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THE ACTIVE SITE OF RABBIT MUSCLE PYRUVATE KINASE

EVIDENCE FOR A SITE COMMON TO THE OXALACETATE DECARBOXYLASE AND PYRUVATE KINASE REACTIONS

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Summary

Rabbit muscle pyruvate kinase has been shown to catalyze the decarboxylation of oxalacetate (Creighton, D.J. and Rose, I.A. (1976) J. Biol. Chem. 251, 61). Noncovalent and covalent modifiers of the enzyme have been used to assess whether the decarboxylase and kinase reactions take place at a common site. Phospho*enol*-α-ketobutyrate, an analog of the substrate phospho*enol*-pyruvate, inhibits decarboxylase and kinase competitively and with nearly identical K_i values (5.7 μM and 4.8 μM, respectively). Oxalate, an analog of *enol*-pyruvate, inhibits each competitively and with similar K_i values (11 μM and 4.7 μM, respectively). Both activities are lost in parallel upon reaction with dithionitrobenzoate, which is active-site specific. These results indicate that the two activities share a common site on the enzyme. But, effects of the following modifiers suggest that different amino acid residues at that site participate in the two reactions: phenylalanine inhibition and fructose 1,6-bisphosphate activation are more effective with the decarboxylase; iodoacetamide preferentially inactivates decarboxylase while trinitrobenzenesulfonate preferentially inactivates kinase.

Introduction

In its primary, physiologically important reaction pyruvate kinase transfers the phosphoryl group of phospho*enol*pyruvate to ADP forming ATP and pyru-

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vate. The enzyme also catalyzes numerous other reactions [1—6]. Recently, the enzyme has been found to be an oxalacetate decarboxylase as well [7,8]. In view of the similarities of the substrates and products for kinase and decarboxylase it is likely that a common site is involved in these catalyses. Creighton and Rose have made such a case for the enzyme from cod fish [8]. The best characterized pyruvate kinase, isolated from rabbit muscle [9], catalyzes the decarboxylase reaction with *enol*pyruvate a likely intermediate [7] as also shown for the kinase reaction [10]. But indications are that the sites are not identical because rates of enolization of enzyme-bound pyruvate generated from oxalacetate and from phospho*enol*pyruvate differ (enolization is much more rapid in the former case) and because $NaBH_4$ can trap enzyme-bound pyruvate generated from oxalacetate, but not that generated from phospho*enol*pyruvate [7]. Furthermore, differences in the active sites are suggested by preliminary results reported by Creighton and Rose [8] that iodoacetamide completely inactivated the decarboxylase activity with no loss in pyruvate kinase activity when phospho*enol*pyruvate, K^+ and Mg^{2+} were present.

Accordingly, this paper assesses the latter observation and examines the sensitivities of the two activities of the crystalline rabbit muscle enzyme to modification by covalent and noncovalent modifiers. A preliminary report of this work has been made [11].

Materials and Methods

Enzymes

Crystalline rabbit muscle pyruvate kinase and lactate dehydrogenase were purchased as suspensions in $(NH_4)_2SO_4$ from Boehringer Mannheim Corp. The enzymes were dialyzed against 20 mM TES (pH 7.5)/0.1 mM EDTA before use. An enzyme diluent containing 20 mM TES (pH 7.5)/1 mg per ml bovine serum albumin/0.1 mM EDTA was used unless otherwise noted. The concentration of pyruvate kinase was determined spectrophotometrically, using the extinction coefficient at 280 nm of 0.54 $mg^{-1} \cdot ml \cdot cm^{-1}$ [9]; however, when trinitrobenzenesulfonate was present, protein was determined by the Lowry procedure [12]. In calculating subunit concentrations, a molecular weight of 237 000 and 4 subunits were used [9].

