Inhibition by Oxalomalate of Rat Liver Mitochondrial and Extramitochondrial Aconitate Hydratase

By A. ADINOLFI, V. GUARRIERA-BOBYLEVA, S. OLEZZA AND A. RUFFO
*Impresa Enzimologia; C.N.R., c/o Istituto di Chimica Biologica, Università di Pavia,
27100 Pavia, Italy, and Istituto di Patologia Generale, Università di Modena,
41100 Modena, Italy*

(Received 5 August 1971)

1. The effect of oxalomalate on the oxidation of citrate and *cis*-aconitate in rat liver mitochondria, and on the activity of mitochondrial and cytoplasmic aconitate hydratase, has been investigated. 2. Oxalomalate that was added to intact rat liver mitochondria at high concentrations (2mM) produced complete inhibition of citrate and *cis*-aconitate oxidation, but lower concentrations (0.1–0.25mM) inhibited oxidation of citrate more than that of *cis*-aconitate. 3. Aconitate hydratase that was either extracted from mitochondria or soluble in the cytoplasm, was strongly inhibited by low concentrations of oxalomalate (0.01–0.2mM), the mitochondrial enzyme being more sensitive than the soluble one. 4. Oxalomalate, when added together with citrate, produced competitive inhibition; the K_i values calculated were 1×10^{-6} M for the mitochondrial and 2.5×10^{-6} M for the cytoplasmic enzyme. 5. With both the enzymic preparations oxalomalate added together with the substrates inhibited the initial rate of the reaction citrate $\rightarrow$ *cis*-aconitate more than that of the reaction isocitrate $\rightarrow$ *cis*-aconitate. 6. After 2min of preincubation of the inhibitor with either of the enzymic preparations the inhibition increased tenfold and became irreversible; under these conditions both the reactions were inhibited to the same extent. 7. The inhibition by oxalomalate of aconitate hydratase appeared to be similar in many respects to that produced by fluorocitrate on the same enzyme.

Oxalomalate, a tricarboxylic acid obtained by chemical condensation of glyoxylate and oxaloacetate, is a competitive inhibitor of aconitate hydratase (EC 4.2.1.3) purified from pig heart (Ruffo, Testa, Adinolfi & Pelizza, 1962). Because oxalomalate inhibits the oxidation of citrate in rat liver mitochondria (Ruffo, Adinolfi & Malcovati, 1966) it seems that the bound mitochondrial enzyme is also sensitive to the inhibitor.

Guarriera-Bobyleva & Buffa (1969) showed that fluorocitrate, another specific inhibitor of aconitate hydratase (Peters, 1961; Fanshier, Gottwald & Kun, 1962, 1964), is more inhibitory with the bound mitochondrial enzyme than with that either extracted from mitochondria or that soluble in the cytoplasm. Moreover, irrespective of the enzymic preparation used, fluorocitrate inhibited the reaction citrate $\rightarrow$ *cis*-aconitate to a greater extent than the reaction isocitrate $\rightarrow$ *cis*-aconitate. This finding, considered together with the observation (Peters & Shorthouse, 1969) that the storage of the enzyme at low temperature inactivates only the first reaction without affecting the second, raises the

question as to whether aconitate hydratase possesses more than one active centre.

The evidence in favour of a single catalytic site (Dickman, 1961) received further support from the crystallographic studies of Glusker (1968), who proposed a catalytic mechanism involving the formation of stereospecific co-ordination complexes between the ferrous ion of the enzyme and the three substrates. Also the crystallographic identification of the inhibitory isomer of fluorocitrate, which is able to form a complex with the enzyme similar to that formed by the substrates, argues for the existence of only one active site (Carrell *et al.* 1970).

To obtain further information on the catalytic mechanism of aconitate hydratase, we have investigated the inhibitory effect of oxalomalate on the oxidation of citrate and *cis*-aconitate in rat liver mitochondria, and also its effect on the aconitate hydratase extracted from mitochondria and the enzyme that is soluble in the cytoplasm. With both of these enzyme preparations we compared the inhibition of the conversion of citrate into *cis*-aconitate with that of isocitrate into *cis*-aconitate.

MATERIALS AND METHODS

Materials. Trisodium DL-isocitrate, ADP and ATP (sodium salts), cytochrome *c* and hexokinase (type III) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Trisodium citrate dihydrate and sucrose were obtained from E. Merck A.-G., Darmstadt, Germany. *cis*-Aconitate was obtained either from Sigma or from Fluka A.-G., Buchs, Switzerland. All the other reagents were of analytical grade from C. Erba, Milan, Italy.

