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PHOSPHO*ENOL*PYRUVATE EFFLUX FROM KIDNEY CORTEX MITOCHONDRIA OF RABBIT

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(1) The relationship between phospho*enol*pyruvate formation and its accumulation in kidney cortex mitochondria of rabbit was studied in the presence of glutamate as substrate. (2) In mitochondria incubated in either State 4 or under uncoupled conditions, both 1,2,3-benzenetricarboxylate and atractyloside resulted in a marked elevation of the intramitochondrial phospho*enol*pyruvate accompanied by a 2–4-fold decline in production of this compound. The same effect was induced by *n*-butylmalonate in uncoupled mitochondria, while both phospho*enol*pyruvate efflux and its production were inhibited to a smaller extent in mitochondria incubated with 1,2,3-benzenetricarboxylate in State 3. (3) Citrate, malate or 2-phosphoglycerate caused a fast displacement of phospho*enol*pyruvate from atractyloside-inhibited mitochondria to the reaction medium. In contrast, on the addition of ATP to mitochondria incubated with 1,2,3-benzenetricarboxylate, the rate of phospho*enol*pyruvate efflux was lower than that induced by either malate or citrate. (4) Despite the presence of both 1,2,3-benzenetricarboxylate and atractyloside, arsenite and rotenone plus antimycin resulted in a leakage of phospho*enol*pyruvate from the mitochondria, probably via a carrier-independent mechanism. (5) Based on the present results it seems that depending on the metabolic condition, the tricarboxylate carrier and the adenine nucleotide translocase are functioning to different extents in the efflux of phospho*enol*pyruvate from rabbit renal mitochondria to the surrounding medium.

Introduction

The transport of phospho*enol*pyruvate across the mitochondrial membrane has been commonly studied as the exchange of this metabolite for intramitochondrial citrate and malate [1–3] or ATP [4]. Robinson [5] reported that in ox liver mitochondria exhibiting a high phospho*enol*pyruvate carboxykinase activity [6,7], phospho*enol*pyruvate synthesized intramitochondrially is transported to the cytosol via the tricarboxylate-transporting system. There are no data, however, on the contribution of adenine nucleotide translocase to the phospho*enol*pyruvate efflux from mitochondria.

The experiments presented in this communication were carried out in rabbit kidney cortex mitochondria, which, like ox liver mitochondria, contain phospho*enol*pyruvate carboxykinase [8] and synthesize efficiently phospho*enol*pyruvate when both substrate level phosphorylation and oxaloacetate are available [9,10].

Both 1,2,3-benzenetricarboxylate, an inhibitor of the tricarboxylate-transporting system [1,5,11], and atractyloside, an inhibitor of adenine nucleotide translocation [12,13], were found to increase the intramitochondrial phospho*enol*pyruvate content in renal mitochondria of rabbit [14,15]. In contrast, agaric acid, reported to inhibit the tricarboxylate carrier in rat liver mitochondria [16,17], affected neither phospho*enol*pyruvate accumulation nor its production in renal mitochondria of rabbit [14]. Thus, the aim of this work was to characterize the efflux of phospho*enol*pyruvate to the surrounding medium.

Abbreviations: FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; TMPD, tetramethyl-*p*-phenylenediamine.

Materials and Methods

Mitochondrial preparation and incubation

Mitochondria were prepared from kidney cortex of white male rabbits (about 2–3 kg in weight) by a minor modification of the method of Schneider and Hogeboom [18] as described previously [9].

The standard incubation mixture contained 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 75 mM Tris-HCl buffer, 10 mM potassium phosphate buffer, 10 mM potassium glutamate, 0.2 mM aminooxyacetate, about 2–3 mg of mitochondrial protein/ml and 15 mM sucrose (derived from the mitochondrial preparation); the final pH was 7.4. State 3 was induced by the following additions to the basic buffer: 20 mM glucose, 0.1 mM ADP and hexokinase (2–5 U/ml). Uncoupled mitochondira were produced by the addition of 0.1–0.2 μM FCCP. When 1,2,3-benzenetricarboxylate was added to the reaction medium, corresponding amounts of Tris-HCl were omitted. Reactions were started by the addition of the substrate followed by incubation of the mitochondrial suspension for 3 min in the reaction medium. Mitochondrial incubations were performed in chambers maintained at 30°C. Oxygen was blown over the surface of the stirred reaction medium. For measurements of the distribution of phospho*enol*pyruvate between mitochondria and the medium, the silicone layer technique was applied [19].

Assays

Determination of phospho*enol*pyruvate in both extramitochondrial and intramitochondrial spaces was done fluorimetrically according to the method of Bucher et al. [20]. Mitochondrial protein was determined by the biuret method [21] as described by Cleland and Slater [22].

Enzymes and chemicals

Pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) used for the phospho*enol*pyruvate assay were purchased from Reanal (Budapest, Hungary) and Polskie Odczynniki Chemiczne (Gliwice, Poland), respectively. $(NH_4)_2SO_4$-free hexokinase (EC 2.7.1.1) was obtained from Koch-Light Laboratories Ltd. (Colnbrook, U.K.) while atractyloside and antimycin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 1,2,3-Benzenetricarboxylate was obtained from Merck-Schuchardt (München, F.R.G.). FCCP and rotenone were the generous gifts of Drs. P. Heytler and J.Howland, respectively. All other chemicals were of analytical grade.

