Succinyl phosphonate inhibits α-ketoglutarate oxidative decarboxylation, catalyzed by α-ketoglutarate dehydrogenase complexes from *E. coli* and pigeon breast muscle

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Abstract **Effects of a set of α-ketoglutarate phosphoanalogues on the activity of α-ketoglutarate dehydrogenase (EC 1.2.4.2) complexes from *E. coli* and pigeon breast muscle, as well as on α-ketoglutarate dehydrogenase isolated from the pigeon breast muscle, have been studied. α-Ketoglutarate phosphoanalogues (succinyl phosphonate and its monomethyl ester) were found to be effective inhibitors of α-ketoglutarate oxidative decarboxylation, catalyzed by both muscle and bacterial α-ketoglutarate dehydrogenase complexes, as well as muscle α-ketoglutarate dehydrogenase. The ability of glutamate phosphoanalogues to inhibit α-ketoglutarate oxidative decarboxylation has been shown in *E. coli* extract and a model system.**

Key words: α-Ketoglutarate dehydrogenase; α-Ketoglutarate phosphoanalogue; Glutamate phosphoanalogue; *E. coli* extract; Transamination

1. Introduction

α-Keto acids, especially those produced from natural amino acids, are of considerable importance for the cell metabolism. Thus pyruvic acid (Pyr) is a connecting link between the nitrogen and carbohydrate pathways of metabolism, whereas oxaloacetic (Oxa) and α-ketoglutaric (α-Kglu) are intermediates of the tricarboxylic acid cycle. The interconversions of α-keto acids and amino acids play an important role in nitrogen metabolism.

The Pyr and α-Kglu oxidative decarboxylation supplies the bulk of carbon atoms, involved in the Krebs cycle as acetyl- and succinyl-CoA, and for this reason the problem of selective inhibition of these processes is important. A perspective approach to creation of oxidative decarboxylation inhibitors can be usage of phosphonic analogues of α-keto acids, in which carboxylic group is replaced with an acidic phosphorus containing fragment and in which the phosphorus-carbon bond is resistant to action of amino acid metabolism enzymes. These substances can be synthesized and used as such in isolated systems or arise in vivo from metabolic precursors, such as phosphoanalogues of α-amino or hydroxy acids. The validity of this approach has been demonstrated by effective inhibition of acetyl-CoA formation in vitro and in vivo by phosphoanalogues of Pyr [1–5] and corresponding amino acid precursor [6–9]. The feature of inhibition of pyruvate dehydrogenase was investigated by phosphoanalogues of Pyr, modified at phosphorus containing fragment [5].

In this work we have studied the effect of a set of α-Kglu phosphoanalogues (succinyl phosphonate, Suc-P, and appropriate esters) on the activity of α-ketoglutarate dehydrogenase complexes (α-KGDC) from *E. coli* and pigeon breast muscle, as well as on α-KGD isolated from the pigeon breast muscle. In addition, we have studied the possibility of metabolic transformation of the glutamate phosphoanalogues, 1-amino-3-carboxypropylphosphonic (Glu-P) and -phosphinic (Glu-P_H) acids into dehydrogenase inhibitors in a model system and extract of *E. coli*.

2. Materials and methods

2.1. Chemical compounds

NAD^+, thiamine pyrophosphate (TPP), cysteine hydrochloride, α-ketoglutarate, and oxaloacetate were purchased from Sigma (USA). Phosphoanalogues of pyruvate, α-ketoglutarate, and glutamate (see Fig. 1) were obtained according to [10] and [11,12], respectively. The other chemicals were of the purest grade available.

2.2. Enzyme preparations and their activity determination

Extract of *E. coli* was prepared according to the method described in [8]. Isolation of α-KGDC from *E. coli* and its activity determination by NAD^+ reduction were carried out using a modified technique [13]. The α-KGDC and α-KGD isolation from pigeon breast muscle and determination of their activities were done by the earlier described methods [14]. The isolation of aspartate aminotransferase (EC 2.6.1.1) from pig hearts and the enzyme activity determination by accumulation of oxaloacetic acid were carried out according to [12]. In all cases the initial rate of enzymatic reaction was measured.

In the case of phosphoanalogue of α-keto acids and α-KGDC and α-KGD the reaction was initiated by the enzyme addition. Preincubation of α-Kglu phosphoanalogues with α-KGD (5 min) was carried out in a cell for the activity determination in 0.05 M K-phosphate buffer, pH 6.3. In this case the enzymatic reaction was initiated by the simultaneous addition of α-Kglu and ferricyanide up to final concentrations of 0.5 mM and 0.6 mM, respectively.