Preparation of mitochondria and determination of O_2 uptake. Mitochondria were prepared from livers of young Wistar strain albino rats, as described by Guarriera-Bobyleva & Buffa (1969). The O_2 uptake was determined by using either the conventional Warburg apparatus or the Clark-type polarographic electrode. For the manometric determination the isolated mitochondria were washed twice in 0.25 M-sucrose as described by Ruffo *et al.* (1966). The composition of incubation media are given with the individual experiments (Table 1 and Fig. 1).

Preparation of enzymes and assay method. Soluble cytoplasmic aconitase was prepared by centrifuging rat liver homogenates at 139000g in 0.25 M-sucrose as described by Guarriera-Bobyleva & Buffa (1969). Mitochondrial aconitase was extracted by the freezing-and-thawing method of Dickman & Speyer (1954) as modified by Guarriera-Bobyleva & Buffa (1969). The aconitase hydratase activity was determined by the method of Racker (1950), with either citrate (30 mM) or DL-isocitrate (20 or 10 mM) as substrate; the formation of *cis*-aconitate was recorded at 240 nm with a Beckman DK-2 spectrophotometer.

Preparation and incubation of oxalomalate. Sodium oxalomalate trihydrate was synthesized as described by Ruffo *et al.* (1962). The freshly prepared solution was added to the assay mixture in the spectrophotometer cuvette, and the reaction was started by adding the enzyme. In some experiments the inhibitor was added to the cuvette containing the enzyme with the substrate 2 min after the beginning of the enzymic reaction. In

preincubation experiments oxalomalate was added together with the enzyme to the spectrophotometer cuvette, and the reaction was started 2 min later by addition of the substrate.

Citrate determination. Citrate was determined by the method of Ettinger, Goldbaum & Smith (1952).

Protein determination. The protein content of intact mitochondria was determined by the micro-Kjeldhal procedure. In the other enzymic preparations proteins were determined either by the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951), or by measuring the E_{280} (Anfinsen, 1955).

RESULTS

Effect of oxalomalate on the oxidation of citrate and cis-aconitate in intact mitochondria

Table 1 shows that 2 mM-oxalomalate inhibited almost completely either citrate or *cis*-aconitate oxidation (Expt. 1). In the first case citrate was not oxidized; in the second its formation was prevented.

Lower concentrations of oxalomalate (Expt. 2) inhibited citrate oxidation to the same extent as in the previous experiment, but the inhibition of *cis*-aconitate oxidation was decreased. When citrate was the substrate, its oxidation was inhibited as before, but when *cis*-aconitate was the substrate, more citrate was found after the incubation than in either the control, or the preceding experiment. Citrate also accumulated when pyruvate plus malate were the substrates.

The effect of oxalomalate on the oxidation of citrate or *cis*-aconitate was further investigated by using the polarographic technique. The ratio of inhibitor to substrate employed was the same as in

Table 1. *Effect of oxalomalate on O_2 uptake of rat liver mitochondria*

In a final volume of 2 ml, each Warburg vessel contained: ATP, 3.5 μ mol; KH_2PO_4 - K_2HPO_4 buffer, pH 7.4, 40 μ mol; cytochrome *c*, 0.022 μ mol; $MgSO_4$, 15 μ mol; glucose, 35 μ mol; 0.7 ml of mitochondrial suspension in 0.25 M-sucrose (corresponding to about 1 mg of N). Hexokinase 100 U (in the side arm) was added to the main chamber after 5 min of equilibration. Substrates (10 μ mol) and oxalomalate were as indicated. Incubation in O_2 as gas phase was for 30 min at 30°C. Deproteinization was done with tungstic acid (2.5%, w/v, final concn.). Citrate ($\Delta \mu$ mol) is the change from the control value.

