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Oxalomalate as inhibitor and substrate of isocitrate (NADP) dehydrogenase

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Biochimica. — *Oxalomalate as inhibitor and substrate of isocitrate (NADP) dehydrogenase.* Nota di ANNA ADINOLFI, GIOVANNI MENNA e ALFREDO RUFFO (*), presentata (**) dal Corrisp. A. RUFFO.

RIASSUNTO. — Nel corso di uno studio sul meccanismo d'inibizione della isocitrato (NADP) deidrogenasi è stato trovato che l'ossalomalato in opportune condizioni viene ridotto dall'enzima ad una velocità pari a quella del substrato naturale. Tuttavia ulteriori risultati escludono che l'inibizione dipenda dalla riduzione del gruppo chetonico e ribadiscono il ruolo preminente del coenzima nel produrre inibizione.

SYNOPSIS

The mechanism of inhibition produced by oxalomalate and by its decarboxylation product hydroxyoxoglutarate has been investigated on a mitochondrial preparation of isocitrate (NADP)-dehydrogenase purified from beef heart. It has been found that both the compounds were reduced by the enzyme at a rate comparable with that of either the natural substrate or of oxaloacetate. The possibility that the enzymic reduction may be responsible for the inhibition is excluded by further results showing that the chemical reduction of the three oxoacids abolished their inhibitive effect. Full inhibition was realized when the oxoacids were preincubated with the enzyme in the presence of the coenzymes suggesting their preminent role in the inhibitory process.

INTRODUCTION

Oxalomalate, a tricarboxylic acid synthetized under physiological conditions of temperature and pH by the reaction of glyoxylate with oxaloacetate, was found to inhibit at very low concentration isocitrate (NADP) dehydrogenase (E. C. 1.1.42) (Ruffo *et al.*, 1967, 1974; Adinolfi *et al.*, 1969). More recently Ingebretsen (1976) in the course of research on the inhibitory effect of either a mixture of glyoxylate plus oxaloacetate or oxalomalate on purified isocitrate (NADP) dehydrogenase, confirmed these results and suggested that the inhibitor affected the enzyme in several ways, probably by interfering with a site different from that of the substrate.

On the other hand oxaloacetate, which structure resembles the molecule of oxalomalate, has been reported either to inhibit (Shio and Ozaki, 1968) or to be reduced by isocitrate dehydrogenase (Illingworth and Tipton, 1970).

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Therefore we investigated whether also oxalomalate may be reduced by the enzyme and extended the investigation to other oxoacids—some of them known to inhibit the enzyme (Peyes and Laties, 1963; Shio and Ozaki, 1968)—in order to establish whether the property of reducing oxoacids could be correlated with a common mechanism of inhibition of isocitrate dehydrogenase. In the present paper we report the results obtained by incubating oxalomalate and the other oxoacids listed in Table I with a purified preparation of the enzyme extracted from beef heart mitochondria. Aliquots of the preincubated samples were assayed for dehydrogenase activity using isocitrate as substrate.

MATERIALS AND METHODS

Oxoacids, D–L-isocitrate (Type 1) were from Sigma (St. Louis, USA); oxidized and reduced coenzyme grade 1 were from Boehringer (Mannheim, Germany). All other reagents were analytical grade from C. Erba (Milano, Italy) or from E. Merck (Darmstat. Germany).

Chemical and analytical methods. 4–Hydroxy–2–oxoglutarate was prepared by acidification of oxalomalate (Ruffo *et al.*, 1967). Chemical reduction of oxalomalate and hydroxyoxoglutarate was obtained by incubating a solution of the oxoacid with 10–fold excess of sodium borohydride. After 2 hours at 4 °C the pH was lowered to 3 to remove the excess of reagent and the solution neutralized before use. Ketonic functions were determined by the colorimetric method of Lardy (1949).

Preparation of the enzyme and assay methods. Isocitrate dehydrogenase was purified from beef heart by fractioning the mitochondrial extract with ammonium sulphate (between 50–80 % saturation), gel filtration on P–200 Biorad and ion exchange chromatography on CM–50 Sephadex. The enzyme eluted from this column by a linear gradient of 30 mM $MgSO_4$ and 90 mM Na_2SO_4 was further purified by gel filtration on P–200 Biorad (Melzi d'Eril *et al.*, 1980). The enzyme activity was measured at 340 nm in a Beckman recording spectrophotometer (Acta III) at 25 °C. The assay mixture contained in the final volume of 3 ml : 1.33 mM D–L–isocitrate; 33 mM Tris–HCl buffer pH 7.4; 0.1 mM NADP; 6.25 mM $MgCl_2$; 1 μM EDTA.

