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Inactivation of α-ketoglutarate dehydrogenase during oxidative decarboxylation of α-ketoadipic acid

V.I. Bunik and O.G. Pavlova

A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russian Federation

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α-Ketoglutarate dehydrogenase was inactivated irreversibly and completely during oxidation of α-ketoadipic acid. The inactivation was revealed both in the model system with ferricyanide and in the overall reaction catalyzed by the α-ketoglutarate dehydrogenase complex. Neither substrate depletion nor product accumulation induced the inactivation. The results obtained were compared with recent data on the enzyme inactivation during oxidation of α-ketoglutaric acid The differences in the inactivation kinetics observed with the two substrates of the enzyme were analyzed. They seem not to reflect the different mechanisms of the inactivation, but, rather, depend on the changes in the rates of the individual stages of the process.

α-Ketoglutarate dehydrogenase; Inactivation during catalysis; α-Ketoadipic acid; Substrate analog; Regulatory site

1. INTRODUCTION

α-Ketoglutarate dehydrogenase (KGD), the first component of the multienzyme KGD complex, catalyses α-ketoglutaric acid (KG) oxidative decarboxylation concomitant with reductive succinylation of lipoic acid covalently attached to the second component of the complex. The activity of individual KGD is assayed in model reactions with artificial electron acceptors (ferricyanide, 2,6-dichloroindophenol). Recently we have shown that KGD from pigeon breast muscle is inactivated during its catalytic action upon KG both in the model system and when the KGD complex functions as a whole, in the absence of ferricyanide [1]. This work deals with the KGD inactivation during reaction with another α-keto substrate, α-ketoadipic acid (KA). The KGD inactivation is shown to proceed also in this case, but the kinetics of the process is different. The data obtained suggest that the differences observed are due to the lower catalytic reactivity of the enzyme intermediate formed in the reaction with KA.

2. MATERIALS AND METHODS

The chemicals used were from the following sources: KG, DTNB, NAD^+ from Serva; KA, malonate and coenzyme A (CoA) from Sigma; ferricyanide from Merck. Succinyl phosphonate (SP) was synthesized according to [2]

Correspondence address. V.I. Bunik, A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russian Federation.

Abbreviations: KGD, α-ketoglutarate dehydrogenase, KG, α-ketoglutaric acid; KA, α-ketoadipic acid; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic) acid; CoA, coenzyme A; SP, succinyl phosphonate.

KGD and the KGD complex were isolated from pigeon breast muscle and their activities were determined as in [3]. The curves of product accumulation were analyzed by the modification of Huggenheim's method [4] as in [5]. Regeneration of the enzyme from the reaction media was performed by the gel-centrifugation technique [6] using Sephadex G-50 fine in 0.04 M potassium phosphate, pH 7.0.

3. RESULTS

Fig. 1a shows that KGD was completely inactivated during oxidation of KA. The decrease in the rate of catalysis was observed during the reaction both in the model system with ferricyanide (curve 1) and in the overall reaction of NADH reduction catalyzed by the KGD complex (curve 2). Linearization of the data from Fig. 1a in a semi-logarithmic Huggenheim's plot [4,5] (Fig. 1b) allows one to determine the inactivation rate constant ($k = 0.37$ min^{-1}), its value being identical for the two reactions: model and overall ones.

The KGD inactivation during reaction with KA, like the process in the system with KG [1], was profound only in the complete reaction media, KA or ferricyanide alone having minor influences on the enzyme activity (Fig. 2).

The experiment on several subsequent enzyme additions to the reaction media containing 1 mM KA and 0.7 mM ferricyanide showed that the inactivation could not be explained by product accumulation: the initial activity ($A = 0.036$ mkmol/min per mg) and the inactivation rate constant ($k = 0.26$ min^{-1}) of the second KGD addition (following the first one after complete inactivation) were the same as the values determined for the first portion of the enzyme ($A = 0.035$, $k = 0.26$).

