REGULATION OF ISOCITRATE DEHYDROGENASE FROM THIOBACILLUS THIOOXIDANS AND PSEUDOMONAS FLUORESCENS

M. L. Hampton and R. S. Hanson

Department of Bacteriology, University of Wisconsin
Madison, Wisconsin

Received June 4, 1969

SUMMARY

The isocitrate dehydrogenases of T. thiooxidans and P. fluorescens have been studied. The apparent K_M's for isocitrate are 1.2×10^{-5} M and 1.5×10^{-5} M respectively. The apparent K_M for NAD^+ is 2.9×10^{-4} M for the enzyme from T. thiooxidans while the apparent K_M for the NADP -specific enzyme of P. fluorescens is 1.7×10^{-5} M. ADP and ATP were found to inhibit the isocitrate dehydrogenases of both organisms. Glyoxalate plus oxalacetate caused concerted inhibition of the enzyme from T. thiooxidans. Glyoxalate, when added alone, was an activator of the enzyme from P. fluorescens but increased the inhibition due to oxalacetate and reduced the K_I for oxalacetate by three orders of magnitude.

The tricarboxylic acid cycle has a dual role in many aerobic organisms. It functions in the biosynthesis of glutamate and in the complete oxidation of pyruvate with the concomitant reduction of pyridine nucleotide which in turn serves as a substrate for ATP generation via oxidative phosphorylation. Some aerobic organisms use the first three enzymes of the tricarboxylic acid cycle for glutamate biosynthesis, although the cycle does not function in energy production (1). Autotrophs also use the first half of the tricarboxylic acid cycle for glutamate biosynthesis although the role of the cycle in oxidation is uncertain (2). We are studying the regulation of the activity of isocitrate dehydrogenase (IDH) from different organisms to determine whether there is a relationship between the regulation of tricarboxylic acid cycle enzymes and their function in the metabolism of organisms of various physiological types. The two reported in this communication are of interest because of their somewhat unique properties and origins.

All eucaryotic systems studied so far possess both NAD^+- and $NADP^+$-linked IDH. Procaryotic systems, on the other hand, have either NAD^+- or $NADP^+$-linked

IDH but never both. Most bacterial IDH's are $NADP^+$-specific but there are a few which are linked to NAD^+ (3, 4, 5). One of these, the enzyme from T. thiooxidans, is described in this communication.

MATERIALS AND METHODS

T. thiooxidans was grown at 30 C in a salts medium containing 0.1% NH_4Cl, 0.05% $MgCl_2$, 0.04% KH_2PO_4, 0.06% K_2HPO_4 and 0.002% $FeCl_3$ (J. London, personal communication). The sole energy source was 1% $Na_2S_2O_3 \cdot 5H_2O$ and CO_2 was the carbon source. P. fluorescens was grown at 30 C in nutrient broth supplemented with 0.2% sodium acetate.

Cells were suspended in 0.01 M tris-maleate buffer (pH 6.3) containing 10^{-3} M $MgCl_2$ and 10^{-4} M EDTA at 0-4 C and broken by passage through a French pressure cell. The extract was then spun at 27,000 X g for 30 min and the pellet discarded. The extract from T. thiooxidans contained NADH oxidase and $(NH_4)_2SO_4$ fractionation was carried out to obtain enzyme preparations free of this activity. When NADPH was added to the extract from P. fluorescens, there was a slow decrease in the optical density at 340 mμ and $(NH_4)_2SO_4$ fractionation was used to rid the extract of this activity.

IDH was assayed by following the reduction of pyridine nucleotide spectrophotometrically at 340 mμ. Since the true substrate of this reaction is d-isocitrate (6, 7), the concentration of isocitrate was determined by running the reaction to completion in the presence of excess NAD^+ (or $NADP^+$) and calculating the amount of isocitrate present by using the molar extinction coefficient of pyridine nucleotide. The IDH from T. thiooxidans reduced NAD^+ but not $NADP^+$ while that from P. fluorescens was active only with $NADP^+$. All reaction mixtures contained 14 mM $MgCl_2$ to satisfy the requirement for a divalent cation. All reaction velocities were measured in 0.01 M tris-maleate buffer at pH 8.0. A unit of activity is defined as the conversion of 1 μmole of substrate/min/mg protein.

RESULTS

Extracts from both organisms were stable when stored at 0-4 C in the same

buffer used for preparation of extracts when 40-50% $(NH_4)_2SO_4$ was present. The pH optimum was 8.0 for both organisms.

