

Chemical modification of lysine and arginine residues of bovine heart 2-oxoglutarate dehydrogenase: Effect on the enzyme activity and regulation^o

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Chemical modification of arginine and lysine residues of bovine heart 2-oxoglutarate dehydrogenase with phenylglyoxal and pyridoxal 5'-phosphate inactivated the enzyme, indicating the importance of these residues for the catalysis. Inactivation caused by pyridoxal 5'-phosphate was prevented in the presence of thiamine pyrophosphate and Mg^{2+} allowing the assumption that lysine residues participate in binding of the cofactor.

2-Oxoglutarate dehydrogenase (EC 1.2.4.2) is the first and most regulated component of the multienzyme 2-oxoglutarate dehydrogenase complex which catalyses the rate-limiting step of the oxidative decarboxylation of 2-oxoglutarate, a key reaction of the Krebs cycle [1]. The anionic nature of the substrate enables us to suggest the existence of positively charged arginine and/or lysine residues located within or close to the active sites of the enzyme which are capable to bind the 2-oxoglutarate carboxyl group [2]. From chemical modification studies an important role of highly reactive arginine residues in the catalysis and regulation of pigeon breast muscle 2-

oxoglutarate dehydrogenase and of bovine kidney pyruvate dehydrogenase has been shown [3, 4]. Besides, arginine residues are frequently involved in adenosine diphosphate binding by the interaction with the phosphate moiety of the nucleotide in a number of ADP-activated enzymes [5].

Pyridoxal 5'-phosphate (PalP) is a lysine-specific reagent which is widely applied for modification of proteins, since it acts as an affinity labelling reagent that is directed towards active sites of the enzymes by its own phosphate group [6]. Chemical modification of lysine residues of bovine glutamate dehydrogenase and of some other enzymes using

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Abbreviations: DCCIP, 2,6-dichlorophenolindophenol; DTT, dithiothreitol; PalP, pyridoxal 5'-phosphate; PG, phenylglyoxal; TPP, thiamine pyrophosphate.

PalP was found to lead to their partial inactivation connected with binding of the reagent to lysines important for the catalysis [6, 7].

We undertook a chemical modification study and determined changes in 2-oxoglutarate dehydrogenase activity after modification of its lysine and arginine residues by treatment of the enzyme with PalP and a specific modifier of the arginine, phenylglyoxal (PG) [8], to elucidate the role of these amino-acid residues for the function of bovine heart 2-oxoglutarate dehydrogenase, the first component of the 2-oxoglutarate dehydrogenase complex. The results show the importance of arginine and lysine residues for the catalytic activity and regulation of the enzyme and suggest the presence of lysine residues in the catalytic sites of 2-oxoglutarate dehydrogenase, binding thiamine pyrophosphate (TPP). They also suggest that arginine residues are located within or close to the site interacting with the allosteric effector ADP.

METHODS

The 2-oxoglutarate dehydrogenase complex was extracted from bovine heart and purified using the method introduced by Stanley and Perham [9]. The complex was obtained simultaneously with the isolation of 2-oxoglutarate dehydrogenase as described in [10]. Freshly prepared solutions of the complex and of its first component, 2-oxoglutarate dehydrogenase, were kept under liquid nitrogen. Sodium dodecyl sulphate polyacrylamide gel electrophoresis showed clear-cut protein bands corresponding to the set of subunits typical for analogous complexes isolated from various animal sources.

2-Oxoglutarate dehydrogenase activity was measured by the rate of reduction of the artificial electron acceptor 2,6-dichlorophenolindophenol (DCPIP) at 600 nm. One unit of the activity was defined as 1 μ mole of DCPIP reduced per min. The standard assay mixture

contained 50 mM Mops buffer (pH 7.0), 1.0 mM $MgCl_2$, 0.2 mM TPP and 1.0 mM 2-oxoglutarate (Na salt). Changes in the concentrations of 2-oxoglutarate (in the case when they were introduced) are shown in the legends to figures. The reaction was started by addition of 1–3 μ g of enzyme protein.

PG and PalP at concentrations up to 100 mM were used as modifiers of the arginine and lysine residues, respectively. The modification of the complex and of its first component was performed by preincubation of 30 μ g to 100 μ g of the enzyme protein with appropriate concentrations of the modifier in 50 mM Mops buffer (pH 7.0). The treatment of the enzyme by PalP was performed in the dark by protecting the samples from light with metal foil. At timed intervals of preincubation at room temperature aliquots of the modified enzyme were added to the assay mixture to start the reaction. Varying concentrations PG and PalP were added to preincubation mixture as shown in the legends to figures. Protection against inactivation by components of the assay mixture was tested by exclusion of one or more components from the preincubation mixture.