Regeneration of the enzyme from the reaction media by the method of gel-centrifugation chromatography

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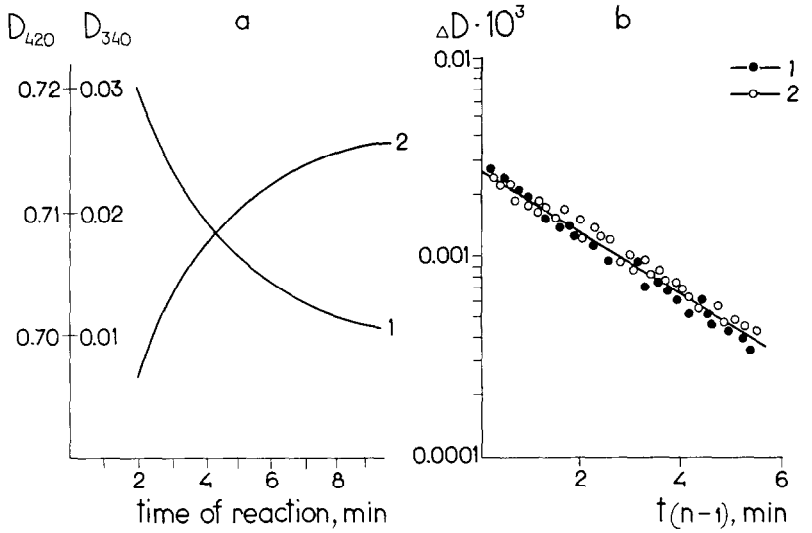

Fig. 1. KGD inactivation during KA oxidation in the model reaction with 1 mM KA and 0.7 mM ferricyanide (D_{420}, curve 1) and in the overall process catalyzed by the KGD complex with 1 mM KA, 0.1 mM NAD^+ and 0.1 mM CoA (D_{340}, curve 2). The records of product accumulation (Fig. 1a) were linearized (Fig. 1b) by modified Huggenheim's method as in [5] with t = 10 s.

[6], as well as the subsequent 1.5 h incubation of the regenerated enzyme in 0.04 M potassium phosphate buffer, pH 7.0, did not restore the enzyme activity even after a short inactivation time (Table I). KG addition to regenerated KGD produced no detectable improvement in the reactivation. The activity was not measurable either with KA or KG. Thus, the KGD inactivation observed during KA oxidation cannot be accounted for by isomerization of the reaction intermediate leading to a slowly dissociating enzyme–product complex. Most likely it is due to irreversible modification of an essential enzyme group.

Fig. 3 shows that the KGD inactivation during KA oxidation (Fig. 3a), unlike the reaction with KG (Fig. 3b), had one stage both at low and high KA concentrations. It should be noted that the initial rate of catalysis observed at 1 mM KA (Fig. 3a, curve 2) approximates to the maximum rate of KA oxidation by KGD, although it is about 30% of the initial rate of the reaction with saturating concentration of KG (Fig. 3b, curve 2). So, the enzyme saturation with KA did not induce a change in the inactivation kinetics, as in case with KG (Fig. 3).

As has been shown earlier [1,7], the first step of the KGD inactivation in the reaction with KG is reversible and observed upon binding the second KG molecule to the KGD subunit. Since the kinetics of the process with KA (Fig. 3a) was similar to that observed at sub-saturating KG concentrations (Fig. 3b, curve 1), the question arose as to whether the absence of the two stages of the KGD inactivation in the reaction with KA was

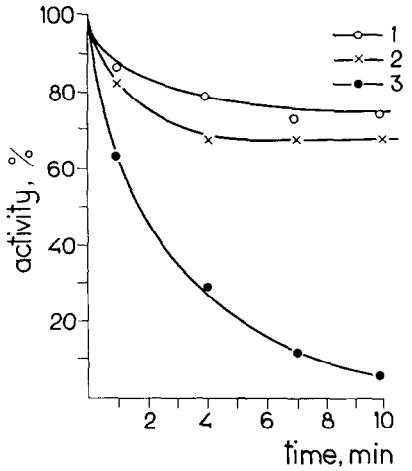

Fig. 2. KGD inactivation during preincubation in 0.05 M potassium phosphate, pH 6.3, with 1 mM KA (1), 0.7 mM ferricyanide (2) and in complete reaction media containing 1 mM KA and 0.7 mM ferricyanide (3). The protein concentration was 0.06 mg/ml

