Inhibition by alloxan of mitochondrial aconitase and other enzymes associated with the citric acid cycle

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Considerable variations were found in the in vitro effect of alloxan on mouse liver enzymes associated with the citric acid cycle. The following approximative alloxan concentrations induced 50% inhibition of enzyme activity: 10^{-6} M for aconitase, 10^{-4} M for NAD-linked isocitrate dehydrogenase, glutamate dehydrogenase, α-ketoglutarate dehydrogenase, succinyl-CoA synthetase and fumarase, and 10^{-3} M for citrate synthase and NADP-linked isocitrate dehydrogenase. Pyruvate dehydrogenase, succinate dehydrogenase and malate dehydrogenase were not inhibited by 10^{-3} M alloxan. The inhibition of aconitase was competitive both when using mouse liver and purified porcine heart enzyme. The K_i values for the purified enzyme in the presence of 5 μM alloxan were 0.22 μM with citrate, 4.0 μM with *cis*-aconitate and 0.62 μM with isocitrate as substrate. The high sensitivity of aconitase for inhibition by alloxan probably plays a prominent role for the toxic effects of alloxan.

Aconitase *Alloxan* *Citric acid cycle* *Enzyme* *Mitochondria*

1. INTRODUCTION

Alloxan is taken up in vivo and in vitro in mitochondria of different mouse organs, including those from the endocrine pancreas which show significantly highest in vivo uptake [1,2], and affects different mitochondrial functions, e.g., the accumulation and retention of different ions [3–5]. Isolated mouse liver mitochondria incubated with alloxan exhibit enhanced state 4 respiration with succinate and inhibited state 4 respiration with pyridine-linked substrates, whereas state 3 respiration is decreased with both kinds of substrates [5]. Moreover, inhibition of the oxidation of pyridine-linked substrates, but unaffected or only slightly decreased oxidation of succinate has been observed in mouse islets isolated 10 min following the injection of alloxan [6], and in rat kidney homogenate exposed to alloxan in vitro [7].

Since divergent effects of alloxan on FAD-linked and pyridine-linked respiration inter alia might be due to different inhibitory influences on pyruvate dehydrogenase and the enzymes directly associated with the citric acid cycle, this work was initiated to study whether alloxan affects the activity of those enzymes. The activities of the pyruvate, succinate and malate dehydrogenases were not affected by alloxan, whereas most other enzymes investigated were inhibited, the inhibition being especially marked for aconitase which for this reason was studied in more detail.

2. METHODS

The enzyme activities were determined in sonically disrupted isolated liver mitochondria of 3–4-month-old C57BL-KsJ − +/+ mice of either sex, starved for 21 h. Additionally purified aconitase from porcine heart (Sigma, St Louis, MO) was used. The determinations were carried out at 25°C.

Fe^{2+} and reductants activate aconitase [8]. Based on pilot experiments performed because reductants might affect the action of alloxan [4], 1 mM Fe^{2+} and 50 μM cysteine were chosen for activation.

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Since those experiments showed high activity of aconitase in fresh mitochondrial preparations not treated with Fe^{2+} or reductant, non-activated aconitase activity was also studied to avoid the possible interaction of reductants with the inhibitory effects of alloxan. Corrections were made for the influence of alloxan on the determinations at 232, 235 and 240 nm.

The aconitase activity was measured by following the appearance and disappearance of *cis*-aconitate at 240 nm with citrate, *cis*-aconitate and isocitrate as substrates [9]. Fumarate formation was similarly studied at 240 nm [9]. NAD- and NADP-linked isocitrate dehydrogenase, and glutamate, α-ketoglutarate, malate and pyruvate dehydrogenases were all measured by following the reduction of pyridine nucleotides at 340 nm; the measurement of α-ketoglutarate dehydrogenase being carried out after ultracentrifugation at 120000 × *g* for 1 h in the presence of CoA, and the measurement of pyruvate dehydrogenase being carried out in the presence of CoA and thiamine pyrophosphate, all additions made at a concentration of 1 mM [10]. The activity of citrate synthase was determined at 232 nm [11], that of succinyl-CoA synthetase was followed at 235 nm [12] and that of succinate dehydrogenase was studied at 600 nm [13].