Expt. no.	Substrate	Addition	O_2 (μ g-atom)	Inhibition (%)	Citrate	
					(μ mol found)	($\Delta \mu$ mol)
1	Citrate	None	12.7	—	3.7	+5.3
		Oxalomalate, 2 mM	1.3	90	9.0	
	<i>cis</i> -Aconitate	None	13.9	—	3.3	-2.4
		Oxalomalate, 2 mM	1.1	92	0.9	
2	Citrate	None	16.1	—	3.1	+6.1
		Oxalomalate, 0.25 mM	1.2	92	9.2	
	<i>cis</i> -Aconitate	None	14.9	—	3.6	+0.9
		Oxalomalate, 0.25 mM	9.7	35	4.5	
	Pyruvate + malate	None	17.8	—	2.3	+4.6
		Oxalomalate, 0.25 mM	10.1	43	6.9	

the preceding experiment. The results (Fig. 1) show that low concentrations of oxalomalate inhibited the oxidation of the two substrates to a different degree. The addition of 0.1 mM-oxalomalate produced 46% inhibition of citrate oxidation (curve I), whereas with *cis*-aconitate as substrate (curve III) the same degree of inhibition was obtained only by increasing the concentration of oxalomalate six-fold. With 0.1 mM-oxalomalate (curve IV) the oxidation of *cis*-aconitate was inhibited by only 17%.

Fig. 1. Oxygen electrode tracings of the effect of oxalomalate on the oxidation of citrate and *cis*-aconitate by rat liver mitochondria. The reaction medium contained in a 3.6 ml final volume: 10 mM-sodium-potassium phosphate -10 mM-tris-chloride buffer, pH 7.4; 100 mM-NaCl; 10 mM-MgCl₂; 14 mM-sucrose; 8.3 mg of mitochondrial protein; mitochondria in all the experiments were equilibrated with the medium at 25°C for 1 min before recording the O₂ uptake. The arrows indicate the addition of: A, 2 mM-citrate; B, 2 mM-*cis*-aconitate; C, 0.55 mM-ADP; D, oxalomalate, 0.1 mM in experiments I and IV and 0.6 mM in experiment III. Control (curve II): respiratory control index 5.14; O₂ uptake in state 3 = 33.4 μl/h per mg of protein; ADP/O ratio 2.28. Control (curve V): respiratory control index 8.0; O₂ uptake in state 3 = 59.4 μl/h per mg of protein; ADP/O ratio 2.43.

Effect of oxalomalate on mitochondrial and cytoplasmic aconitate hydratase. The effect of oxalomalate on both the preparations was studied with either citrate or isocitrate as substrate. The results (Figs. 2 and 3) show that the addition of 0.2 mM-oxalomalate to the mitochondrial or to the cytoplasmic enzyme inhibited the conversion of both citrate and isocitrate into *cis*-aconitate.

The mitochondrial enzyme (Fig. 2) is more rapidly inhibited than the cytoplasmic enzyme (Fig. 3). Thus 1 min after the addition of oxalomalate the amount of *cis*-aconitate formed from citrate by the mitochondrial enzyme was 80% less than the amount formed in the control experiment (Fig. 2) and the corresponding value for the cytoplasmic enzyme was 50% less than the control (Fig. 3). The results of experiments comparing the amount of *cis*-aconitate formed from isocitrate were similar to the above, i.e. 60% less than the control in the mitochondrial (Fig. 2) and 25% less in the cytoplasmic preparation (Fig. 3). A comparison of the

Fig. 2. Effect of oxalomalate on solubilized rat liver mitochondrial aconitate hydratase. Each incubation mixture, prepared directly in the quartz cuvettes of a Beckman spectrophotometer, contained 0.05 M-phosphate buffer, pH 7.4, 30 mM-citrate or 10 mM-DL-isocitrate. The reaction was started by adding 0.1 ml of enzyme preparation corresponding to 0.32 mg of protein. Oxalomalate (0.2 mM, final concentration) was added 2 min after the start of the reaction (arrow). The final volume was 3 ml. ●, DL-Isocitrate; ○, effect of oxalomalate; ▲, citrate; △, effect of oxalomalate.

Fig. 3. Effect of oxalomalate on rat liver extramitochondrial aconitate hydratase. Experimental conditions were as in Fig. 2. The reaction was started by adding 0.1 ml of cytoplasmic enzyme corresponding to 0.43 mg of protein. Oxalomalate (0.2 mM final concentration) was added 2 min after the start of the reaction (arrow). ●, DL-Isocitrate; ○, effect of oxalomalate; ▲, citrate; △, effect of oxalomalate.

results during the successive minutes (3–6 min of Figs. 2 and 3), indicates that the inhibition by oxalomalate increased more rapidly with time in the mitochondrial (Fig. 2) than in the cytoplasmic (Fig. 3) preparation. Therefore it was expected that a preincubation of the aconitate hydratase with oxalomalate would increase the inhibitory effect.