Table I

The activity of KGD inactivated during oxidative decarboxylation of KA and after regeneration from the reaction media

Treatment of KGD	Remaining activity (%) at the catalysis duration for	
	2.5 min	8 min
Inactivation during the reaction with KA	42	9
Regeneration after catalysis	44	6
Regeneration after catalysis with subsequent incubation for 1.5 h at 0°C	12	9

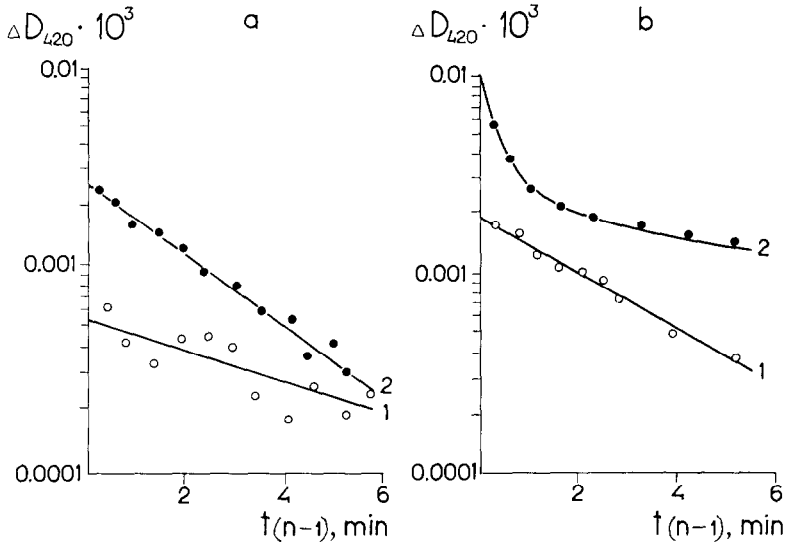

Fig. 3. The dependence of KGD inactivation during catalysis on the concentration of KA (a) or KG (b). Curves 1 and 2 were obtained with t = 24 s at 0.01 and 1 mM concentration of α-ketoacids, respectively. The protein concentration was 0.02 mg/ml.

due to the failure of KA binding to the second enzyme site. If KGD did not bind KA in the non-catalytic site, the first stage of the inactivation would not be observed in the reaction with KA. Then the enzyme inactivation during KA oxidation should be analogous to the second inactivation step in the reaction with KG (the slow phase of curve 2 in Fig. 3b). Indeed, the inactivation is irreversible in both cases, as shown above for the system with KA and in [1] for the slow inactivation step in the reaction with KG.

On the other hand, the difference in the inactivation kinetics observed in the reaction with KA can be due not only to the absence of KA interaction with the non-catalytic site but also to the increased involvement of the reactive intermediate in the irreversible inactivation. In fact, when KGD is saturated with its substrate, the inactivation rate characterized by a slope of the line in a semi-logarithmic Huggenheim's plot is higher in the reaction with KA (Fig. 3a, curve 2), than in the irreversible phase of the inactivation during the reaction with KG (the slow stage on curve 2 of Fig. 3b). Thus, the partially active KGD form could be reversibly produced in the reaction with KA according to the same mechanism as in the process with KG, however, it is possible that this form is inactivated irreversibly before its involvement in catalysis. In this case it would not contribute to product accumulation and the process should become monophasic.

To distinguish between these possibilities we undertook the following experiments.

Let us suggest that the first reversible stage of the inactivation (the rapid phase on curve 2 of Fig. 3b) is not seen in the reaction with KA (Fig. 3a, curve 2) due to the failure of KA binding to the non-catalytic site. Then the KGD activity with KA should not be sensitive to the compounds, which inhibit preferentially the enzyme with the substrate bound to the non-catalytic site. As shown earlier [5], this takes place in the case of the KGD inhibition by malonate and SH-reagents, however, Table II shows that the KA dehydrogenase activity of KGD was sensitive to malonate and DTNB to the same extent as the KG dehydrogenase one. Since malonate and SH-reagents appear to influence the KGD activity decreasing the activatory effect of the substrate bound to the non-catalytic site [5], their inhibition of the KA dehydrogenase reaction is indicative of the KA occupation of this site.