Chemicals

The inactivators and effectors were: 5,5'-dithiobis(2-nitrobenzoic acid), phospho*enol*pyruvate (monocyclohexylammonium salt), and fructose 1,6-bisphosphate (tetracyclohexylammonium salt), Sigma Chemical Co.; 2,4,6-trinitrobenzenesulfonic acid, Pierce Chemical Co.; 2-iodoacetamide, Eastman Kodak Co.; oxalacetic acid, Calbiochem; oxalic acid, J.T. Baker Chemical Co.; L-phenylalanine, Nutritional Biochemical Corp.; DL-alanine, Merck and Co. Phospho*enol*-α-ketobutyrate (cyclohexylammonium salt) was a kind gift of Dr. D.B. Sprinson; the compound was an isomeric mixture, $E/Z = \frac{3}{4}$ [13]. *N*-tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES) and tetramethylammonium hydroxide were obtained from Calbiochem. All other chemicals were the best grade commercially available and all buffers were adjusted with tetramethylammonium hydroxide. All assays and modifications were conducted at 25° C.

Pyruvate kinase and oxalacetate decarboxylase activities

The velocity of the primary reaction of pyruvate kinase was measured in the presence of 100 mM TES (pH 7.5)/150 mM KCl/5 mM $MgCl_2$/2 mM ADP/5 mM phospho*enol*pyruvate/0.2 mM NADH/15 units of lactate dehydrogenase, and an appropriate dilution of pyruvate kinase in 1 ml. The standard assay for the decarboxylase reaction contained 100 mM TES (pH 7.5)/150 mM KCl/1 mM $MgCl_2$/1 mM fructose bisphosphate/0.5 mM NADH/3.5 mM oxalacetate/15 units of lactate dehydrogenase, and an appropriate dilution of the enzyme in 1 ml. While not required for the decarboxylase assay, fructose bisphosphate and KCl were added to give optimum activity. The decrease in absorbance at 340 nm was followed continuously on a Gilford 240 recording spectrophotometer; corrections for non-enzymic reaction were made. Kinetic data were analyzed using the computer program of Cleland [14] and an IBM 360/75 computer.

Covalent modifications of pyruvate kinase

For modification by dithionitrobenzoate, 1.2 mg enzyme (20 μM subunits) was incubated in 1.0 ml 100 mM TES (pH 7.5)/30 μM dithionitrobenzoate for 30 min. The reaction was stopped by adding 1 ml enzyme diluent, mixing, and immersing the test tubes in ice. For reversal of this inactivation, 0.05 ml 10 mM dithiothreitol was added to 0.45 ml inactivated enzyme and incubated for an additional 30 min. For alkylation by iodoacetamide, 1.2 mg enzyme was incubated in 1.0 ml 100 mM TES (pH 7.5) with iodoacetamide, 10 mM (kinase) or 2.5 mM (decarboxylase), for 30 min. The reaction was stopped as above. When enzyme was protected from inactivation by phospho*enol*pyruvate, it was essential to dialyze extensively against 20 mM TES (pH 7.5)/0.1 mM EDTA at 4° C before assaying residual decarboxylase activity because phospho*enol*pyruvate is such a potent inhibitor. Trinitrobenzenesulfonate modification was achieved by incubating 1.2 mg enzyme in 1.0 ml 100 mM sodium phosphate buffer (pH 8.0) and trinitrobenzenesulfonate, either 0.037 mM (kinase) or 2.25 mM (decarboxylase), for 30 min in aluminum-foil-covered test tubes. The reation was stopped as above. When determining the stoichiometry of this reactivation, the enzyme (20 μM subunits) was incubated in 2.0 ml 100 mM sodium phosphate buffer (pH 8.2) and either 0, 0.04, 0.4, or 4 mM trinitrobenzenesulfonate for 2 h as above. After stopping the reaction, each mixture was dialyzed 3 times for 3 h against 500 ml 20 mM TES (pH 7.5)/0.1 mM EDTA at 4° C. After the dialysis, 0.8 ml each solution was mixed with 0.1 ml 10% SDS/0.1 ml 1.4 M HCl, and the absorbance at 346 nm was measured. Moles of TNP-lysyl residues were determined from this absorbance (corrected for protein and residual inactivator) using the extinction coefficient of $1.45 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [15].