TABLE 1

INHIBITION CONSTANTS OF ADENINE NUCLEOTIDES

	K_I (M)	
Inhibitor	T. thiooxidans	P. fluorescens
AMP	No inhibition	7×10^{-3}
ADP	7×10^{-4}	2×10^{-3}
ATP	6.4×10^{-3}	5×10^{-3}

Reaction mixture: 3 ml final volume, 14 mM $MgCl_2$; 0.01 M tris-maleate, (pH 8.0), 1.6 mM NAD^+, extract (0.056 mg T. thiooxidans protein, 0.052 mg P. fluorescens protein). The concentrations of isocitrate and inhibitors were varied to generate plots similar to those in Fig. 5. The K_I for each compound was determined from these plots.

TABLE 2

INHIBITION OF ISOCITRATE DEHYDROGENASE OF T. THIOOXIDANS

Addition	Concentration (mM)	Relative velocity
None	-	1.0
Oxalacetate	1.0	0.84
Glyoxalate	0.25	0.84
Oxalacetate + glyoxalate	1.0, 0.25	0.13
Oxalomalate	1.0	0.82
Hydroxy-α-ketoglutarate	1.0	0.57

Reaction mixture: 3 ml final volume, 14 mM $MgCl_2$, 1.6 mM NAD^+, 0.16 mM isocitrate, 0.01 M tris-maleate (pH 8.0), extract (0.056 mg protein) and inhibitors.

Plots of initial velocity versus isocitrate or NAD^+ concentration for the enzyme from T. thiooxidans are hyperbolic and the apparent K_M's, determined from double reciprocal plots, were 1.2×10^{-5} M for isocitrate and 2.9×10^{-4} M for

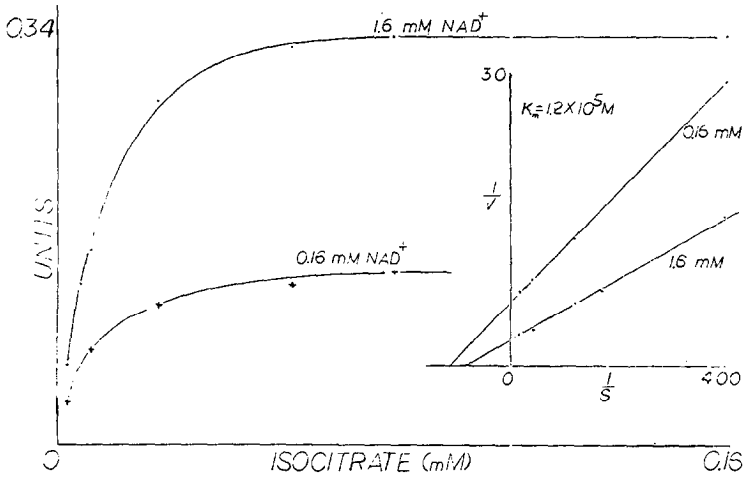

Fig. 1. Initial velocity vs. isocitrate concentration for IDH of T. thiooxidans. Reaction mixture: 3 ml final volume, 14 mM $MgCl_2$, 0.01 M tris-maleate (pH 8.0), 0.056 mg protein, NAD^+ and isocitrate as indicated.

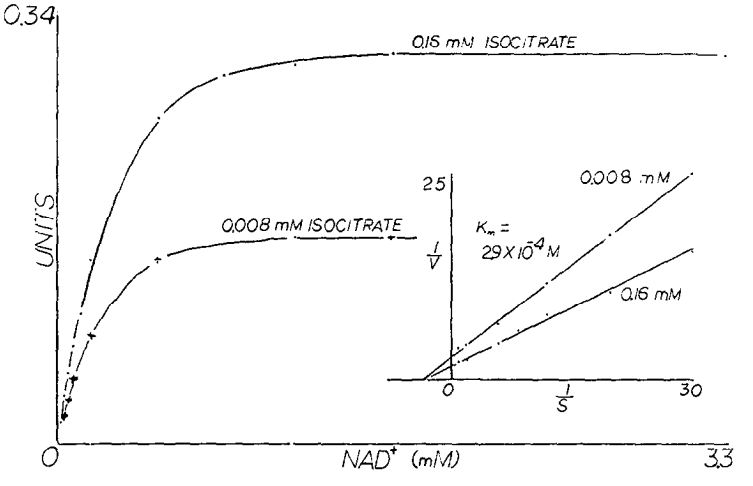

Fig. 2. Initial velocity vs. NAD^+ concentration for IDH of T. thiooxidans. Reaction mixtures identical to those in Fig. 1.

