Volume 278, number 2, 147-150 FEBS 09342 January 1991
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ADONIS 001457939100069Q

Effect of α-ketoglutarate and its structural analogues on hysteretic properties of α-ketoglutarate dehydrogenase

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Received 23 November 1990

The burst of product accumulation during the KGD reaction was investigated. It has been shown not to be the obligatory feature of catalysis but appears when increasing the enzyme saturation by KG. Structural analogues of KG and the SH group modification suppress the initial burst without preventing catalysis. The results obtained are in favour of the existence of the regulatory site for binding KG and its structural analogues essential for hysteretic properties of KGD.

α-Ketoglutarate dehydrogenase; Hysteretic property; α-Ketoglutarate analogue; SH group; Regulatory site

1. INTRODUCTION

KGD, a component of the multienzyme KGD complex, catalyses the first and rate-determining step of KG oxidative decarboxylation. Individual KGD catalyses the reaction in the presence of artificial electron acceptors. Both in the model system and when the KGD complex functions as a whole there is a decrease in KGD activity, caused neither by the substrate depletion and the product accumulation nor by changing the oligomeric structure of KGD [1]. Two independent mechanisms of enzyme inactivation have been established corresponding to two stages of the process [1]. The slow one is more expressed in the model system, where irreversible inactivation due to hexacyanoferrate is observed. On the contrary, the fast stage manifests itself to the same degree both in the model reaction and in the natural one; it may be reversed and proceeds only during catalysis. This inactivation leads to the burst in the product accumulation during KG oxidative decarboxylation, suggesting the kinetically slow transition of KGD during catalysis. Such enzymes are referred to as hysteretic ones and the slow changes in their properties are supposed to be important for regulating the complex processes in vivo [2]. In the present investigation the hysteretic properties of KGD were shown to be induced by increasing KG concentration and suppressed in the presence of structural analogues of KG. This regulation appears to be realized through the binding of KG or its structural analogues to the site, different from the catalytic one.

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Abbreviations: KGD, α-ketoglutarate dehydrogenase; KG, α-ketoglutarate

2. MATERIALS AND METHODS

The chemicals used were obtained from the following sources: KG from Serva, potassium hexacyanoferrate from Merck, the structural analogues of the substrate from Sigma. The compounds were of the finest grade available.

KGD was isolated from pigeon breast muscle as in [3]. KGD activity was determined with the artificial electron acceptor hexacyanoferrate (0.7 mM) in 0.05 M potassium phosphate buffer, pH 6.3. The curves of product accumulation were analyzed by the modification of Huggenheim's method [4]. According to it they were divided in n time intervals of t = 20 s. When the inactivation during KGD reaction was not described by the kinetics of the first order reaction, the method of Ray and Koshland [5] was used. The computations were performed on an IBM compatible personal computer with the 'MathCAD' program. The initial rate of product accumulation was determined by extrapolating the curve obtained with the calculated constant values to the zero time. SH-groups of KGD were modified by a 3-min incubation with an equimolar quantity of 4-hydroxymercuribenzoic acid in 0.1 M potassium phosphate, pH 6.0.

3. RESULTS

The semilogarithmic plots of hexacyanoferrate reduction during KGD reaction with different KG concentrations are shown in Fig. 1. According to the method of Huggenheim (see section 2), changes in optical density at 420 nm during the fixed consecutive time intervals are plotted versus reaction time. It can be seen from Fig. 1 that the quantity of the product synthesized in the same time (t = 20 s) is decreased in the course of the reaction (with increasing n). Two stages of the process are evident at high concentrations of KG (Fig. 1, lines 3-5), which is in accordance with earlier experiments [1]. But decreasing the KG concentration leads to the disappearance of the fast stage of inactivation in such a manner that at small substrate concentration practically only the slow stage is displayed (Fig. 1, line 1). This stage appears to be induced by hexacyanoferrate, as was shown earlier [1].