All experiments were carried in triplicate; the computation was performed by using linear least square regression [11]; kinetic parameters were calculated using Lineweaver-Burk, Hill and Huggenheim's plots [11, 12].

The chemicals used were obtained from the following sources: 2-oxoglutarate (sodium salt) and Mops from Serva (Heidelberg, Germany); CoA, PalP, PG, thiamine pyrophosphate (TPP) and dithiothreitol (DTT) from Sigma (St. Louis, MO, U.S.A.); NAD from Reanal (Budapest, Hungary); Sepharose 2B for gel electrophoresis from Pharmacia (Uppsala, Sweden).

RESULTS

Preincubation experiments showed a significant decrease of the 2-oxoglutarate dehydro-

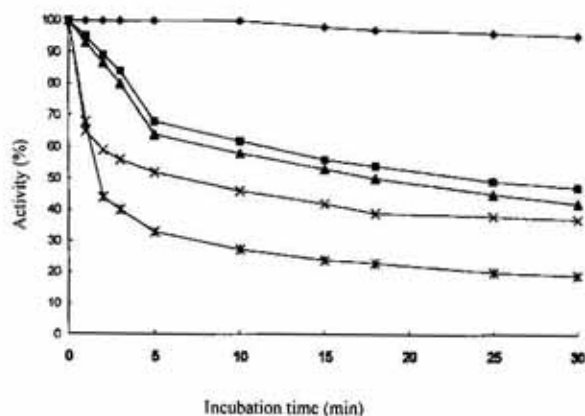


Figure 1. Time dependence of the inactivation of 2-oxoglutarate dehydrogenase by phenylglyoxal (PG).

2-Oxoglutarate dehydrogenase was preincubated with the following compounds before the reaction was started: (◆), none; (■), 0.05 mM PG; (▲), 0.1 mM PG; (×), 1.0 mM PG; (☆), 1.0 mM PG and 0.5 mM 2-oxoglutarate. Activities are expressed as percentage of the initial activity of the unmodified enzyme.

genase activity in the presence of phenylglyoxal (PG) (Fig. 1). The process of inactivation was time-dependent and could be characterised as biphasic, with fast and slow stages. The extent of the inhibitory effect correlated with PG concentrations: 40% of the enzyme activity was lost during 10 min of preincubation with 0.1 mM PG. With 1.0 mM PG the same degree of inactivation was observed after only 2 min.

Using another chemical reagent, pyridoxal 5'-phosphate (PalP), we have taken advantage of a two-step modification process that took place in a time-dependent manner when the enzyme preparation was incubated with the excess of the modifier (Fig. 2). The sensitivity of the enzyme to modification of lysine residues by PalP was also dependent on the modifier concentrations.

Linearization of the data obtained after preincubation of the enzyme with various concentrations of PG (from 0.05 mM up to 2.5 mM) and PalP (from 0.1 mM up to 1.0 mM) in semi-logarithmic Huggenheim's plots allows to determine, from the slopes of the in-

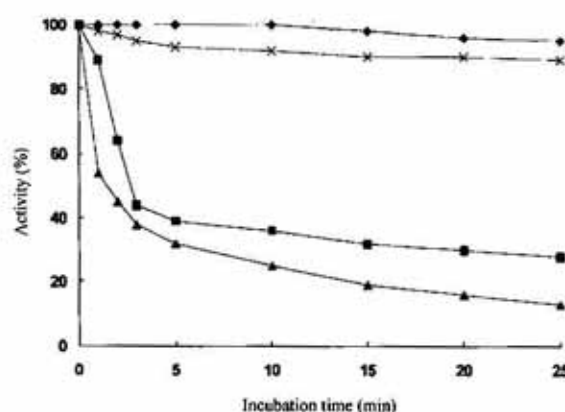


Figure 2. Time dependence of the inactivation of 2-oxoglutarate dehydrogenase by PalP.