NAD^+ (Figs. 1 and 2). Similar experiments with the enzyme from P. fluorescens indicated apparent K_M's of 1.5×10^{-5} M for isocitrate and 1.7×10^{-5} M for $NADP^+$ (Figs. 3 and 4).

The effect of AMP, ADP and ATP on these two enzymes was examined because

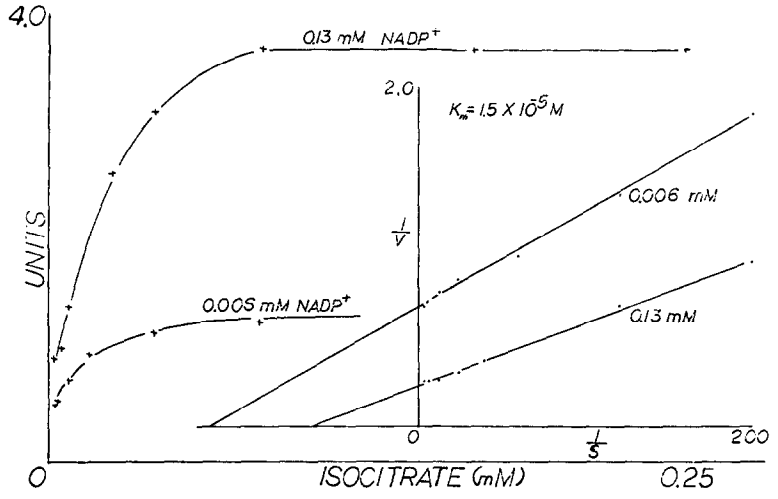

Fig. 3. Initial velocity vs. isocitrate concentration for IDH of P. fluorescens. Reaction mixture: 3 ml final volume, 14 mM $MgCl_2$, 0.01 M tris-maleate (pH 8.0), 0.052 mg protein, $NADP^+$ and isocitrate as indicated.

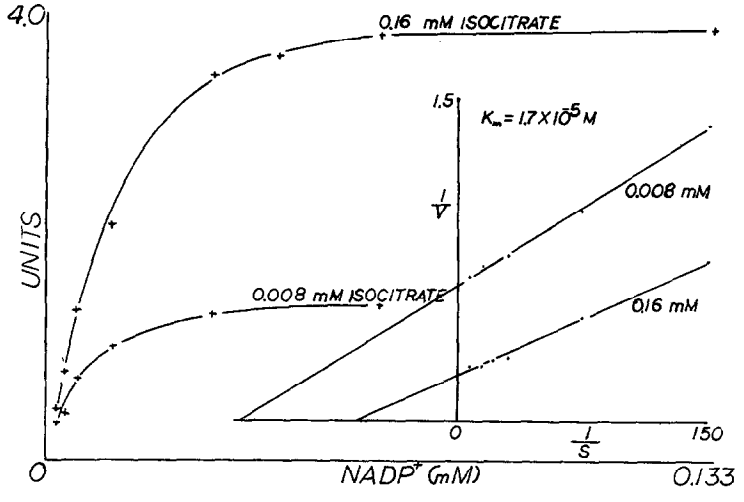

Fig. 4. Initial velocity vs. $NADP^+$ concentration for IDH of P. fluorescens. Reaction mixtures identical to those in Fig. 3.

---

it has been shown that adenine nucleotides modify this reaction when catalyzed by enzymes from other organisms (8, 9, 10). The IDH from T. thiooxidans was insensitive to AMP but was inhibited by ADP (Fig. 5) and ATP. All three adenine nucleotides were inhibitors of the enzyme of P. fluorescens. The inhibition

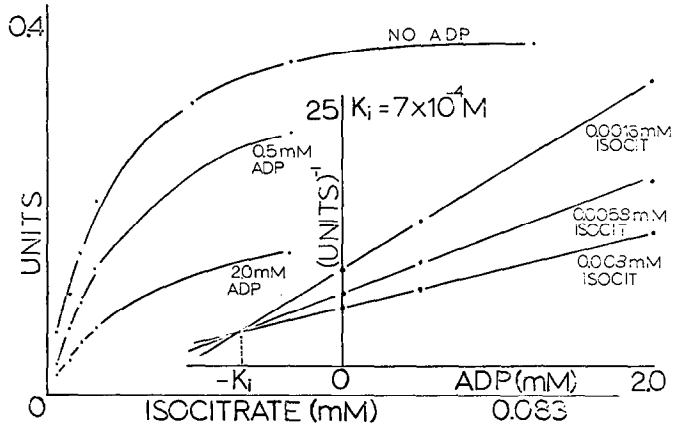

Fig. 5. Effect of ADP on IDH of T. thiooxidans. Reaction mixtures identical to those in Fig. 1; all reactions contained 1.6 mM NAD^+ and ADP as indicated.