3. RESULTS AND DISCUSSION

Determination of the concentration of alloxan inducing 50% inhibition of different mitochondrial enzymes showed that aconitase was inhibited at a concentration of 10^{-6} M, whereas higher concentrations were needed for inhibition of citrate synthase, succinyl-CoA synthetase, and the NAD- and NADP-linked isocitrate and the glutamate, α-ketoglutarate and malate dehydrogenases (table 1). The activities of the succinate, malate and pyruvate dehydrogenases were not at all inhibited at concentrations of alloxan up to 10^{-3} M.

These data are consistent with the finding that alloxan inhibits the respiration with glutamate plus malate in isolated liver mitochondria [5], and the oxidation of some pyridine-linked substrates by kidney homogenates [7], and prevents the raise in oxygen uptake in isolated pancreatic islets after addition of glucose, citrate or oxaloacetate [6]. The present data also agree with the observation that rather high concentrations of alloxan are needed for induction of changes in the handling of ions, membrane potential and volume of isolated liver and islet mitochondria when the substrate is succinate [3–5], whereas lower concentrations of alloxan induce such changes when pyridine-linked substrates are used (unpublished).

Since the data presented in table 1 indicated that aconitase was especially sensitive to inhibition by alloxan, this inhibition was studied more thoroughly. Both when using mouse liver mitochondrial aconitase and the purified enzyme from porcine heart, alloxan behaved essentially as a competitive inhibitor regardless of the substrate (figs 1 and 2). The K_i values for the different substrates in the presence of 5 μM alloxan show that the reaction started with *cis*-aconitate is less

Table 1

Concentrations of alloxan inducing 50% inhibition of the activity of enzymes associated with the citric acid cycle of mouse liver mitochondria

Enzyme	Substrate	Alloxan concentration (M)
Citrate synthase	oxaloacetate	8.5×10^{-4}
Aconitase	citrate	6.9×10^{-6}
Aconitase	*cis*-aconitate	2.8×10^{-5}
Aconitase	isocitrate	9.2×10^{-6}
NAD-linked isocitrate dehydrogenase	isocitrate	1.5×10^{-4}
NADP-linked isocitrate dehydrogenase	isocitrate	1.2×10^{-3}
Glutamate dehydrogenase	glutamate	3.8×10^{-4}
α-Ketoglutarate dehydrogenase	α-ketoglutarate	2.8×10^{-4}
Succinyl-CoA synthetase	succinate	3.2×10^{-4}
Succinate dehydrogenase	succinate	no inhibition[a]
Fumarase	malate	1.4×10^{-4}
Malate dehydrogenase	oxaloacetate	no inhibition[b]
Pyruvate dehydrogenase	pyruvate	no inhibition[b]

[a] Highest concentration studied: 5 mM
[b] Highest concentration studied: 1 mM

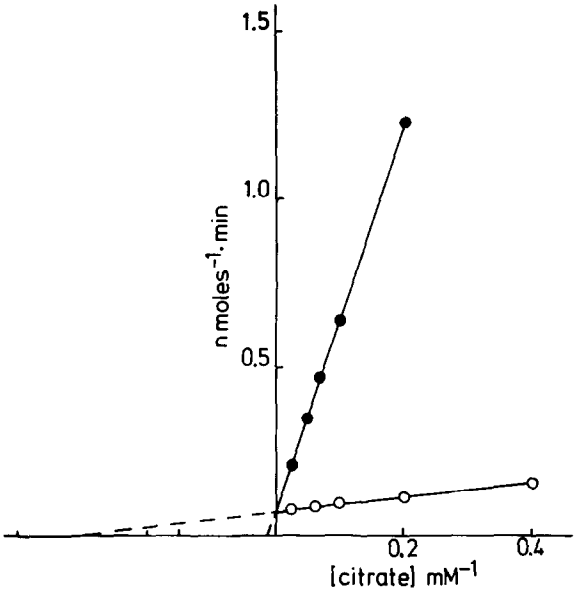

Fig.1. Lineweaver-Burk plot of porcine heart aconitase in the absence (○—○) and presence (●—●) of 5 μM alloxan.