The enzyme was preincubated as follows: (◆), without additions; (■), with 0.1 mM PalP; (▲), with 1.0 mM PalP; (×), with 1.0 mM PalP, 0.2 mM TPP and 1.0 mM Mg^{2+} .

activation lines, apparent inactivation rate constants (k_{app}) for each of the two steps of the inactivation (figures not shown). The values of k_{app} for 1.0 mM PG were 0.336 min^{-1} and 0.064 min^{-1} for the fast and the slow steps, respectively. Corresponding parameters determined for 1.0 mM PalP were 0.133 min^{-1} and 0.029 min^{-1} . It is thus evident that PG was a more effective inhibitor. Plotting the logarithm of k_{app} against the logarithm of the modifier concentration the rate coefficients for the first order reaction could be calculated as 0.86 and 0.76 for the fast steps and 1.2 and 1.0 for the slow steps of PG- and PalP-dependent inactivation, respectively. This means that the fast and the slow stages of the process may be characterised as pseudo first order reaction. A linear relationship between $k_{app} \text{ min}^{-1}$ and the modifier concentration was obtained which shows a simple bimolecular reaction.

Activation of the enzyme by 0.01–1.0 mM ADP is shown in Fig. 3. Modification of arginine residues by PG did not change the shape of the activation curve but significantly decreases the activatory effect of ADP.

As shown in Fig. 4A, the modifier of arginine residues significantly changed the ki-

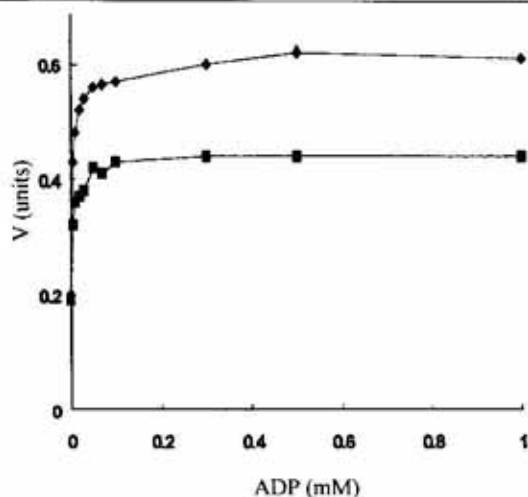


Figure 3. Initial rate of the reaction catalysed by 2-oxoglutarate dehydrogenase as a function of ADP concentration. (◆), Unmodified enzyme; (■), after 3 min preincubation with 1.0 mM PG.

netics of the initial rate of the reaction catalysed by 2-oxoglutarate dehydrogenase as illustrated by the double reciprocal plot. The Hill coefficients calculated from the Hill plots (Fig. 4B) were determined as 0.597 and 0.535 in the absence and presence of PG, respectively.

The co-substrates and other components of the assay medium were tested for their ability to protect bovine heart 2-oxoglutarate dehydrogenase. A marked protection against the inhibition caused by PalP was found when all the co-substrates and components of the assay medium were present. Even after 40 min preincubation with 2.5 mM PalP the enzyme retained its full activity. A similar effect was observed for some other enzymes when even 40 min preincubation with 2.5 mM PalP and 1.0 mM PG did not inactivate the enzyme completely [13]. The effects of individual components of the reaction mixture were studied by excluding one or more of them from the preincubation mixture. Preincubation of 2-oxoglutarate dehydrogenase with PG in the presence of 2-oxoglutarate accelerated the inactivation rate and resulted in the loss of about 80% of the enzyme activity during 25 min (Fig. 1). None of the components of the reaction mixture either alone or in different combinations were fully protective against

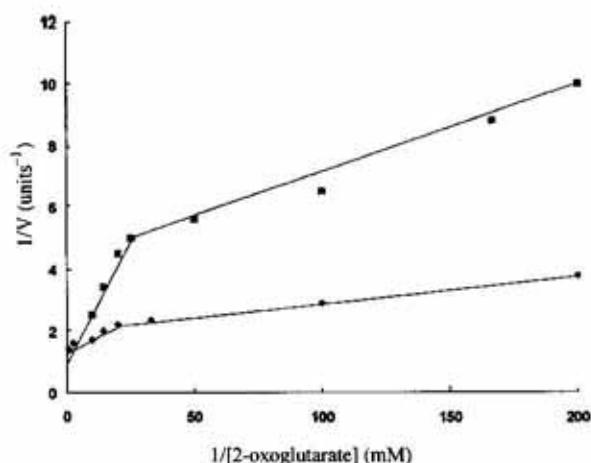


Figure 4A. Lineweaver-Burk plots of the initial rate of 2-oxoglutarate dehydrogenase reaction as a function of 2-oxoglutarate concentration.

(◆), In the absence of modifiers; (■), after 3 min preincubation with 0.1 mM PG. The points are means of triplicate measurements.