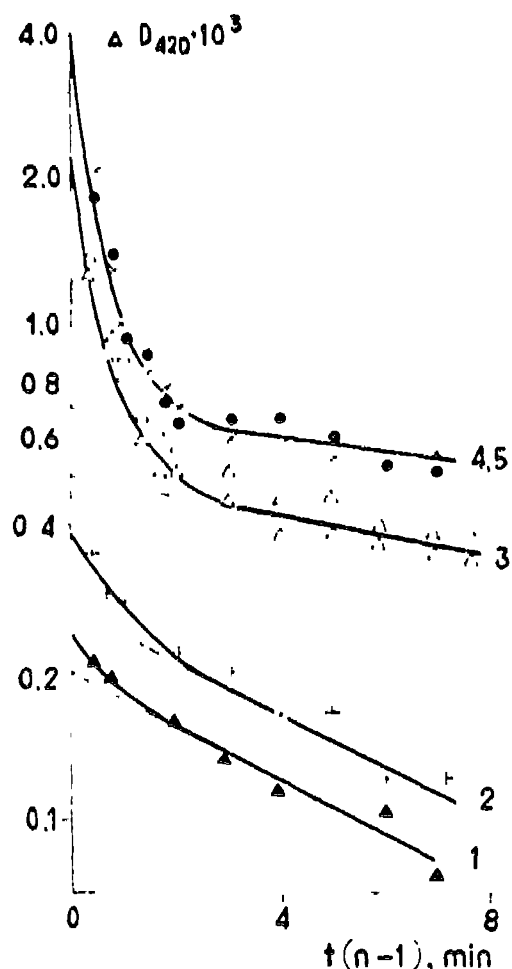

Fig. 1. Huggenheim's plot of product accumulation during KGD reaction at KG concentrations of 0.005 (1), 0.01 (2), 0.05 (3), 0.1 (4) and 1 (5) mM

The fast inactivation step was established to depend neither on the rate of product formation nor to be defined by the depletion of a KG form [1]. The rate of this stage does not allow one to consider it as corresponding to the steady-state achievement So, more and more pronounced with increasing KG concentration, the first stage of the inactivation must correspond to the inactivation of the KGD form, induced by KG. This form interconverts slowly, if at all, with another one, inactivated with a lower rate and existing predominantly at low KG concentration (Fig. 1, lines 1,2) The slow (compared to the inactivation) rate of the interconversion is the necessary condition for observing the biphasicity of the inactivation curve In the case of the rapid interconversion of the two forms, inactivated with different rates, only the change in the inactivation rate would occur [6]. Therefore, increasing the quantity of the catalytic complex of KGD with KG cannot account for the increased contribution of the fast inactivation step when KG concentration grows, since the catalysis rate and consequently, the rate of the interconversion between the catalytic enzyme substrate complex and the free enzyme is much greater than the rate of the fast inactivation

Recently we have shown two forms of KGD to exist [3,7,8] One has the interacting active sites at substrate binding [7], while subunits of another function independently [8]. KG changes the kinetics of inactivation during the KGD reaction, catalysed by both the forms. Moreover, the initial burst is inherent in the immobilized monomer of KGD too [1]. Hence, its appearance is not caused by the subunit cooperation.

All this provides evidence, that fast-inactivated KGD is the enzyme form with the monomers having bound the second KG molecule, in addition to the molecule participating in the catalysis.

Since the fast-inactivation step proceeds only during the enzyme reaction [1], it must be intimately related to the mechanism of catalysis So, its appearance means new catalytic properties of KGD to arise If it is the second KG molecule which induces the enzyme form catalysing the reaction in a different way, the plot of KGD activity versus KG concentration may be non-hyperbolic. In fact, KGD is shown to be characterized by the complex kinetic behavior [3] Analysis of the curves from Fig. 1 allows one to correlate a deviation from the Michaelian kinetics with the appearance of the burst in the product accumulation. As seen from Fig 1 (lines 1 and 2) when the burst is not essential, a 2-fold change of KG concentration from 0.005 to 0 01 mM, induces an 1 5-fold increase in the reaction rate In accordance with a hyperbolic dependence of the enzyme saturation with KG one may expect a lesser rate of activity growth due to the subsequent augmentation of KG concentration But it is not so indeed: the subsequent 5-fold increase in KG concentration (to 0.05 mM) produces more than a 6-fold change in the initial rate of the reaction as seen from lines 2 and 3 of Fig 1 Simultaneously the burst in product accumulation appears. Thus, the change in the catalytic properties of KGD at KG saturation is accompanied by the additional activation of the enzyme compared to the one expected from the saturation of active sites. The analysis above suggests, that this is a result of binding the second KG molecule to the KGD monomer Therefore not having decided beforehand on the question of separating this site from the active one, it may be regarded as a regulatory center for KG activating KGD In the course of catalysis the activated enzyme is turning comparatively slowly (during several minutes, Fig. 1, lines 3-5) into the lower activity state. This is displayed as an initial burst of KGD activity during the reaction So, the burst magnitude may characterize the quantity of KGD activated by KG and measure the regulatory site filling with KG

The suggestion about the activatory site for substrate binding is supported by experiments with KG structural