The binding of KA to the KGD non-catalytic site was further supported by the KA-induced reactivation of the enzyme inhibited by SP, the synthetic analog of the KGD substrate, having the phosphonate moiety instead of the α-carboxyl group. Recently we have established that the KGD preincubation with SP leads to isomerization of the enzyme–inhibitor complex, analogous to the first inactivation step during KG oxidation: the enzyme preincubated with SP did not show an initial burst of

Table II

The sensitivity of the initial rate of the KA and KG dehydrogenase reactions to malonate and DTNB

Influencing factor	Remaining activity (%)	
	With KA	With KG
10 min incubation with 0.2 mM DTNB in 0.1 M potassium phosphate, pH 7.0	69	69
1 mM malonate at 0.4 mM α-ketoacid	54	56

activity with KG [7], however, the incubation of KGD with KG, following treatment with SP, restored the burst of activity without the full activity recovering. This indicated the KG binding to the non-catalytic site, since the level of saturation of the catalytic one with SP, testified by remaining inhibition, was unchanged during the enzyme preincubation with KG [7].

We ran an analogous experiment to test the possibility of KA binding to the enzyme with its catalytic site filled with SP. If the enzyme–inhibitor complex did not bind KA, the latter would not induce the initial burst of KG dehydrogenase activity of KGD preincubated with SP, however, Fig. 4 shows this was not the case. The burst eliminated due to isomerization of the KGD–SP complex, observed during preincubation of the enzyme with the inhibitor (Fig. 4, curve 2), was restored after the following interaction of the complex with KA (Fig. 4, curves 3 and 4). The complete reversal of the SP-induced isomerization was reached in 12 min (Fig. 4, curve 4). After this incubation KGD was characterized by the same burst amplitude (the burst amplitude is defined as (A1 − A2)/A1, where A1 is the initial activity and A2 is the activity reached after finishing the burst) (77%) as in control curve 1 of Fig. 4 (74%), however, under the condition of complete burst recovery the level of the catalytic sites filled with SP was still very high. Comparing curves 1 and 4 of Fig. 4 one can see that about half of the catalytic sites are obviously occupied by SP after KA interaction with KGD: this is manifested in approximately 50% remaining inhibition of the KG dehydrogenase reaction, both in the initial and slow phases of product accumulation. In fact, during the preincubation with KA the activity in the slow phase of the KG dehydrogenase reaction was not influenced so much as the initial activity was. This disproportion points to the independent regulation of the activity itself and of the burst amplitude. In other words, KA restores the burst by not replacing SP at the catalytic site. Consequently, it interacts with the enzyme–inhibitor complex, providing the conformational transition of the complex through binding to a site other than catalytic.

Thus, the data obtained (Table II, Fig. 4) contradict the assumption that monoexponential inactivation of KGD during the reaction with KA is due to the failure of KA binding to the non-catalytic site, arguing for the other explanation of the differences observed in the inactivation kinetics.

4. DISCUSSION

This work has shown that another KGD substrate, namely KA, can also occupy the KGD site different from the catalytic one in a manner similar to that established earlier for KG [5,7]. Nevertheless, this does not induce the biphasic product accumulation curves in the catalytic process with KA (Figs. 1 and 3a), inherent in the reaction with KG when KG is bound at the non-catalytic site (Fig. 3b [5,7]).