Results and Discussion

Noncovalent modifiers

If kinase and decarboxylase occur at a common site, one would expect the substrate of one to be a competitive inhibitor of the other. Phospho*enol*pyruvate, a substrate of the kinase, inhibits decarboxylase competitively with respect to oxalacetate with K_i = 14 μM. This compares with phospho*enol*pyruvate K_m = 50 μM. The affinity of the enzyme for phospho*enol*pyruvate is simi-

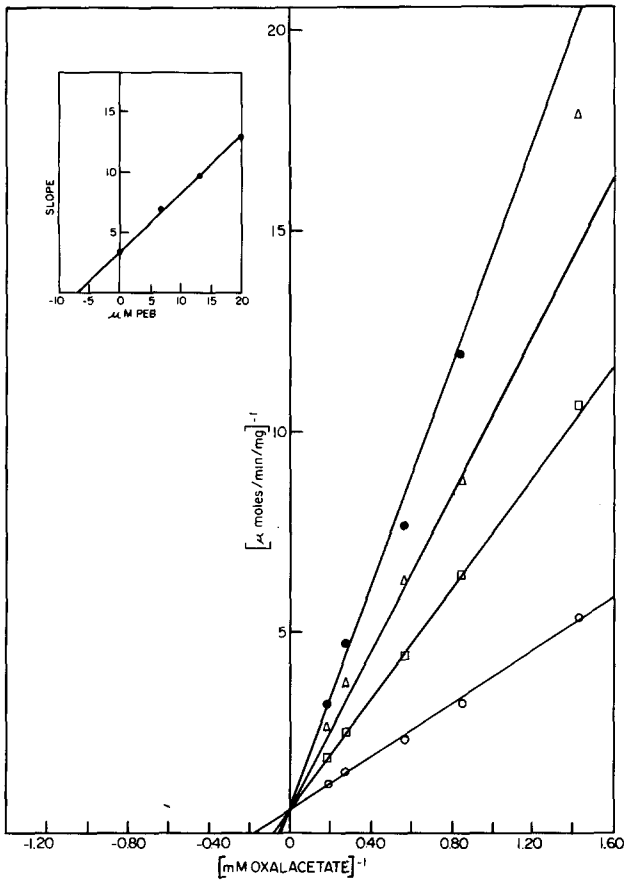

Fig. 1. Phospho*enol*-α-ketobutyrate inhibition of decarboxylase activity. Assay conditions were as described in Methods with addition of (○) no, (□) 7 μM, (△) 13 μM, or (●) 20 μM phospho*enol*-α-ketobutyrate (PEB).

lar whether it is a substrate for the kinase reaction or an inhibitor for the decarboxylase reaction; the K_m and K_i values are unlikely to be identical because ADP is a co-substrate only for kinase. Oxalacetate, substrate for decarboxylase, is an inhibitor of the kinase activity, but inhibition constants cannot be obtained because of rapid non-enzymic decarboxylation of oxalacetate under the conditions of the kinase assay.

Inhibition of the two activities by phospho*enol*-α-ketobutyrate, an analog of phospho*enol*pyruvate, was compared. Michaelis-Menten plots (Figs. 1 and 2) show that this analog is a competitive inhibitor of both activities. Inhibition constants calculated by computer were essentially the same, K_i (decarboxylase) = 5.7 μM and K_i (kinase) = 4.8 μM. The compound had previously been reported to be a potent competitive inhibitor of kinase activity with K_i = 65 μM [16] and 10 μM [17]. The value obtained in this study agrees with the latter value. Phospho*enol*-α-ketobutyrate is a poor substrate in the kinase reaction with a maximum velocity 0.1% that of phospho*enol*pyruvate [13,17—19] but a similar K_m [18,19]. Thus, this compound binds at the active site of the kinase