2.3. The effect of Glu-P and Glu-P_H on dehydrogenase activity in E. coli extract and in a model system

50 μl of fresh *E. coli* extract was added to 50 μl of incubation mixture that contained 50 mM of K-phosphate buffer, pH 7.4, 2.6 mM L-cysteine, 1 mM $MgCl_2$, and 2.0 mM Pyr. In some cases 0.1 mM of aminooxy acetate was added. The mixture also contained 5 mM of one of gluphosphoanalogues. The incubation was carried out at 37°C for 6 h. An aliquot (10 μl) was taken at definite intervals of time to determine α-KGDC activity using the above method.

In the model system purified enzyme preparations of aspartate aminotransferase from pig hearts and α-KGDC from *E. coli* were used. The Glu phosphoanalogues were incubated with aspartate aminotransferase under conditions similar to those described in [12], then aliquots were taken and added to the incubation mixture for determination of α-KGDC activity.

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Abbreviations: Pyr, pyruvate; α-Kglu, α-ketoglutarate; Oxa, oxaloacetate; Suc-P, succinyl phosphonate; Suc-P^H, succinyl phosphinate; Ac-P, acetyl phosphonate; Ac-P_H, acetyl phosphinate; Glu-P, glutamyl phosphonate; Glu-P_H, glutamyl phosphinate; α-KGDC, α-ketoglutarate dehydrogenase complex; α-KGD, α-ketoglutarate dehydrogenase.

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Table 1
Inhibition of α-KGD and α-KGDC from pigeon breast muscle and α-KGDC from *E. coli* by α-Kglu phosphoanalogues[a]

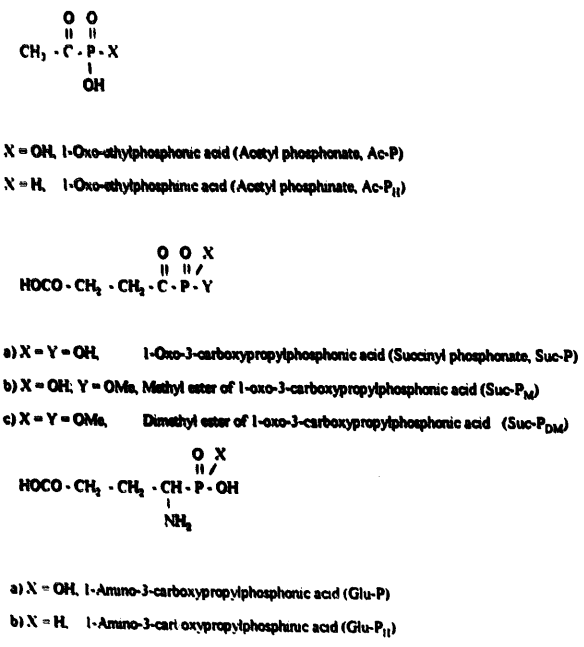
Source of enzyme	Enzyme	Experiment number	Compound	Concentration of analogue (mM)	Relative activity (%)
E. coli	α-KGDC	1	Suc-P	0.02	5
			$Suc\text{-}P_M$	0.02	50
			$Suc\text{-}P_{DM}$	0.02	100
			Suc-P+2 mM α-Kglu	0.02	33
	α-KGDC	2	Suc-P	0.06	2
			$Suc\text{-}P_M$	0.06	25
			$Suc\text{-}P_{DM}$	0.06	100
Pigeon breast muscle	α-KGDC	3	Suc-P	0.06	38
			$Suc\text{-}P_M$	0.06	67
			$Suc\text{-}P_{DM}$	0.06	98
	α-KGD	4	Suc-P	0.06	20
			$Suc\text{-}P_M$	0.06	40
			$Suc\text{-}P_{DM}$	0.06	100
	α-KGD	5[b]	Suc-P	0.02	4
			$Suc\text{-}P_M$	0.02	40
			$Suc\text{-}P_{DM}$	0.02	92
			Suc-P + 0.5 mM α-KGlu	0.02	25

[a]Activity of enzymes was determined in the presence of 0.5 mM α-Kglu. The K_m value for α-Kglu in the case of *E. coli* α-KGDC is equal to 0.07 mM. The K_{diss} value for α-Kglu in the case of muscle α-KGD is 0.02 mM [14].
[b]Activity of α-KGD was determined after preincubation for 5 min in the presence of analogue.

3. Results and discussion

3.1. *Succinyl phosphonate inhibits oxydative decarboxylation of α-ketoglutarate*

At the first step we have studied the influence of Suc-P and its esters on α-Kglu oxydative decarboxylation, catalyzed by α-KGD and α-KGDC from pigeon breast muscle, as well as by α-KGDC from *E. coli*. The Suc-P interaction with complexes was competitive in relation to the substrate, i.e. an increase of α-Kglu concentration resulted in the inhibition extent decrease. Thus, the residual activity of *E. coli* α-KGDC was equal to 33% in the presence of 0.02 mM Suc-P and of 2 mM of α-Kglu, whereas it made up only 5% in the case of 0.5 mM of α-Kglu (Table 1, experiment 1). This competition was also shown for α-KGD (Table 1, experiment 5): in the presence of α-Kglu 25% of activity was preserved, while preincubation with Suc-P in the absence of α-Kglu caused practically complete inactivation of α-KGD.