Effect of the preincubation of inhibitor with the enzyme

The preincubation experiments were performed in a spectrophotometer cuvette by mixing the enzyme solution with increasing concentrations of oxalomalate. The reaction was started 2 min later by the addition of the substrate and in the control experiments the reaction was started by the enzyme.

The results (Fig. 4) show that, without preincubation (a and c) and irrespective of the origin of the enzyme, oxalomalate, when added together with the substrates, inhibited the reaction citrate → *cis*-aconitate more than the isocitrate → *cis*-aconitate reaction. After preincubation (b and d) the

difference in degree of inhibition with the two substrates disappeared, and an inhibition comparable with that without preincubation was obtained at a tenfold lower concentration of oxalomalate.

With regard to the sensitivity of the two preparations to the inhibitor, the results confirm those of Figs. 2 and 3, i.e. that oxalomalate, particularly at the lower concentrations, inhibited the mitochondrial more than the soluble enzyme.

We have also investigated whether preincubation affected the mechanism of inhibition with citrate as substrate. The results (Fig. 5) confirm that the inhibition by oxalomalate of mitochondrial or soluble aconitate hydratase is competitive, as previously found with non-preincubated enzyme from pig heart (Ruffo *et al.* 1962). The K_i calculated from the results of Fig. 5 is 2.5×10^{-6} M for the soluble and 1×10^{-6} M for the mitochondrial enzyme. In contrast, when oxalomalate was preincubated for 2 min with either the mitochondrial or the soluble enzyme, the plot (Lineweaver & Burk, 1934) of the results showed that the inhibition, with respect to the substrate, was not of the competitive type.

DISCUSSION

The results reported in the first part of the present paper confirm that oxalomalate inhibits bound mitochondrial aconitate hydratase and show that, at low concentrations of the inhibitor, the oxidation of citrate is inhibited to a greater extent than that of *cis*-aconitate. They also show that the enzymic preparations, either extracted from mitochondria or soluble in the cytoplasm, are inhibited by oxalomalate, the mitochondrial enzyme being more sensitive than the soluble one. These results are similar to those obtained with fluorocitrate and analogous preparations of aconitate hydratase (Guarriera-Bobyleva & Buffa, 1969) and suggest a different sensitivity of the two forms of the enzyme.

With regard to the efficiency of the inhibitors, fluorocitrate inhibited aconitase in intact mitochondria more than oxalomalate (Guarriera-Bobyleva & Buffa, 1969), but oxalomalate was a more powerful inhibitor than fluorocitrate of the enzyme either extracted from mitochondria or soluble in the cytoplasm.

The results reported in the second part show that after preincubation the inhibitory effect of oxalomalate on both the enzymic preparations increases tenfold. The inhibition, which was of a competitive type with respect to citrate without preincubation, became irreversible after preincubation of the enzyme with the inhibitor. This result is also similar to that obtained with fluorocitrate. The observations of Morrison & Peters (1954) and

Fig. 4. Effect of preincubation with oxalomalate on the inhibition of aconitate hydratase. Each cuvette contained in 3 ml of 0.05 M-phosphate buffer, pH 7.4, 30 mM-citrate (●) or 20 mM-DL-isocitrate (○), oxalomalate at the concentrations indicated and 0.40 mg of mitochondrial protein (a and b) or 0.64 mg of cytoplasmic protein (c and d). In (b) and (d) preincubation was performed in the cuvette by allowing oxalomalate to incubate for 2 min with mitochondrial (b) or cytoplasmic (d) enzyme before addition of the substrate. In (a) and (c) the reaction was started by adding the mitochondrial (a) or the cytoplasmic enzyme (c) to the mixture containing the inhibitor and the substrate. The ΔE_{240} between 0 and 3 min was taken as a measure of the enzymic activity.

Peters (1961), that have been confirmed and extended (Fanshier *et al.* 1964; Dummel & Kun, 1969) show that the inhibition of aconitate hydra-

tase by fluorocitrate is competitive only when determined in the first minutes of incubation. After 3–4 min a progressive time-dependent in-

Fig. 5. Inhibition by oxalomalate of aconitate hydratase from rat liver mitochondria and cytoplasm. Experimental conditions were as in Figs. 2 and 3. The ΔE_{240} between 2 and 3.5 min was taken as a measure of the enzyme activity. (a) Mitochondrial enzyme; (b) cytoplasmic enzyme. Concentration of citrate: $\circ$, 20 mM; Δ , 10 mM; $\square$, 5 mM.

inhibition occurs, which cannot be reversed by the addition of substrate. Kun (1969) suggested that the inactivation of the enzyme produced by fluorocitrate depends on the alkylation of the active centre of the enzyme. According to Carrell *et al.* (1970) the alkylation may be produced by cleavage of the C-F bond of the inhibitory isomer (1R:2R) of fluorocitrate linked to the enzyme in a complex similar to that of the substrates.