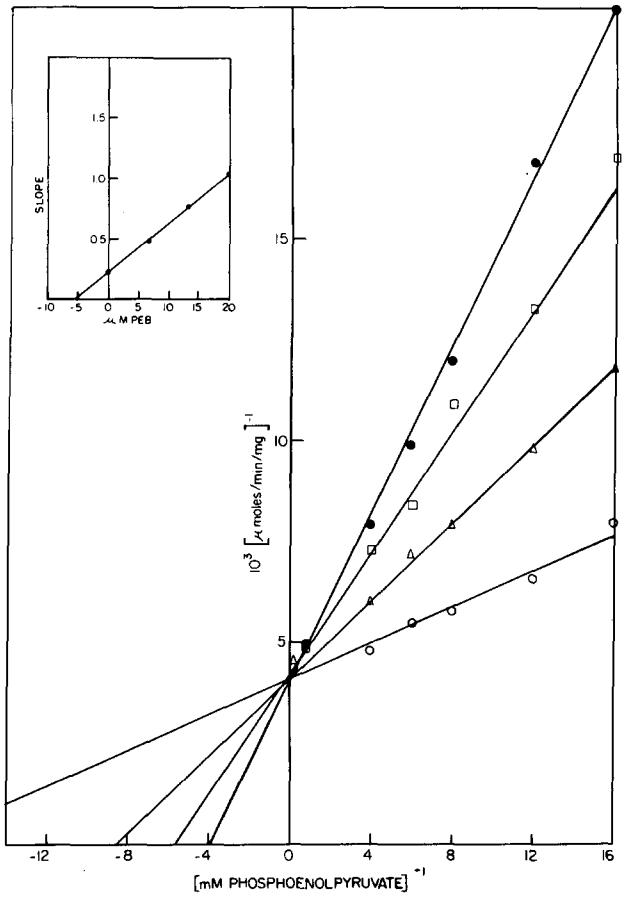

Fig. 2. Phospho*enol*-α-ketobutyrate inhibition of kinase activity. (○) no, (△) 7 μM, (□) 13 μM, or (●) 20 μM phospho*enol*-α-ketobutyrate (PEB).

reaction and the similarity of competitive inhibitor constants suggests the site probably accommodates either oxalacetate or phospho*enol*pyruvate.

Oxalate, an analog of *enol*pyruvate, an intermediate in both the kinase and decarboxylase reactions, has been shown to be a competitive inhibitor of rabbit muscle pyruvate kinase with K_i = 6.0 μM [20] and of cod fish oxalacetate decarboxylase with K_i = 10 μM [21]. Oxalate inhibition of the rabbit muscle enzyme is competitive and similar inhibition constants are obtained for both, 4.7 μM (kinase) and 11.1 μM (decarboxylase). This is consistent with the *enol*-pyruvate intermediate of the two reactions binding at a common site.

Phenylalanine, an allosteric inhibitor of kinase activity [22—24] exhibits similar kinetics toward the decarboxylase activity. Inhibition of the decarboxylase activity occurs with Mg^{2+} as divalent cation activator but not with Mn^{2+} and is reversed by the addition of alanine as had been shown for kinase [22,23]. However, some differences were noted: 20-fold more phenylalanine was required for inhibition of kinase activity than for inhibition of decarboxylase and the latter resulted from decrease in V and increase in K_m for oxalace-

tate; the effect on kinase is strictly an effect on K_m for phospho*enol*pyruvate [22—24]. These differences indicate that while the two activities may share a common site, different amino acid residues are important to each activity. A similar conclusion can be drawn from our study of the noncovalent effector, fructose bisphosphate; it stimulated the maximum velocity of the decarboxylase activity by 42%, but did not affect the kinase reaction.

Covalent modifiers

Dithionitrobenzoate reacts with pyruvate kinase to form a mixed disulfide of thionitrobenzoate and a cysteinyl residue; a subsequent displacement of thionitrobenzoate by the sulfhydryl of another cysteinyl residue yields an intramolecular disulfide bond [25]. As monitored by kinase activity, this modification is active site selective [25,26]. Upon reaction with dithionitrobenzoate, kinase and decarboxylase activities are lost in parallel and both are similarly restored by dithiothreitol. This suggests that the inactivator affects sulfhydryl residues that are equally important to each activity and is consistent with a common active site. For enzyme inactivated about 60% by dithionitrobenzoate, K_m values obtained, 0.06 mM for phospho*enol*pyruvate and 3.7 mM for oxalacetate, are the same as those for unmodified enzymes (Table I). Flashner et al. [25] had previously noted that the K_m values for phospho*enol*pyruvate and ADP were not changed and suggested that the activity of partially modified enzyme is due to enzyme still in its native form. As decarboxylase is similarly affected, a common site for both activities is indicated.