The assay medium for the reduction of oxoacids contained in 3 ml of final volume: 10 mM K–phosphate buffer pH 6.8; 0.125 mM NADPH; 25 mM $MgCl_2$ and 1 mM substrates, in the presence and absence of 0.2 ml of 0.6 M $KHCO_3$ saturated with CO_2.

Proteins were determined at 280 and 260 nm according to the method of Kalkar (1947). The final enzyme preparation had a specific isocitrate-dehydrogenase activity which ranged between 30 and 40 I.U./mg protein and gave only one band on disc electrophoresis in SDS.

Results

Reduction of oxoacids. The reduction of oxoacids by isocitrate dehydrogenase was determined following the experimental conditions previously described as optimal for the reduction of oxaloacetate (Illingworth and Tipton, 1970), except that pH was increased to 6.8 to prevent spontaneous decarboxylation of oxoacids. In Table I the rate of reduction of the oxoacids tested is compared with that of 2–oxoglutarate, the physiological substrate of the enzyme.

TABLE I

Reductive activity of mitochondrial isocitrate dehydrogenase.

Incubation mixture contained in the final volume of 3 ml: 10 mM K–phosphate buffer pH 6.8; 1.25 mM NADPH; 25 mM $MgCl_2$; 1 mM oxoacids in the presence or in the absence of 0.2 ml of 0.6 M $KHCO_3$ saturated with CO_2; 0.02 ml of the enzyme solution corresponding to 0.34 I.U. were added last to start the reaction. Readings at 340 nm were taken for 3 min. and the decrement of O.D. between 30 sec. and 1 min. 30 sec. was chosen to determine the activity.

Substrates (1 mM)	ΔE/min		Δ%
	no $KHCO_3$	+ $KHCO_3$	
2 oxoglutarate	0	0.100	—
Oxalomalate	0.160	0.070	— 56
Hydroxyoxoglutarate	0.180	0.085	— 53
Oxaloacetate	0.110	0.050	— 54
Glyoxylate	0.025	—	—
Other oxoacids (*)	0	0	0

(*) acetoacetic; acetopyruvic; 2-oxobutyric; 2-oxogulonic; oxomalonic; 2–oxovaleric; 4–oxovaleric (levulinic); pyruvic.

The results show that among them only oxaloacetate, oxalomalate, hydroxyoxoglutarate and to a lesser extent glyoxylate were reduced by the enzyme. The slight rate of reduction of glyoxylate did not increase even when the concentration of enzyme increased four times. The presence of CO_2, which was required for the reduction of oxoglutarate, inhibited by about 50 % the reduction of the other oxoacids. NADH did not replace NADPH and no reduction was observed in the absence of Mg^{2+}. NADP was identified as the product of the reaction by adding an excess of isocitrate which regenerated NADPH. The concentration of oxoacids in the deproteinized filtrates determined by colorimetric assay of ketonic functions showed a decrease of 10 %,

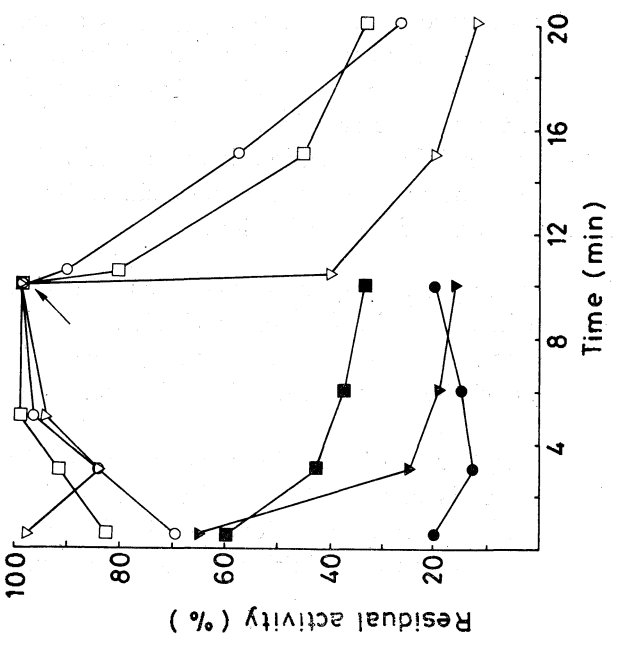

Fig. 1 *a*.

Fig. 1 *b*.