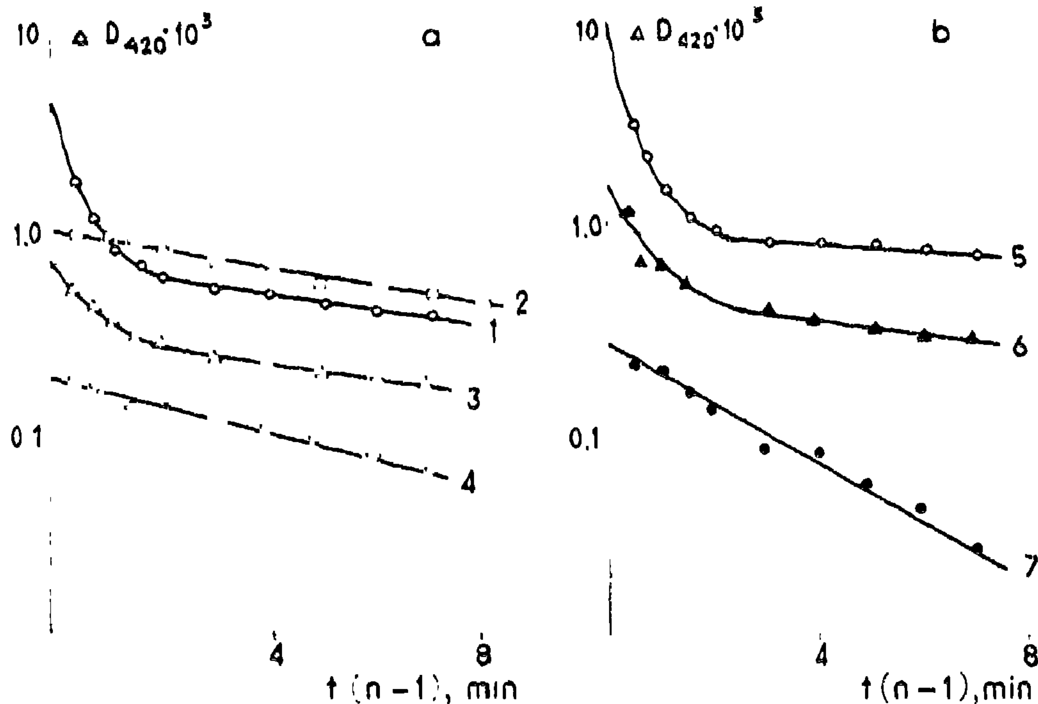

Fig. 2. The product accumulation curves of KGD reaction at 0.02 (a) and 0.2 (b) mM KG without inhibitors (1, 5) and in the presence of 5 mM malonate (2), 1 mM succinate (3), 0.4 mM ketomalonate (4), 3 mM glutarate (6) and 2 mM oxaloacetate (7).

analogues. As can be seen from Fig. 2, these compounds inhibit KGD activity. Two features of the process must be emphasized. First, any of the analogues tested inhibits the initial rate of reaction more than the delayed ones (i.e. reached after finishing the burst). This is evidence for the different catalytic properties of KGD at the initial moment of reaction and after the first inactivation stage is completed. Secondly, the comparison of the effects of the compounds used shows that inhibition of the delayed activity does not correlate with suppression of the burst. In fact, malonate eliminates completely the fast inactivation step without affecting the delayed activity (Fig. 2a, lines 1 and 2). On the contrary, succinate (Fig. 2a, line 3) and glutarate (Fig. 2b, line 6) affect the burst to a lesser extent, but decrease the delayed activity. As for oxaloacetate and ketomalonate (Fig. 2b, line 7 and Fig. 2a, line 4), they strongly influence both the burst and the delayed activity. The absence of correlation between the effects of the analogues on the initial burst of activity and on the activity level reached after the fast inactivation allows one to consider these effects as conditioned by two types of independent interactions of KGD with the compounds used. So, substrate analogues like KG appear to bind with KGD in two ways. The competition with KG at the regulatory site decreases reaction rate through eliminating the activatory effect of the substrate and is displayed in suppressing the initial burst of activity. As for the interaction with the active center, it does not change the kinetics of inactivation during the reaction, since it affects to the same degree both the initial activity and the delayed one. Then we may consider the inhibitory effect of malonate as the elimination of KG activation through the analogue binding to the regulatory site. The absence of the influence on the delayed activity in this case suggests that malonate does not interact with the catalytic site. This is in accordance with the absence in malonate of the α-keto group essential for the formation of the catalytic complex [9]. Therefore, malonate binding should not prevent the catalysis because it may proceed only in the regulatory site. Indeed, the inhibition by malonate is not complete, which manifests itself in the convex curves when analyzing its effect in Dixon's plot (Fig. 3a). However, having more structural similarity to KG, oxaloacetate can compete with the substrate in two centers. Binding at the regulatory one eliminates the activatory effect of KG which is displayed in the complete suppression of the initial burst (Fig. 2b, line 7). The active site binding causes an additional inhibition revealed in the decrease in the delayed activity. Interaction of more than one oxaloacetate molecule with KGD is supported by a parabolic inhibition pattern in Dixon's plot (Fig. 3b).