---

constants of the adenine nucleotides with respect to isocitrate were calculated by the method of Dixon (11, Fig. 5) and are given in Table 1. In all cases the inhibition is competitive. It is interesting to note that for both enzymes ADP is a better inhibitor than ATP. It has been suggested that inhibition by ATP might be caused by a decrease in Mg^{+2} concentration due to chelation (12), but if this were the case, ATP should be a better inhibitor than ADP. In addition, decreasing the concentration of $MgCl_2$ to 7 mM does not give increased inhibition as would be the case if a reduction in the concentration of the divalent cation due to chelation was the cause.

Shiio and Ozaki (13, 14) have reported that glyoxalate plus oxalacetate gives concerted inhibition of IDH from Brevibacterium flavum, Escherichia coli, Bacillus subtilis and pig heart. We tested the effect of these compounds on the enzymes of T. thiooxidans and P. fluorescens. The behavior of the enzyme from T. thiooxidans is the same as that reported by Shiio (Table 2). It is possible that the apparent concerted inhibition is actually due to condensation to form oxalomalate or hydroxy-α-ketoglutarate. When these compounds were tested, however, the inhibition produced by 1 mM of either compound was less than that caused by 0.25 mM glyoxalate plus 1 mM oxalacetate (Table 2). The K_I's for

TABLE 3

INHIBITION OF ISOCITRATE DEHYDROGENASE OF P. FLUORESCENS

Addition	Concentration (mM)	Relative velocity
None	-	1.0
Oxalacetate	1.0	0.86
Glyoxalate	1.0	1.79
Oxalacetate + glyoxalate	1,0, 1.0	0.20

Reaction mixture: 3 ml final volume, 14 mM $MgCl_2$, 0.16 mM isocitrate, 0.13 mM $NADP^+$, 0.01 mM tris-maleate (pH 8.0) and extract (0.152 mg protein) and inhibitors at the concentrations indicated above.

TABLE 4

INHIBITION CONSTANTS OF GLYOXALATE AND OXALACETATE

	K_I (mM)	
Inhibitor	T. thiooxidans	P. fluorescens
Glyoxalate	6×10^{-4}	No inhibition
Glyoxalate + 1 mM oxalacetate	5×10^{-6}	5×10^{-6}
Oxalacetate	1.4×10^{-3}	2×10^{-3}
Oxylacetate + 1 mM oxalacetate	4×10^{-6}	2.5×10^{-6}

The reaction mixtures were identical to those indicated in Table 1.

glyoxalate and oxalacetate in the absence and presence of the other compound were determined (Table 4). The presence of 1 mM glyoxalate greatly increases the ability of oxalacetate to inhibit the reaction; i.e., the K_I of oxalacetate is decreased by a factor of 10^3 by the addition of 1 mM glyoxalate. The presence of oxalacetate causes a similar effect on the K_I for glyoxalate. When these compounds were tested using the IDH of P. fluorescens, we found that glyoxalate, instead of inhibiting, caused a considerable increase in the reaction

TABLE 5

ACTIVATION OF ISOCITRATE DEHYDROGENASE OF P. FLUORESCENS

Compound	Structure $R\text{-}C(=O)COOH$ R-	% Activation
Glyoxalate	H-	79
Pyruvate	H_3C-	35
α-ketobutyrate	H_3CCH_2-	23
α-ketovalerate	$H_3C(CH_2)_2$-	13
α-ketocaproate	$H_3C(CH_2)_3$-	17
α-ketoisovalerate	$(H_3C)_2CH_2$-	35
α-ketoisocaproate	$(H_3C)_2(CH_2)_2$-	19

The reaction mixture was identical to that indicated in Table 3.