Fig. 1. – Dependence of inhibition on the presence of coenzymes. Sample preincubated at 25 °C contained in 3 ml: 10 mM K-phosphate buffer pH 6.8; 25 mM $MgCl_2$; 1 mM oxoacids; 1.8 I.U. of the enzyme in the presence (filled symbols) and in the absence (open symbols) of 1.25 mM coenzymes. At the time indicated aliquots of 0.1 ml were taken for determining the activity of isocitrate dehydrogenase. After 10 minutes (see arrow) 1.25 mM NADPH (*a*) or $NADP^+$ (*b*) were added to the samples preincubated without coenzymes. The assays for the dehydrogenase activity contained: 33 mM Tris–HCl buffer pH 7.4; 0.1 mM $NADP^+$; 6.25 mM $MgCl_2$; 1 μM EDTA and 1.33 mM D-L-isocitrate. The results are expressed as percentage of residual activity with respect to a control preincubated without oxoacids. (○ and ●) Oxalomalate; (□ and ■) hydroxyoxoglutarate; (△ and ▲) oxaloacetate.

in good correlation with the observed oxidation of the coenzyme. However neither D-malate, indicated by Illingworth and Tipton (1970) as a product of enzyme reduction of oxaloacetate nor the compounds prepared by chemical reduction with $NaBH_4$ from both oxalomalate and hydroxyoxoglutarate were oxidized by the enzyme in the reverse reaction.

In order to investigate the possible correlation between reduction of oxoacids and inhibition of the enzyme we determined the residual isocitrate dehydrogenase activity in the course of the reduction.

Dependence of inhibition on the presence of coenzyme. Sample of composition described in Table I were incubated for the intervals indicated in Fig. 1. Aliquots were taken to measure the residual isocitrate dehydrogenase activity, in comparison with the samples of the same composition but without the oxoacids.

The results in Fig. 1 *a* show that isocitrate dehydrogenase activity is strongly inhibited when the enzyme is incubated, even for short periods of time, with oxalomalate, hydroxyoxoglutarate or oxaloacetate in the presence of NADPH. In the absence of coenzyme the inhibition is scarce and quickly

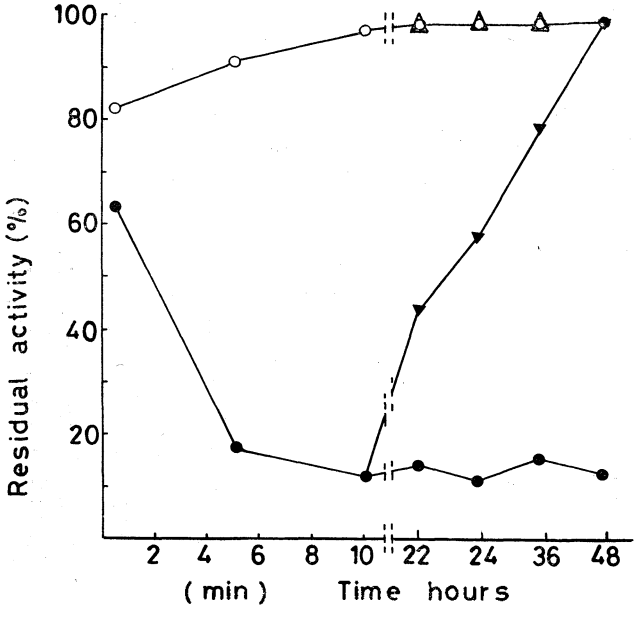

Fig. 2. – Reactivation of the inhibited enzyme by dialysis. A sample containing in 3 ml of 10 mM K-phosphate buffer pH 6.88, 25 mM $MgCl_2$ and 1 mM oxalomalate was preincubated at 25 °C with 3.5 I.U. of enzyme in the absence (open symbols) and in the presence (filled symbols) of 1 mM NADP. After 10 minutes the sample was divided into two parts. One (△ and ▲) was dialyzed against the same buffer in the cold room while the other was left for the same period without dialysis (○ and ●). At the time indicated aliquots of 0.05 ml were added to the assay mixtures for determining the activity of isocitrate-dehydrogenase (see Fig. 1). The results are expressed as residual activity with respect to a control preincubated without oxalomalate and dialyzed for the same period.

disappearing but it is immediately restored by the addition of NADPH. Similar results are obtained by substituting NADPH with NADP, as reported in Fig. 1 *b*. This rules out the possibility that the inhibition may be due to the hydroxyacids formed by the enzymic reduction of the oxoacids. Moreover, neither D–malate nor the hydroxyacids prepared by chemical reduction of either oxalomalate or hydroxyoxoglutarate inhibited the isocitrate dehydrogenase activity. The concentration of coenzymes required to observe inhibition is very low. For instance the inhibition produced by 1 mM oxalomalate rose from 8 % to 50 % when preincubation was carried out in the presence of either NADP or NADPH both 0.05 μM. Neither NAD^+ nor NADH replaced the specific coenzymes in producing inhibition. The addition of 1 mM isocitrate to the preincubation mixture containing oxalomalate and NADPH abolished the inibition while oxoglutarate at a concentration 10 times higher protected only partially.