The ability of several reaction components to protect both activities from dithionitrobenzoate was also examined (Table II). When tested individually at concentrations used in the kinase assay, ADP and K^+ give no protection to either kinase or decarboxylase, whereas Mg^{2+} provides partial protection to kinase and extensively protects the decarboxylase. The protective effect of the other components in the presence of Mg^{2+} are not much different from the protection afforded by Mg^{2+} alone. The protection afforded the kinase assay is qualitatively that previously observed [25]. While the results indicate that the

TABLE I

EFFECT OF PARTIAL INACTIVATION ON KINASE AND DECARBOXYLASE KINETICS

Incubation and assay conditions were as described in Materials and Methods with: Dithionitrobenzoate at 0.015 mM 15 min for kinase and 30 min for decarboxylase; iodoacetamide at 10 mM for kinase and 1 mM for decarboxylase (30 min each); trinitrobenzenesulfonate at 0.037 mM for kinase and 3.0 mM for decarboxylase (30 min each). K_m is with respect to phospho*enol*pyruvate for kinase and oxalacetate for decarboxylase.

Inactivator	Reaction	V (units/mg)	K_m (mM)
None	Kinase	270	0.06
	Decarboxylase	1.7	3.7
Dithionitrobenzoate	Kinase	90	0.06
	Decarboxylase	0.60	3.7
Iodoacetamide	Kinase	100	0.40
	Decarboxylase	1.2	3.8
Trinitrobenzenesulfonate	Kinase	66	0.05
	Decarboxylase	1.7	25

TABLE II

PROTECTION AGAINST DITHIONITROBENZOATE MODIFICATION OF PYRUVATE KINASE

Incubation and assay conditions were described in Materials and Methods the kinase activity was assayed with 0.25 mM phospho*enol*pyruvate. The final concentrations of added components were as follows: 2 mM ADP, 150 mM KCl, 5 mM $MgCl_2$.

Component added	Percent residual activity	
	Kinase	Decarboxylase
None	12	19
ADP	11	14
K^+	15	19
Mg^{2+}	43	75
ADP, K^+	17	17
ADP, Mg^{2+}	53	88
ADP, K^+, Mg^{2+}	43	92

divalent cation provides protection against this inactivation for both the kinase and decarboxylase activities, the extent of protection is not identical suggesting that the sulfhydryl residues modified by dithionitrobenzoate may be slightly more important for kinase than for decarboxylase activity.

Iodoacetamide modification of pyruvate kinase has been poorly characterized and appears to lack the specificity of an active site reagent [27]. The decarboxylase activity is more sensitive to this alkylating reagent. Incubating the enzyme with 5 mM iodoacetamide for 30 min leaves 54% of the kinase but only 8% of the decarboxylase activity. Further, the kinetics are altered in different ways. Inactivation of decarboxylase results from a decrease in maximum velocity, whereas inactivation of kinase is due to both a decrease in V and an increase in K_m for phospho*enol*pyruvate (Table I). The protective effects of substrates and cofactors against iodoacetamide inactivation were examined (Table III). To obtain similar levels of inactivation for each activity, four times more iodoacetamide was used to inactivate kinase than to inactivate decarboxylase, but protection afforded against inactivation is remarkably similar; Mg^{2+} + ADP and $K^+ + Mg^{2+}$ are the most effective. The addition of phospho*enol*pyru-

TABLE III

PROTECTION AGAINST IODOACETAMIDE INACTIVATION OF PYRUVATE KINASE

Conditions were as described in Materials and Methods. The final concentration of added components were as follows: 150 mM KCl, 5 mM (kinase) or 1 mM (decarboxylase) $MgCl_2$, 10 mM phospho*enol*pyruvate, and 2 mM ADP.

Component added	Percent residual activity	
	Kinase	Decarboxylase
None	12	14
K^+	53	78
Mg^{2+}	50	67
Mg^{2+}, ADP	89	95
K^+, Mg^{2+}	94	94
K^+, Mg^{2+}, phospho*enol*pyruvate	90	93

TABLE IV

CHARACTERISTICS OF TRINITROBENZENESULFONATE MODIFICATION OF PYRUVATE KINASE

Conditions were as described in Materials and Methods.

