CONCERTED INHIBITION OF A $NADP^+$-SPECIFIC ISOCITRATE DEHYDROGENASE AND THE IMPLICATIONS FOR METABOLIC REGULATION[1]

J. Joseph Marr[2] and Morton M. Weber

Department of Microbiology, St. Louis University School of Medicine
St. Louis, Missouri 63104

Received February 10, 1969

A possible physiologic role for the concerted inhibition of a $NADP^+$-specific isocitrate dehydrogenase by oxalacetate and glyoxylate is presented. The significance of this inhibition is also discussed with respect to the "induced fit" theory of enzyme-substrate interaction. However, since numerous structural analogues of these two compounds were without effect, it is believed that these two components are not acting as a "synthetic" substrate.

The functions of the NAD^+- and $NADP^+$-specific isocitrate dehydrogenases (IDH) have been in question for several years. Because of the localization of the NAD^+-specific enzyme in the mitochondria and the $NADP^+$-specific enzyme primarily in the cytoplasm (1-3), it is believed that the former is concerned with oxidative metabolism and the latter with the generation of reducing equivalents for biosynthetic processes. This concept was drawn from the work of Chen and Plaut (4), among others, and the more recent investigations of Sanwal and coworkers (5,6) and Atkinson and coworkers (7,8). These investigators have shown that the NAD^+-linked IDH is a regulatory enzyme subject to the heterotropic modifiers AMP or ADP, depending on the source, while the $NADP^+$-linked enzymes from the same sources were not subject to this type of regulation. Stein et al. (9) have postulated that the $NADP^+$-linked IDH may act via a transhydrogenase to furnish NADH for the electron transport system.

---

[1] This work was supported by Grant AI-03046 from the National Institutes of Health, United States Public Health Service.

[2] Postdoctoral fellow, PF-280, of the American Cancer Society.

It has recently been shown, however, that in some organisms the $NADP^+$-linked IDH has some importance in oxidative metabolism (3). Marr and Weber (10, 11) have shown it to be inhibited by ATP, and Shiio and Ozaki (12) have shown it to be inhibited by oxalacetate and glyoxylate. This work confirms the findings of these latter authors and proposes a possible physiologic function for this concerted inhibition.

This effect of ATP and other nucleotides has been demonstrated in both Salmonella typhimurium (10) and in the protozoan Crithidia fasciculata (11). The specific mechanisms of inhibition are different in the two organisms, the nucleotide competing with the coenzyme in the former case and acting at a site separate from the active site in the latter. This report describes the inhibition of a $NADP^+$-specific IDH (E. C. 1. 1. 1. 42) from the protozoan Crithidia fasciculata by oxalacetate and glyoxylate. The inhibition is of a concerted type, first described by Datta and Gest (13) for homoserine dehydrogenase. The chemical structure of the inhibitors and some of the kinetic parameters suggest that this inhibition may be an example of the "induced fit" theory of Koshland (14). This possibility and that of a biologically meaningful feedback inhibition are discussed.

MATERIALS AND METHODS

Crithidia fasciculata was grown and harvested by a previously described procedure (15) and the enzyme was purified as shown in Table I.

The sodium salt of threo-D_sL_s-isocitrate was prepared by dissolving the lactone, obtained from Calbiochem, in 0. 1 M NaOH (3. 5 moles per mole of lactone) and heating in a boiling water bath for 10 minutes. The pH was adjusted to 7. 0 after cooling. All other chemicals were purchased from the Sigma Chemical Company. Oxalacetate was prepared immediately prior to

TABLE I

PURIFICATION PROCEDURE

Step	Procedure	Volume ml	Total activity units	Total protein mg	Specific activity	Recovery %
1.	supernatant fluid from 105, 000 x g for 70 min.	70.0	1820	658.0	2.8	100
2.	precipitate from 70-80% $(NH_4)_2SO_4$	5.5	660	30.2	21.9	36
3.	2 hr. dialysis against 0.01 M Tris-HCl, pH 7.7	6.0	708	25.3	28.0	39
4.	0.05 M potassium phosphate buffer, pH 7.7, elution from calcium phosphate gel (10 mg dry wt. gel to 1 mg protein)	5.2	327	10.0	32.7	18

each experiment. Over a time period of one hour there was a noticeable decrease in capacity for inhibition, probably due to spontaneous decarboxylation of the compound.

Reactions were started by the addition of enzyme, and the reduction of $NADP^+$ was measured at 340 mμ in a Gilford recording spectrophotometer equipped with a Beckman DU monochromator. Reactions were carried out at 25°.

Protein was determined by the method of Lowry et al. (16) or Warburg and Christian (17).

