	93.2
BaCl ₂	103.5
MgSO ₄	117.2
Sr (NO ₃) ₂	29.6
CaCl ₂	45.3

TABLE 3. Changes of Mn^{2+} -activation and Ca^{2+} -inhibition of the *S. lutea* NADP-specific isocitrate dehydrogenase as a function of pH.

The reactions were performed in 67 mM of different buffers. Mn^{2+} -activation was expressed as per cent of the activity obtained without Mn^{2+} . Ca^{2+} -inhibition was as described in the legend to Table 1.

pH	Mn^{2+} -activation (%)	Ca^{2+} -inhibition (%)
6.0	1,100	0.0
6.5	500	57.1
7.0	400	61.3
7.4	180	65.8
8.5	400	65.0
9.0	320	75.4
9.5	360	84.5
10.0	380	73.1
<i>E. coli</i>		
6.0	0.0	0.0

TABLE 4. Changes of the dependency on Mn²⁺ and inhibition by Ca²⁺ and by ATP of the *S. lutea* isocitrate dehydrogenase.

Reaction mixture was the same as in Table 1. NaCl or EDTA was added to the incubation mixture in a final concentration of 330 mM or 20 μM, respectively. Relative activity was expressed as per cent.

Addition	Relative activity (%)		
	Control	NaCl (330 mM)	EDTA (20 μM)
Mn ²⁺	100	100	100
Mn ²⁺ +Ca ²⁺	73.8	100	56.3
Mn ²⁺ +ATP	100	66.2	78.1
None	48.0	1.3	1.3

acteristics of the enzyme proteins. When the *S. lutea* enzyme was assayed in the presence of 0.33 M NaCl or LiCl, both the loss of the inhibition by Ca²⁺ and the increment of the dependency on Mn²⁺ were observed as in the case at pH 6.0. Further experiments were done whether the loss of the inhibition by Ca²⁺ and the increment of the dependency on Mn²⁺ could be observed simultaneously under other conditions. Addition of 20 μM EDTA in the assay mixture caused the high dependency on Mn²⁺ but the inhibition by Ca²⁺ was still remained (Table 4). Above results suggest that the changes of the degree of the inhibition by Ca²⁺ may be introduced by the modification of the enzyme protein by NaCl or LiCl, and the enzyme have different sites for Ca²⁺ and Mn²⁺. The slight inhibition by ATP alone might be due to its chelating effect on Mn²⁺, though considerable amount of Mn²⁺ was required for the reaction in the presence of NaCl, LiCl and EDTA. When NaCl was removed by dialysis, Ca²⁺-inhibition and Mn²⁺-activation became the same level as the original. Urea had no effect to diminish the Ca²⁺-inhibition.

Discussion

Recently the recognition on the regulation of the NADP-specific ICDH's was renewed by some investigations which concerned with the concerted inhibitions of glyoxylate and oxaloacetate (13-15), the allosteric regulation by folate (16) and the inhibition by ATP (5-7). Although the concerted inhibition has been widely observed in enzymes of different sources, the inhibition by ATP was observed only in bacterial or protozoan enzymes. Furthermore it has been reported that some bacterial NADP-specific ICDH's

were not inhibited by ATP (8). This was confirmed again in our experiments regarding on the ATP-inhibition among five species of bacteria (Table 1). Among the enzymes tested, only the *P. vulgaris* enzyme was inhibited by 670 μ M of ATP. These results may suggest that the different types of NADP-specific ICDH's are obtained from different organisms. It has been known that most bacterial ICDH's are NADP-specific. It is physiologically interesting to study on the bacterial NADP-specific ICDH's whether they exhibit different regulatory mechanisms.

The strong requirement for other bivalent metals than Mn^{2+} or Mg^{2+} was not reported in both the NAD- and NADP-specific ICDH's. It is still unresolved how the bivalent metals act on the enzyme proteins. We examined the effects of bivalent metals, Ca^{2+} , Co^{2+} , Ba^{2+} , Cd^{2+} , Sr^{2+} and Mg^{2+} , on the enzymes and found that the *S. lutea* enzyme was inhibited to a great extent by Ca^{2+} or Sr^{2+} (Table 2). The requirement for Mn^{2+} of the enzymes from *S. lutea*, *P. vulgaris* and *E. coli* were all the same level, while the susceptibilities to Ca^{2+} were different. The degrees of the inhibition by Ca^{2+} in the *S. lutea* enzyme were determined in the presence of various concentrations of Mn^{2+} , and 50% inhibition was attained when the ratio of Ca^{2+} to Mn^{2+} was about 1 (Table 1, Fig. 3). When the *S. lutea* enzyme was assayed at pH 6.0, the enzyme activities were remained about 30% of that obtained at neutral pH, but the inhibition by Ca^{2+} were disappeared and the requirements for Mn^{2+} were remarkably increased. However, the requirement for Mn^{2+} in the *E. coli* enzyme was lost at pH 6.0. These results seem to present more reliable proof on the presence of different types among bacterial NADP-specific ICDH's. It has been reported that the changes of pH caused reversible changes of the conformational states of some enzyme proteins (17-19). The susceptibilities to Ca^{2+} might be also accompanied with the conformational changes of the enzyme proteins. When the enzymes were assayed in the presence of appropriate concentrations of NaCl or LiCl, the susceptibilities to Ca^{2+} were completely disappeared without any change of the enzyme activities (Table 4). Although EDTA caused the remarkable activation by Mn^{2+} as NaCl or LiCl, it did not overcome the Ca^{2+} -inhibition. The main difference between the effects of the salts and urea on the enzyme proteins was that the former could lead to the desensitization to the Ca^{2+} -inhibition but the latter could not. The sensitive antagonistic effects of Ca^{2+} to Mn^{2+} , which was observed only in *S. lutea*, might suggest that these bivalent metals effect the tertiary or quarternary structures of enzyme proteins. The clear interpretations of this suggestion must await the studies of the interaction of the molecular weight and the ligands with highly purified

enzyme proteins.

Summary

NADP-specific isocitrate dehydrogenases (EC 1.1.1.42) were partially purified from *Proteus vulgaris*, *Escherichia coli*, *Sarcina lutea*, *Pseudomonas aeruginosa*, *Acetobacter xylinum* and pig heart muscle. ATP-inhibition was examined in these NADP-specific enzymes and it was found that only the *P. vulgaris* enzyme was inhibited significantly. Strong antagonistic effect of Ca²⁺ to Mn²⁺ was observed only in the *S. lutea* enzyme. This antagonistic effect was more stressed in the presence of ATP. The antagonistic effect of Ca²⁺ was diminished when the enzymes were assayed at pH 6.0, and in the presence of 330 mM NaCl or LiCl at pH 7.4. While a part of the enzyme activity was lost at pH 6.0, complete activity was kept in the presence of 330 mM NaCl or LiCl. The enzymes dialyzed against 1 M urea failed to overcome the antagonistic effect of Ca²⁺. In the *S. lutea* enzyme the requirement of Mn²⁺ for its maximal activity was increased at pH 6.0, while the requirement in the *E. coli* enzyme was lost completely. A few considerations on the interactions of the enzyme proteins with NaCl, LiCl, urea and bivalent metals are included here.

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