Scheme 1

The inhibition of α-Kglu oxidative decarboxylation by phosphoanalogues was selective, since millimolar concentrations of Ac-P and $Ac\text{-}P_H$ did not inhibit α-KGDC and α-KGD activities. We have found in experiments with isolated α-KGD (Table 1, experiments 4 and 5) that the level of enzyme inhibition by Suc-P increases upon preincubation with inhibitor. A similar effect was observed during Pyr phosphoanalogues interaction with pyruvate dehydrogenase from *E. coli* and this was explained by enzyme-inhibitor complex (E-I) isomerization [15]. A similar level of inhibition of α-KGD, preincubated with analogue (Table 1, experiment 5), and α-KGDC from *E. coli* without preincubation (Table 1, experiment 1), could be interpreted as a difference in the stability of E-I complexes of muscle α-KGD and bacterial enzyme. This correlates with the possibility of conformational alterations of the muscle α-KGD during catalysis [16], which is typical for bacterial enzymes. The difference in the properties of E-I complexes may explain the lower sensitivity of muscle α-KGDC towards inhibitors (Table 1, experiment 3), as of bacterial one (Table 1, experiment 2). It should be noted that the substrate affinities for the muscle dehydrogenase and bacterial enzyme are close to each other and are equal to 0.02–0.04 mM [14] and 0.07 mM, respectively.

The data in Table 1 show that the inhibitory activity of Suc-P in all cases was higher than that of monomethyl ester[1] of the same compound. The opposite situation has been observed for pyruvate dehydrogenase complex inhibition by pyruvate phosphoanalogues. According to the literature [2] and our own data [4,7], the most effective inhibition of pyruvate dehydro-

[1] A related fluorescent substance, monomethyl ester of pyrene butyl phosphonate, has a sufficient affinity to the enzyme [13].

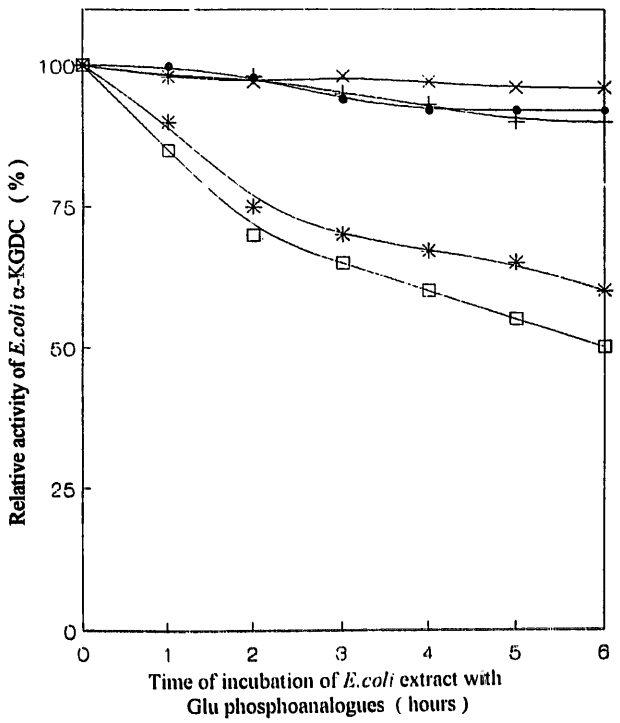


Fig. 1. Inhibition of α-KGDC activity in *E. coli* extract after incubation with Glu phosphoanalogues. For incubation conditions see section 2. α-KGDC activity was determined at 37°C in 0.99 ml of K-phosphate buffer, pH 7.4, containing 2.6 mM L-cysteine, 1 mM $MgCl_2$, 0.2 mM TPP, 2.5 mM NAD, 0.1 mM CoA, and 2 mM α-Kglu. The reaction was initiated by addition of a 10 μl aliquot of incubated *E. coli* extract containing: (●) 2 mM Pyr; (*) 2 mM Pyr+5 mM Glu-P; (□) 2 mM Pyr+5 mM Glu-P_H; (×) 2 mM Pyr+5 mM Glu-P+0.1 mM aminooxy acetate; (+) 2 mM Pyr+5 mM Glu-P_H+0.1 mM aminooxy acetate.

genase complex was caused by acetyl phosphonate monomethyl ester. Dimethyl ester of Suc-P, lacking a negative charge on its phosphonate fragment, was inactive.