RESULTS

Demonstration of Concerted Inhibition

Table II shows the addition of various compounds in the presence of

TABLE II

CONCERTED INHIBITION OF IDH BY GLYOXYLATE AND OXALACETATE

ADDITION (1 mM)	% INHIBITION	
	Glyoxylate (1 mM)	Oxalacetate (1 mM)
None	0	6
Oxalacetate	97	14
Glyoxylate	0	97
α-Ketoglutarate	15	14
L_s-Malate	16	10
D_s-Malate	0	11
Succinate	13	0
Fumarate	7	3
Pyruvate	0	10
Acetate	0	3
Acetaldehyde	4	5
Glycolic acid	8	0
Glycolaldehyde	6	0
Oxalate	0	8
Ethanol	0	8
Formate	0	8
Formaldehyde	0	0

Reaction mixtures contained 0.5 mmole of Tris HCl, pH 7.7, 0.2 μmole of $MnCl_2$, 0.3 μmole of $NADP^+$, 0.2 μmole of isocitrate, and water to a final volume of 3 ml. Reactions were carried out with either oxalacetate or glyoxylate in a final concentration of 1 mM, and all other inhibitors were added to the same final concentration. Reactions were started by the addition of 38 μg of extract protein.

either oxalacetate or glyoxylate. Significant inhibition was achieved only when glyoxylate and oxalacetate were present together in the reaction mixture. Doubling the concentration of either compound was without significant effect. Furthermore, though not shown here, the successive addition of all α-keto compounds did not result in an additive inhibition of the enzyme. Although the concentrations of the inhibitors are given in Table II as 1 mM, the combination of oxalacetate and glyoxylate was effective at much lower concentrations (see Fig. 1).

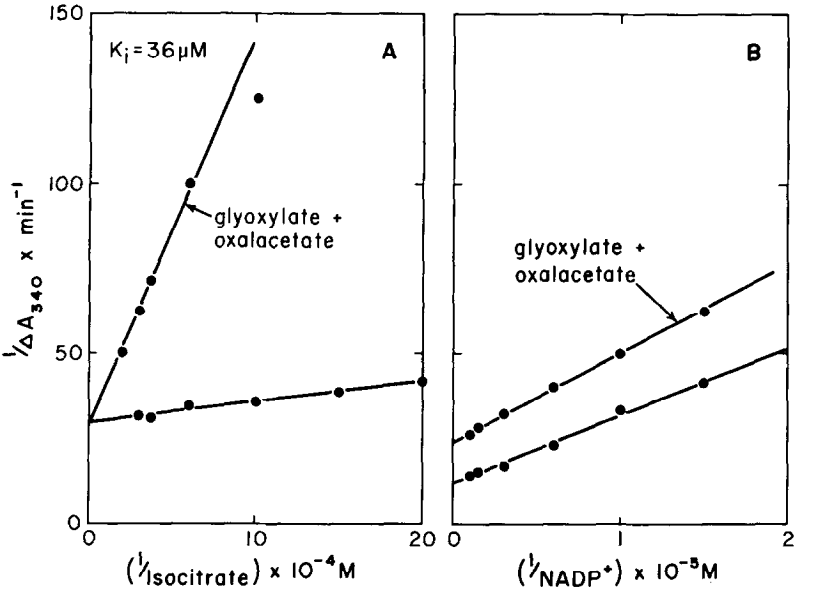

Fig. 1. Inhibition of the $NADP^+$-specific IDH by oxalacetate and glyoxylate. Reaction mixtures contained 0.5 mmole of Tris-HCl, pH 7.7, 0.2 μmole of $MnCl_2$, 0.3 μmole of $NADP^+$, isocitrate as indicated, and water to a final volume of 3 ml. Oxalacetate and glyoxylate were each present in a final concentration of 17 μM, and were prepared in advance and frozen until used in the experiment. Reactions were started by the addition of 38 μg of extract protein.

Kinetics of Inhibition

The kinetics of inhibition of the reaction was examined with respect to the individual substrates. Although not shown, oxalacetate was a competitive inhibitor of isocitrate and had a K_i of 2.74 mM. Glyoxylate gave no inhibition at concentrations up to 3 mM. However, as seen in Fig. 1, when the two compounds were examined together in the reaction there was a marked inhibition which was competitive with isocitrate and noncompetitive with $NADP^+$. The K_i with respect to isocitrate was 36 μM. The ratio of the $K_i^{oxal}/K_i^{oxal+glyox}$ was about 76. The apparent K_m for isocitrate was 2.27 μM.

The possibility that the actual inhibitor of the reaction might be oxalomalate was considered (18), but was ruled out because of the long period of time required to form the compound (2-3 hours) and the short duration of our enzymatic reaction (30-60 seconds). This possibility was also examined in some detail by Shiio and Ozaki (12) and found not to be the case.