Besides KG analogues the modification of SH-groups can suppress the activation by KG. Fig. 4 shows the results of the KGD treatment by equimolar quantity of 4-hydroxymercurybenzoic acid. It is seen that such a limited modification (no more than one thiol in the

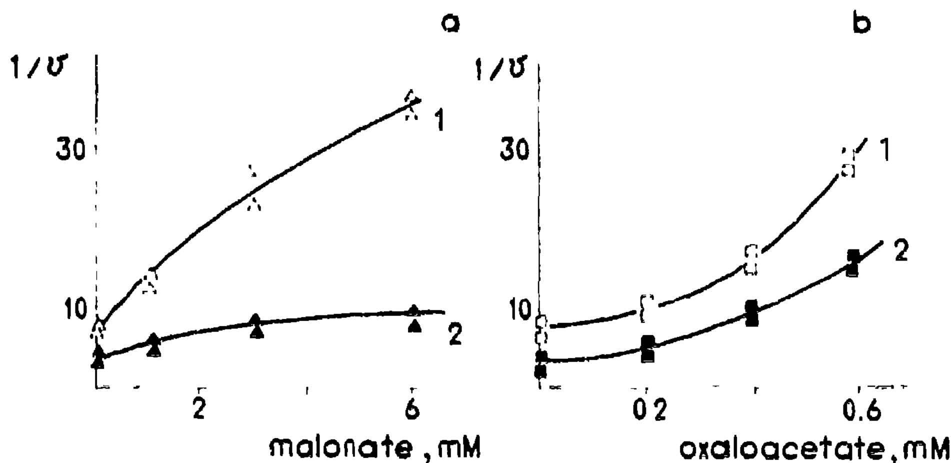

Fig 3 Dixon's plot of the inhibitory effects of malonate (a) and oxaloacetate (b) at 0 02 (1) and 0 2 (2) mM KG

monomer) by reagent, not so dangerous for the disturbance of protein structure, leads to the essential decrease in the quantity of KGD with catalytic properties changed, which is manifested in the substantial reduction of the initial burst of KGD activity (Fig. 4, line 2) This suggests KGD cysteine residue involvement in the enzyme activation by KG The modification of this residue desensitized KGD to the activatory effect of the substrate

4. DISCUSSION

The results obtained show the possibility of a short-time activation of KGD when KG concentration increases The activation appears to be induced by binding the second KG molecule to the KGD monomer. This allows one to regulate the enzyme activity by dicarbonic acids, which cannot effectively inhibit the reaction competing with KG in the catalytic site, since KG interaction with the holoenzyme is highly specific [9]. Besides, the revealed regulation of hysteretic properties of KGD functioning at the intercept of sugar and protein metabolic pathways is in good agreement with Frieden's suggestion [2] that hysteretic effects may be important for regulating metabolic pathways, where several enzymes compete for the common intermediate Noteworthy is that the studied properties of KGD allow one to distinguish a short-time increase in KG concentration and a prolonged one. Indeed, the momentary reaction of KGD to the augmentation of KG in a medium is the activation leading to more effective utilization of KG in the Krebs cycle. The prolonged increase in KG concentration, however, should have another effect In this case KGD inactivation, observed during the reaction, may contribute to the involvement of more KG in transamination, leading to glutamic acid. All this argues for the regulatory role of the effects revealed.

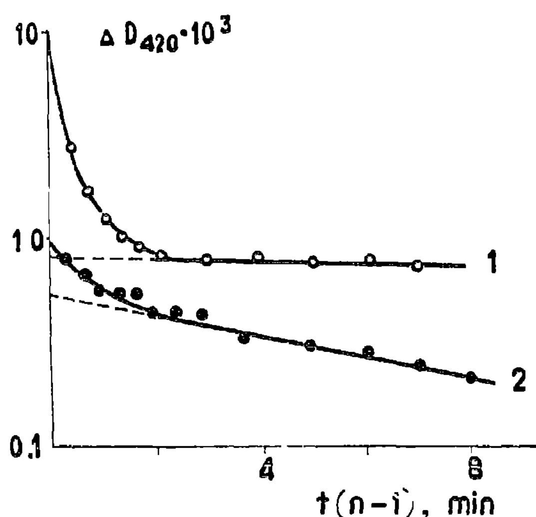

Fig 4 The product accumulation curves of KGD reaction before (1) and after (2) KGD treatment with 4 hydroxymercuribenzoic acid

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