Inactivation conditions (mol trinitrobenzenesulfonate per mol enzyme subunit)	Percent residual activity		Covalent modification of enzyme (mol TNP-lysyl residues per mol enzyme subunit)
	Kinase	Decarboxylase	
2	13	90	0.95
20	6	34	4.82
200	<0.01	<0.01	19.1

vate to the latter binary combination affords no added protection. Assay of residual decarboxylase activity when protected by phospho*enol*pyruvate requires dialysis to remove it, a potent inhibitor of that activity (K_i = 14 μM). Failure to remove it or correct for it will lead to the erroneous conclusion that phospho*enol*pyruvate does not protect decarboxylase activity from iodoacetamide inactivation but may protect kinase. It is suggested that this may account for the preliminary observation (see ref. 8) that $K^+ + Mg^{2+}$ + phospho*enol*pyruvate appeared to protect kinase but not decarboxylase from iodoacetamide inactivation. The present study of iodoacetamide inactivation indicates that this non-specific modifier differentially affects the two activities with decarboxylase being more sensitive than kinase to this alkylating agent.

Trinitrobenzenesulfonate reacts with one lysyl ϵ-amino group per pyruvate kinase subunit to inactivate the primary reaction and it likely reacts at the adenine nucleotide site on the enzyme [15,26]. The decarboxylase activity shows less sensitivity to this covalent modifier than kinase does (Table IV). When enzyme is incubated with a 2-fold molar excess of trinitrobenzenesulfonate for 30 min, nearly one mole of lysyl residues are modified per enzyme subunit, little kinase activity but most decarboxylase activity remains. When incubated with a 20-fold excess of inactivator the enzyme still retains 5 times more decarboxylase than kinase. Upon increasing the trinitrobenzenesulfonate to 200 times the enzyme subunit concentration, over half the 37 lysyl residues per subunit [28] have reacted and both activities are eliminated. The decarboxylase activity is clearly less affected by this inactivator, requiring modification of more than 5 lysyl residues per subunit to inactivate 90% of that activity. Enzyme partially modified by trinitrobenzenesulfonate shows differences in kinetics for each of the reactions (Table I). The inactivation of kinase results from a decrease in V (as noted previously [15]) whereas loss of decarboxylase activity results from increased K_m for oxalacetate. The results indicate that binding of one inactivator molecule per active site disrupts residues more important for the kinase activity. Modification of the adenine nucleotide site which is not necessary for the decarboxylase reaction is further supported.

Conclusion

Evidence has been presented to support the hypothesis that the oxalacetate decarboxylase and pyruvate kinase reactions occur at a common site. The sub-

strate of each activity inhibits the other. Phospho*enol*-α-ketobutyrate, a substrate analog for kinase, inhibits both reactions competitively and with the same K_i value. Oxalate, an analog for *enol*pyruvate which is a likely intermediate in both reactions, inhibits both reactions competitively and with similar K_i values. Dithionitrobenzoate, an active site specific alkylating agent, inactivates both reactions in parallel; the inactivation is reversed to similar extent by dithiothreitol and partial inactivation leads to similar kinetics for both reactions. Recently, Wyatt and Colman have reported that 5'-fluorosulfonylbenzoyl adenosine, an active site directed reagent, inactivates both activities with the same time dependence [29]. This lends further support for a site common to both activities.

Some differences in the effects of noncovalent and covalent modifiers on the two activities indicate that not all amino acid residues at this site are shared. Fructose bisphosphate increases the velocity of the decarboxylase reaction but has no effect on kinase. Much lower levels of phenylalanine are required to inhibit decarboxylase than kinase. Iodoacetamide preferentially inactivates decarboxylase and in such a way as to decrease its V; higher levels of iodoacetamide are required to alter the kinase activity and the K_m is altered in addition to the V. Trinitrobenzenesulfonate preferentially inactivates kinase and in such a way as to decrease its V; higher levels are required to alter decarboxylase and only K_m is affected. While a common site is involved in both reactions, these different sensitivities of the two activities to covalent and noncovalent modification indicates that some amino acid residues at the site are necessary only for the kinase activity and others are solely required for the decarboxylase reaction. This is consistent with the differences in substrate structures and, in the case of kinase, in requirement for ADP as a co-substrate.

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