DISCUSSION

The significance of the concerted inhibition of the $NADP^+$-linked IDH by oxalacetate and glyoxylate cannot be interpreted with certainty. However, several possibilities are evident. The striking decrease in the K_i for the glyoxylate and oxalacetate combination for isocitrate implies that the combination of inhibitor molecules may be similar in configuration to the substrate and can fit the active site with a similar affinity. This is a situation which would be predicted by the "induced fit" theory of Koshland (14), and is currently under active investigation. Indeed, constructed models of these two inhibitors can be fitted so as to mimic the configuration of threo-D_s-isocitrate. However, the combinations of D_s-malate and glyoxylate, oxalacetate

and glycolate, and oxalacetate and glycolaldehyde were without effect, and this is not what would have been predicted on the basis of chemical structure. Models of these latter compounds have also been constructed, and from their configurations these combinations should have been as effective as oxalacetate and glyoxylate. The fact that none of these was inhibitory tends to make this hypothesis less acceptable, but by no means disproven.

An alternate explanation, which would not necessarily be obviated by the above, is that this represents a form of feedback inhibition. Concerted inhibition by oxalacetate and glyoxylate has been shown to occur in several bacterial species, in pig heart, in Salmonella typhimurium (our unpublished observations), and now in the protozoan C. fasciculata. The isocitrate lyase catalyzes the production of glyoxylate from isocitrate; oxalacetate is also accumulated via this anapleurotic glyoxylate cycle (19) as well as by fat catabolism through the action of the acetyl-CoA activated pyruvate carboxylase (20). The activating effect of isocitrate and citrate on fat biosynthesis is well known (21, 22), and inhibition of this soluble IDH by products of fat catabolism such as oxalacetate and ATP would provide a method for restoration of the cell economy. It has also been shown that the $NADP^+$-specific IDH in these two organisms is inhibited by ATP (10, 11), and it may be that the $NADP^+$-linked IDH is involved in oxidative metabolism by way of a transhydrogenase. Inasmuch as the IDH reaction is not readily reversible, and it is the first point at which reduced pyridine nucleotides are shunted to the electron transport system, and since isocitrate can enter alternate pathways at this point, it is conceivable that this cytoplasmic IDH may serve as a branch point in carbohydrate metabolism. This hypothesis is also presently under investigation.

ACKNOWLEDGMENT

The authors would like to thank Miss Susan Dorris for her excellent technical assistance.

REFERENCES

1. Hogeboom, G. H. and Schneider, W. C., J. Biol. Chem. 186, 417 (1950).
2. Ernster, L. and Navazio, F., Exp. Cell Res. 11, 483 (1962).
3. Muller, M., Hogg, J. F., and de Duve, C., J. Biol. Chem. 243, 5385 (1968).
4. Chen, R. F. and Plaut, G. W. E., Biochemistry 2, 1023 (1963).
5. Sanwal, B. D. and Stachow, C. S., Biochim. Biophys. Acta 96, 28 (1965).
6. Sanwal, B. D., Stachow, C. S., and Cook, R. A., Biochemistry 4, 410 (1968).
7. Hathaway, J. A. and Atkinson, D. E., J. Biol. Chem. 238, 2875 (1963).
8. Atkinson, D. E., Hathaway, J. A., and Smith, E. C., J. Biol. Chem. 240 2682 (1965).
9. Stein, A. M., Stein, J. H., and Kirkman, S. K., Biochemistry 6, 1370 (1967).
10. Marr, J. J. and Weber, M. M., J. Biol. Chem. 243, 4973 (1968).
11. Marr, J. J. and Weber, M. M., J. Biol. Chem. (in press) 244, April (1969).
12. Shiio, I. and Ozaki, H., J. Biochem. 64, 45 (1968).
13. Datta, P. and Gest, H., Nature 203, 1259 (1964).
14. Koshland, D. E., Cold Spring Harbor Symp. Quant. Biol. 28, 473 (1963).
15. Toner, J. J. and Weber, M. M., Biochem. Biophys. Res. Commun. 28, 821 (1967).
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).
17. Warburg, O. and Christian, W., Biochem. Z. 310, 384 (1941).
18. Ruffo, A., Testa, E., Adinolfi, A., and Pelizza, G., Biochem. J. 85, 588 (1962).
19. Kornberg, H. L., Ann. Rev. Microbiol. 13, 49 (1963).
20. Utter, M. F. and Keech, D. B., J. Biol. Chem. 238, 2603 (1963).
21. Waite, M. and Wakil, S. J., J. Biol. Chem. 237, 2750 (1962).
22. Vagelos, R. P., Alberto, A. W., and Martin, D. B., J. Biol. Chem. 238, 533 (1963).