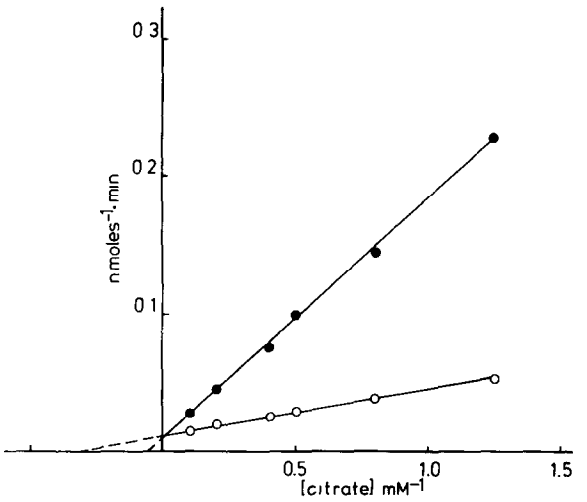

Fig.2. Lineweaver-Burk plot of aconitase activity in mouse liver mitochondria in the absence (○—○) and presence (●—●) of 5 μM alloxan.

sensitive to inhibition by alloxan than that started with either citrate or isocitrate (table 2). The K_m and K_i values for the inhibition of the purified enzyme were not very different from the apparent K_m and K_i values for the liver mitochondrial enzyme. No significant difference in inhibitory effect of alloxan was found between activated and non-activated aconitase.

The reason why the aconitase reaction starting with *cis*-aconitate is less sensitive to inhibition by alloxan than that starting with citrate or isocitrate is not known. A similar difference has been reported for fluorocitrate [14–16], which represents a potent aconitase inhibitor. That difference has been suggested to be due to a greater affinity of *cis*-aconitate for the enzyme than the enzyme affinity of citrate or isocitrate [14,15]. Since a simple binding of inhibitor to enzyme would be expected to give the same K_i value regardless of the substrate, the present K_i values may indicate a complex interaction between aconitase and alloxan, which is well known to react, e.g., with thiol groups and metal ions.

The K_i values obtained indicate that alloxan, compared with other known inhibitors [17], is a very potent inhibitor of mitochondrial aconitase.

Table 2

Kinetic values for activated purified porcine aconitase and apparent values for mouse liver mitochondrial aconitase

Substrate	Purified porcine aconitase			Mouse aconitase		
	K_m (mM)	k_{cat} (mkat·kg^{-1})	K_i (μM)	K_m (mM)	k_{cat} (mkat·kg^{-1})	K_i (μM)
Citrate	3.3	92	0.22	3.0	2.4	1.1
cis-Aconitate	0.12	76	4.0	–	–	–
Isocitrate	0.88	132	0.62	0.90	3.9	0.92

5 μM alloxan was used for determination of K_i. The values represent the means of data obtained from Lineweaver-Burk and Eadie-Hofstee plots and direct computerized calculation

Our early electron microscopic studies of the insulin-producing cells in the pancreas of mice injected with alloxan suggested that alloxan diabetes might be due to mitochondrial damage [18,19], and subsequent biochemical investigations supported this opinion [1–5]. The present data indicate that the primary target for the toxic action of alloxan may be mitochondrial aconitase. Interestingly, there are a few reports of disturbed glucose metabolism, hyperglycemia and ketonemia in animals in which aconitase has been inhibited by fluorocitrate [20,21], although other symptoms predominate in fluorocitrate poisoning.

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