TABLE 6

EFFECT OF KETOACIDS ON OXALACETATE INHIBITION OF ISOCITRATE DEHYDROGENASE OF P. FLUORESCENS

Addition	Relative velocity
1 mM oxalacetate	1.0
1 mM oxalacetate + 1 mM glyoxalate	0.2
1 mM oxalacetate + 1 mM pyruvate	1.65
1 mM oxalacetate + 1 mM α-ketobutyrate	1.23

The reaction mixture was identical to that indicated in Table 3.

rate, although oxalacetate was again an inhibitor and glyoxalate plus oxalacetate caused greater inhibition than oxalacetate alone (Table 3). The K_I of

oxalacetate is decreased by a factor of 10^3 by the addition of 1 mM glyoxalate and is similar to the T. thiooxidans enzyme in this respect.

Several other compounds were tested as modifiers of the reaction. Pyruvate had no effect on the enzyme from T. thiooxidans but, like glyoxalate, stimulated the reaction catalyzed by the P. fluorescens enzyme. Because of the chemical similarities between glyoxalate and pyruvate, other α-keto-monocarboxylic acids were tested (Table 5). Glyoxalate, pyruvate, α-ketobutyrate, α-ketovalerate,, α-ketocaproate, α-ketoisovalerate and α-ketoisocaproate all activated the enzyme and, with the exception of α-ketoisovalerate, there appeared to be a general decrease in the degree of activation with increase in the molecular weight of the activator. Only glyoxalate caused an increase in inhibition when added to reaction mixtures containing oxalacetate (Table 6). No other α-ketodicarboxylic acids would replace oxalacetate in producing concerted inhibition with glyoxalate.

Several other compounds were tested for their ability to modify the reaction of these two enzymes. Citrate is a competitive inhibitor of the enzyme from both sources. Succinate and alanine slightly inhibit the enzyme from T. thiooxidans at concentrations of 5 mM and 1 mM respectively, but have no effect on the P. fluorescens enzyme. Both α-ketoglutarate and ketomalonate inhibit the enzyme from P. fluorescens. Glycine, glutamate and phosphoenolpyruvate had no effect on either enzyme.

DISCUSSION

It has been suggested that inhibition of IDH by ATP is a means by which an organism is able to reduce the activity of the tricarboxylic acid cycle when energy production exceeds the demand for ATP. It is interesting that in T. thiooxidans, where the tricarboxylic acid cycle has been postulated to function mainly as a biosynthetic pathway, nucleotides still decrease the reaction velocity. There is some doubt concerning the role of the tricarboxylic acid cycle in ATP generation in this organism, however (2, 15). It seems reasonable to assume that in the interest of economy acetate carbon would be utilized exclusively for biosynthesis because its formation from CO_2 requires

more energy than is made available by its reoxidation in the tricarboxylic acid cycle.

It has been suggested that the concerted inhibition of IDH by glyoxalate plus oxalacetate serves to apportion carbon between the tricarboxylic acid cycle and the glyoxalate bypass. The activation of IDH by glyoxalate has not been reported previously to our knowledge. It is conceivable that the enzyme modification, caused by glyoxalate, which increases the affinity of the enzyme for the inhibitor oxalacetate, promotes its catalytic ability in the absence of oxalacetate.

This project was supported in part by a grant (GB-7627) from the National Science Foundation.

REFERENCES

1. Stern, J. R. and Bambers, G. (1966) Biochemistry 5, 1113.
2. Smith, A. J., London, J. and Stanier, R. Y. (1967) J. Bacteriol. 94, 972.
3. Ragland, T. E., Kawasaki, T. and Lowenstein, J. M. (1966) J. Bacteriol. 91, 236.
4. Burchall, J. J., Niederman, R. A. and Wolin, M. J. (1964) J. Bacteriol. 88, 1038.
5. Hampton, M. L. and Hanson, R. S. (1969) Bacteriol. Proc., p. 118.
6. Pulcher, G. W. and Vickery, H. B. (1946) J. Biol. Chem. 163, 169.
7. Ochoa, S. (1951) The Enzymes, 1st ed. Vol. II, Part 2, p. 929.
8. Atkinson, D. E. (1966) Ann. Rev. Biochem. 35(I), 85.
9. Marr, J. J. and Weber, M. M. (1968) J. Biol. Chem. 243, 4973.
10. Hanson, R. S. and MacKechnie, I. (1969) Spores IV, in press.
11. Dixon, M. (1953) Biochem. J. 55, 170.
12. Goebell, H. and Klingenberg, M. (1964) Biochem. Z. 340, 441.
13. Shiio, I. and Ozaki, H. (1968) J. Biochem. 64, 45.
14. Ozaki, H. and Shiio, I. (1968) J. Biochem. 64, 355.
15. Butler, R. G. and Umbreit, W. W. (1969) J. Bacteriol. 97, 966.