Reactivation by dialysis. Our previous results (Ruffo *et al.*, 1967) and the more recent ones by Ingebretsen (1976) had shown that inhibition of isocitate dehydrogenase by oxalomalate was time–dependent and suggested possible inactivation of the enzyme. To investigate this point we incubated the enzyme with 1 mM oxalomalate and 1 mM NADPH (Fig. 2). After 10 minutes, when the residual isocitrate dehydrogenase activity was only 10 % of that the control incubated without oxoacids, an aliquot of the sample was extensively dialyzed at 5 °C against phosphate buffer pH 7.6 containing 10 % (v/v) glycerol, 0.1 mM mercaptoethanol and 1 mM EDTA.

The results in Fig. 2 show that dialysis restored the isocitrate dehydrogenase activity, which reached after 48 hours the same values as that of the control dialyzed for the same period of time against the same buffer. Inhibition was unchanged in the undialyzed sample.

DISCUSSION

The observation that oxalomalate, hydroxyoxoglutarate and oxaloacetate serve as either substrate or inhibitor of the enzyme, supports the view that the inhibition depends on the interaction of the 3 oxoacids with the active site. Since at the end of preincubation we found that only 10 % of ketonic groups were reduced by the enzyme, it seems confirmed that the presence of the ketonic function is responsible for the inhibition (Ruffo *et al.*, 1967, 1969, 1974). We suggest the formation of a complex between the keto–groups, Mg^{2+} and coenzymes able to be linked by the active site. Accordingly the inhibition disappeared when the enzyme was preincubated either with the oxoacids previously reduced by $NaBH_4$ or in the absence of the coenzymes. The preceding results showing that either isocitrate (Colman, 1972) or oxalomalate (Ingebretsen, 1976) react preferentially with the enzyme when complexed with metal-ions in the presence of the coenzymes, further support the prospected mechanism of inhibition. Also the protection by isocitrate and

the recovery of the activity after dialysis, excluding any irreversible structural change of the enzyme, are in agreement with the suggested interaction of the complex for the catalytic site.

In conclusion, the observation that the three powerful inhibitors of isocitric-dehydrogenase, oxalomalate, hydroxyoxoglutarate and oxaloacetate, by diminishing the value of pH and by increasing the reduced state of the coenzyme may be converted into inactive hydroxyacids by the enzyme itself, suggests that the system oxoacids-coenzymes may be used by mitochondria for autoregulating the enzyme activity.

REFERENCES

ADINOLFI A., MORATTI R., OLEZZA S. and RUFFO A. (1969) – « Biochem. J. », *114*, 513–518.
COLMAN R. F. (1972) – « J. Biol. Chem. », *247*, 215–223.
ILLINGWORTH J. A. and TIPTON K. F. (1970) – « FEBS Letters », *7*, 157–160.
INGEBRETSEN O. C. (1976) – « Biochim. Biophys ». Acta *452*, 302–309.
KALKAR H. M. (1947) – « J. Biol. Chem. », *167*, 461–475.
LARDY A. H. (1949) – in Monometric techniques and tissue metabolism. (Umbreit, W. W., Burris, R. H. and Stanffer, J. F., eds), pp. 162–165, Burgess Publ. Co., Minneapolis.
MELZI D'ERIL G. L., MORATTI R., PASQUINELLI R. and SANTACROCE M. (1980) – « Rend. Acc. Naz. Lincei ». In the press.
PEYES B. and LATIES G. G. (1963) – « Biochem. Biophys. Res. Commun. », *10*, (460–466).
RUFFO A., TESTA E., ADINOLFI A., PELIZZA G. and MORATTI R. (1967) – « Biochem. J. », *103*, 19–23.
RUFFO A., MORATTI R., MONTANI A. and MELZI D'ERIL G. L. (1974) – « Ital. J. of Biochem. *23*, 357–370.
SHIO I. and OZAKI H. (1968) – « J. of Biochem. », *64*, 45–53.