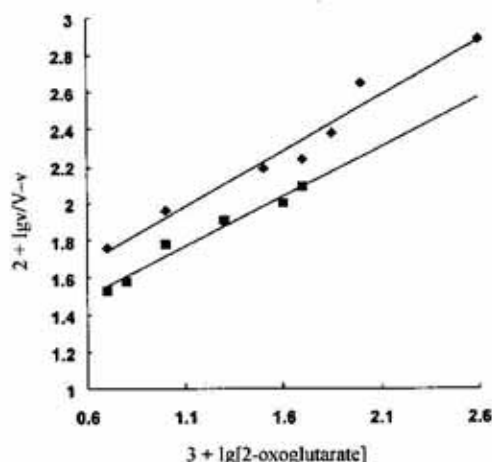


Figure 4B. Hill plots of the dependence of the initial rate of 2-oxoglutarate dehydrogenase reaction on 2-oxoglutarate concentration.

(◆), In the absence of modifiers; (■), after 3 min preincubation in the presence of 0.1 mM PG.

modification by PG. The highest protection compounds against inactivation by PalP was exerted by TPP and Mg^{2+} . Omission of these compounds from the preincubation mixture containing the PalP resulted in a decrease of the enzyme activity (Fig. 2) but the activity was almost completely restored in the presence of 0.2 mM TPP and 1.0 mM Mg^{2+} (Fig. 2). The reducing agent, dithiothreitol (DTT),

at the concentration of 0.1 mM also protected 2-oxoglutarate dehydrogenase against inactivation by PG and PalP (not shown).

DISCUSSION

Advantage has been taken of a two-step modification procedure to preferentially change arginine and lysine residues. The observation of pronounced two pseudo first order reaction stages of 2-oxoglutarate dehydrogenase inactivation with specific amino-acid reagents can be caused by different sensitivity of lysine and arginine residues to chemical modification. The most reactive residues are modified first and the modification of less reactive ones requires the increase of the time of preincubation or the concentration of the modifier.

We have previously reported [14] about the presence of two active sites on bovine heart 2-oxoglutarate dehydrogenase dimers differing in their affinity for the substrate as a result of non-equivalent catalytic efficiency. Kinetic analysis of the enzyme activity has shown that the presence of 2-oxoglutarate in the preincubation medium caused the appearance of attributes of negative cooperativity of the substrate-binding sites and resulted in a non-linear curve shown in double reciprocal plots. The extent of this negative cooperativity (Hill coefficient below 1) was slightly increased in the presence of PG (a decrease of the Hill coefficient). This modifier also caused a decrease of the enzyme affinity for the substrate and of the V_{\max} , indicating involvement of arginine residues in the catalytic function of 2-oxoglutarate dehydrogenase and possibility of conformational changes of the enzyme molecules after modification.

2-Oxoglutarate appeared not to be effective in protecting the enzyme from inactivation by PG and PalP. Arginine residues are even more sensitive towards inactivation by PG in the presence of the substrate, suggesting that functional arginines are located outside the

active site of the enzyme. The failure to observe any protection by 2-oxoglutarate alone indicates that neither lysine nor arginine residues are involved in 2-oxoglutarate binding. Inactivation by PalP was prevented by TPP, a well known participant of thiamine catalysis, binding to the catalytic sites of the enzyme, that allows the assumption that lysine residues may function as TPP-binding ligands. The role of Mg^{2+} has been reported as an intermediate in TPP binding by thiamine pyrophosphate enzymes, since the cofactor anchors generally to the pyrophosphate binding domain *via* a bivalent ion, mostly Mg^{2+} [15].

None of the components of the assay mixture alone was effective in protecting 2-oxoglutarate dehydrogenase against inactivation by PG. Interestingly, a practically complete loss of 2-oxoglutarate dehydrogenase sensitivity to the action of both PalP and PG was found after its preincubation with the reducing reagent DTT. It has been shown for the analogous enzyme isolated from pigeon breast muscle that functional peculiarities of the first component of the 2-oxoglutarate dehydrogenase complex are correlated with the redox state of SH groups and that the enzyme with oxidised thiols does not reveal the cooperative properties and at the same time reducing the enzyme with DTT has led to the subunits interaction [16]. The effect of DTT on the inactivation of bovine heart 2-oxoglutarate dehydrogenase by chemical reagents may be regarded as the evidence of importance of the redox state of the enzyme SH groups as well as of its conformation for effectiveness of the modifier action.

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