So, obtained data show that Suc-P and corresponding monoester are effective inhibitors of α-Kglu oxidative decarboxylation, catalyzed by both muscle and bacterial α-KGDs. The mechanism of inhibition may include the interaction of inhibitor carbonyl group with TPP in the α-KGD active center similarly to the earlier proposed mechanism of pyruvate phosphoanalogues interaction with the pyruvate dehydrogenase complex from *E. coli* [2].

3.2. *Generation of inhibitors of α-Kglu oxidative decarboxylation from glutamate phosphoanalogues (Glu-P and Glu-P_H) in transaminase reaction and extract of E. coli*

Earlier we have shown the possibility of transamination of amino acid phosphoanalogues to corresponding keto acids in vitro [12], in vivo [6,8], and in *E. coli* extracts [7–9].

We applied the same approach in present paper to generate dehydrogenase reaction inhibitors using glutamate phosphoanalogues and *E. coli* extract and aspartate aminotransferase. As is seen from Fig. 1, incubation of Glu-P or Glu-P_H with *E. coli* extract in 50 mM K-phosphate buffer, pH 7.5 in the presence of Pyr results in a drop of α-KGDC activity. Addition to the medium of aminoxy acetate, a specific inhibitor of enzymatic transamination, retained the dehydrogenase activity.

A possible explanation consisted in enzymatic transformation (transamination) of aminoanalogues into ketoinhibitors in *E. coli* extract, like glutamic acid could be a source of α-Kglu in the same system.It has been shown in separate experiments that Pyr, Glu-P, and Glu-P_H have no inhibitory effect on α-Kglu oxydative decarboxylation.

In addition, we have studied the transformation of Glu-P and Glu-P_H into corresponding phosphoanalogues of α-Kglu in the system α-KGDC–aspartate aminotransferase. As is seen from Fig. 2 in such system a decrease of α-KGDC activity observes in the presence of Glu-P or Glu-P_H.

α-KGDC inhibition by Suc-P and the resulting biological effect could be observed in vivo if this compound or its metabolic precursor Glu-P could permeate a cell. However, it is known that phosphonates and aminophosphonates poorly permeate the cell envelope. Using of Glu-P_H may open new possibilities, since the substances of this family are characterized by a good permeability and may transform to the phosphinic inhibitor of α-KGDC, Suc-P_H. The latter compound has not been synthesized yet and a unique possibility of generation of it is metabolic transformation of amino precursor, Glu-P_H.

The obtained results confirm the ability of Glu phosphoanalogues to be metabolic precursors of Suc-P and Suc-P_H, inhibitors of α-Kglu oxidative decarboxylation.

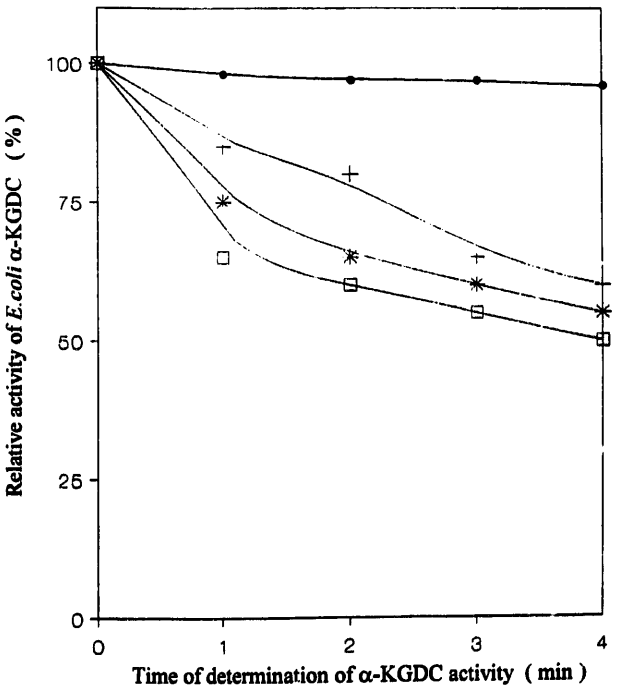


Fig. 2. Effect of α-keto inhibitors, generated during transamination, on the *E. coli* α-KGDC activity. α-KGDC (10 μg/ml) was incubated in conditions analogous those indicated in the legend to Fig. 1. The reaction was initiated by the simultaneous addition of 5 μl of 2 mM α-Kglu and 5 μl of transamination medium containing: (●) 0.3 mM Glu+0.002 mM α-Kglu; (+) 0.3 mM Asp[a]+0.002 mM α-Kglu; (*) 0.2 mM Glu-P+0.002 mM α-Kglu; (□) 0.2 mM Glu-P_H+0.002 mM α-Kglu. Incubation conditions for transamination as in [12]. [a]As a result of aspartate transamination Oxa is formed, the effective inhibitor of *E. coli* α-KGDC (K_i for Oxa = 0.045 mM).

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