Oxalomalate, which in our experiments behaved in many respects like fluorocitrate, also presents two centres of asymmetry, comparable with the carbon atoms 1 and 2 of fluorocitrate (for the absolute configuration of the stereoisomers see the paper of Carrell *et al.* 1970). It is possible that only one of the four isomers of oxalomalate, by analogy with fluorocitrate, fits in the active centre of the enzyme, producing competitive inhibition. In that case the different inhibitory power of oxalomalate on the reactions involving respectively citrate, *cis*-aconitate and isocitrate (Table 1, Figs. 1-4) can be explained by a different efficiency of the three substrates in displacing the inhibitor from the enzyme.

This possibility is consistent with the mechanism of action of aconitase proposed by Glusker (1968),

based on the formation of three-dimensional complexes involving Fe^{2+} between the substrates and the active site of the enzyme.

The mechanism of inhibition of the enzyme by oxalomalate was in many respects similar to that produced by fluorocitrate, but the mechanism of inactivation must be different. In fact oxalomalate is not an alkylating agent, and unlike fluorocitrate (Fanshier *et al.* 1962) produces inactivation also of isocitrate dehydrogenase (Ruffo & Adinolfi, 1963).

In conclusion, the inhibition of either the mitochondrial or the soluble aconitate hydratase by oxalomalate suggests that a complex is formed between the inhibitor and a single active site of the enzyme. This interpretation refers to either the inhibition or the inactivation of the enzyme and is in agreement with the mechanism of aconitate hydratase action proposed by Glusker (1968).

REFERENCES

- Anfinsen, C. B. (1955). In *Methods in Enzymology*, vol. 1, p. 695. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Carrell, H. L., Glusker, J. P., Villafranca, J. J., Mildvan, A. S., Dummel, R. J. & Kun, E. (1970). *Science, N.Y.*, **170**, 1412.
- Dickman, S. R. (1961). In *The Enzymes*, vol. 5, p. 495. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.
- Dickman, S. R. & Speyer, J. F. (1954). *J. biol. Chem.* **206**, 65.
- Dummel, R. J. & Kun, E. (1969). *J. biol. Chem.* **244**, 2966.
- Ettinger, R. H., Goldbaum, L. R. & Smith, L. H. (1952). *J. biol. Chem.* **199**, 531.
- Fanshier, D. W., Gottwald, L. K. & Kun, E. (1962). *J. biol. Chem.* **237**, 3588.
- Fanshier, D. W., Gottwald, L. K. & Kun, E. (1964). *J. biol. Chem.* **239**, 425.
- Glusker, J. P. (1968). *J. molec. Biol.* **38**, 149.
- Guarriera-Bobyleva, V. & Buffa, P. (1969). *Biochem. J.* **113**, 853.
- Kun, E. (1969). In *Citric Acid Cycle*, p. 297. Ed. by Lowenstein, J. M. New York: Marcel Dekker Inc.
- Lineweaver, H. & Burk, D. (1934). *J. Am. chem. Soc.* **56**, 658.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Morrison, J. F. & Peters, R. A. (1954). *Biochem. J.* **58**, 473.
- Peters, R. A. (1961). *Biochem. J.* **79**, 261.
- Peters, R. A. & Shorthouse, M. (1969). *Nature, Lond.*, **221**, 774.
- Racker, E. (1950). *Biochim. biophys. Acta*, **4**, 211.
- Ruffo, A., Testa, E., Adinolfi, A. & Pelizza, G. (1962). *Biochem. J.* **85**, 588.
- Ruffo, A. & Adinolfi, A. (1963). *Biochem. J.* **89**, 50P.
- Ruffo, A., Adinolfi, A. & Malcovati, M. (1966). In *Biochim. biophys. Acta Library*, vol. 7, p. 518: *Regulation of Metabolic Processes in Mitochondria*. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. Amsterdam: Elsevier Publishing Co.