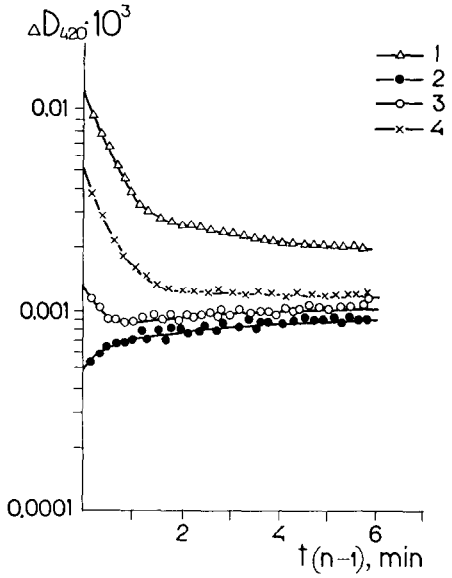

Fig. 4. The KA-induced recovery of the initial activity burst in the KG dehydrogenase reaction, eliminated due to preincubation with 0.003 mM SP (1) Control KGD; (2) KGD preincubated with SP for 3 min; (3) after 3 min KGD preincubation with SP, KA (0.1 mM) was added and incubated further for 1 min; (4) as (3), but the incubation in the presence of KA was for 12 min. The lines were obtained with t = 10 s at 1 mM concentration of KG. The protein concentration was 0.05 mg/ml.

Such a difference can be explained by the different rates of two competitive processes with one intermediate reversibly formed during the enzymatic reaction. On the one hand, this intermediate can be involved in catalysis with the rate of v_{cat}. On the other hand, it can be irreversibly inactivated with the rate of v_{in}. Obviously, when $v_{in} < v_{cat}$, the intermediate will contribute to the product accumulation before it is irreversibly inactivated. Apparently, this contribution causes the slow phase of the p(t) curves in the reaction with high KG concentrations (Fig. 3b, curve 2). When $v_{in} > v_{cat}$, the inactivation becomes monoexponential since the intermediate is irreversibly inactivated before its involvement into the catalytic cycle. This situation seems to take place in the reaction with KA. Really, the observed catalytic rate with KA is lower and the rate of the irreversibly inactivation in the course of this reaction is higher than the analogous values for the reaction with KG (Fig. 3).

The discussed differences in v_{in}/v_{cat} for the two substrates of KGD are consistent also with the fact that KGD binds KA tighter than KG [8]. Indeed, an increase in the KA binding tightness must stabilize the intermediate of the KA reaction subjected to the inactivation. This stabilization had to decrease its catalytic potency, which requires the dissociation of the intermediate into products and free enzyme. Simultaneously the probabil-

ity of the intermediate involvement in the irreversible inactivation is to be increased as compared to the less stable (and consequently more catalytically competent) intermediate formed in the reaction with KG.

Thus, the KGD irreversible inactivation in the course of catalysis is more pronounced in the system with KA. During the reaction with KG it seems to be alleviated by the more effective involvement of the reactive intermediate in catalytic cycle. Obviously, the KGD inactivation during catalysis is connected with the stage of the substrate oxidation, since the enzymatic activity essentially decreases only in the complete reaction media, but not during the preincubation with each substrate alone (Fig. 2, [1]). Nevertheless, the inactivation observed is not inherent in the model reaction with ferricyanide only: there is similar KGD inactivation in the overall reactions with KA (Fig. 1, curve 2) or KG [1], catalyzed by the KGD complex. Thus, the investigated KGD inactivation in the course of catalysis might be a way of muscle KGD regulation.

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REFERENCES

[1] Bunik, V.L., Romasch, O.G. and Gomazkova, V.S. (1990) Biochem. Int. 22, 967–976.
[2] Khomutov, R.M., Osipova, T.I. and Zhukov, Yu.N. (1978) Izvestiya AN SSSR (Russ.) 6, 1391–1394.
[3] Bunik, V I., Buneeva, O A. and Gomazkova, V.S. (1990) FEBS Lett 269, 252–254.
[4] Berezin, I.V. and Klyosov, A.A (1976) The Practical Course of Chemical and Enzyme Kinetics (Russ.), Moscow University edn., Moscow.
[5] Bunik, V.I., Romash, O.G. and Gomazkova, V S. (1991) FEBS Lett. 278, 147–150.
[6] Andersen, K.B. and Vaughan, M.H. (1982) J Chromatogr. 240, 1–8.
[7] Bunik, V.I., Biryukov, A.I. and Zhukov, Yu.N. (1992) FEBS Lett. 303, 197–201.
[8] Bunik, V.I. and Gomazkova, V.S (1987) Biochimiya (Russ.) 52, 1235–1247.