Results and Discussion

The effect of 1,2,3-benzenetricarboxylate, atractyloside and n-butylmalonate on phosphoenolpyruvate formation and intramitochondrial accumulation

As shown in Table I, kidney cortex mitochondria of rabbit producing phospho*enol*pyruvate in either State 4, State 3 or under uncoupled conditions have a low intramitochondrial level of this metabolite (about 1 nmol/mg of mitochondrial protein), suggesting that the phospho*enol*pyruvate-transporting system operates efficiently in these mitochondria.

It is commonly accepted that both the tricarboxylate carrier [1–3] and adenine nucleotide translocase [4] contribute to phospho*enol*pyruvate transport across the mitochondrial membrane of rat liver. Thus, in order to characterize the efflux of phospho*enol*pyruvate from mitochondria, we have applied both 1,2,3-benzenetricarboxylate and atractyloside, at 50 and 1 mM, respectively, since under these conditions they caused both the highest accumulation of intramitochondrial phospho*enol*pyruvate and maximal inhibition of production of this compound [14]. This concentration of 1,2,3-benzenetricarboxylate was found by Robinson [1] to inhibit completely the exchange of phospho*enol*pyruvate with citrate.

The addition of either atractyloside or 1,2,3-benzenetricarboxylate to kidney cortex mitochondria incubated in State 4 and under uncoupled conditions resulted in a marked increase in intramitochondrial phospho*enol*pyruvate (up to about 7 nmol/mg mitochondrial protein) accompanied by a significant decline in the rate of phospho*enol*pyruvate formation (by about 2–4-fold). Presumably, the increase in phospho*enol*pyruvate concentration slows down its synthesis by product inhibition of phospho*enol*pyruvate carboxykinase, so the total phospho*enol*pyruvate synthesized is less when the exit of this compound from the mitochondrial compartment is prevented. In contrast, when 1,2,3-benzenetricarboxylate was added to renal mitochondria incubated in State 3, a decrease in the rate of phospho*enol*pyruvate formation was accompanied by a small increase in its con-

TABLE I

EFFECT OF 1,2,3-BENZENETRICARBOXYLATE AND ATRACTYLOSIDE ON PHOSPHO*ENOL*PYRUVATE FORMATION AND ACCUMULATION IN RABBIT RENAL MITOCHONDRIA INCUBATED UNDER DIFFERENT CONDITIONS

1,2,3-Benzenetricarboxylate and atractyloside were added at 50 mM and 1 mM, respectively, where indicated. The intramitochondrial phospho*enol*pyruvate level was determined after 9 min of incubation. For measurements of the rates of phospho*enol*pyruvate, 1-ml aliquots were removed at 3-min intervals. Values shown are means ± S.E. of five separate experiments.

Incubation conditions	Atractyloside	1,2,3-Benzene-tricarboxylate	Phospho*enolpyruvate* formation (nmol/min per mg protein)	Phospho*enol*pyruvate accumulation (nmol/mg protein)
State 4	–	–	2.1 ± 0.4	1.3 ± 0.4
	+	–	1.2 ± 0.2	4.6 ± 0.3
	–	+	1.4 ± 0.2	6.5 ± 0.5
	+	+	0.6 ± 0.2	7.0 ± 0.4
State 3	–	–	5.8 ± 0.5	0.9 ± 0.3
	–	+	1.5 ± 0.5	1.7 ± 0.5
Uncoupled	–	–	5.9 ± 0.9	0.8 ± 0.1
	+	–	2.6 ± 0.5	7.4 ± 0.5
	–	+	1.5 ± 0.3	6.8 ± 0.4
	+	+	1.1 ± 0.6	8.1 ± 0.7

tent in mitochondria (up to about 2 nmol/mg protein). This effect was not due to an inhibitory effect of 1,2,3-benzenetricarboxylate on phospho*enol*pyruvate formation, since in sonicated mitochondria 50 mM 1,2,3-benzenetricarboxylate did not decrease the phospho*enol*pyruvate production studied with oxaloacetate and GTP as substrates (not shown). Thus, in State 3 the efflux of phospho*enol*pyruvate from mitochondria seems to occur in exchange for the extramitochondrial ATP which is released from mitochondria in exchange for the external ADP via the adenine nucleotide translocase. The exit of phospho*enol*pyruvate from mitochondria via this mechanism implies an increase in the internal adenine nucleotide pool which indeed occurs at a rate of 0.2 ± 0.1 nmol/min per mg mitochondrial protein. However, the rate of phospho*enol*pyruvate efflux from mitochondria incubated in State 3 in the presence of 1,2,3-benzenetricarboxylate is higher than that of adenine nucleotide accumulation under these conditions (cf. Table I). Thus, the possibility of leakage of phospho*enol*pyruvate from mitochondria, probably via a carrier-independent mechanism, cannot also be excluded.

The addition of *n*-butylmalonate, an inhibitor of the dicarboxylate carrier [23], resulted in a decrease in phospho*enol*pyruvate production by about 40% and an increase in intramitochondrial phospho*enol*pyruvate content by about 9-fold (Table II). This may indicate that the efflux of phospho*enol*pyruvate from mitochondria incubated with glutamate as oxa-

TABLE II

THE EFFECT OF *n*-BUTYLMALONATE AND ATRACTYLOSIDE ON PHOSPHO*ENOL*PYRUVATE FORMATION AND ACCUMULATION IN KIDNEY CORTEX MITOCHONDRIA OF RABBIT

2 mM *n*-butylmalonate and 1 mM atractyloside were added where indicated. The intramitochondrial phospho*enol*pyruvate levels were determined after 9 min of incubation. For measurement of the rates of phospho*enol*pyruvate formation, 1-ml aliquots were removed at 3-min intervals up to 9 min of incubation.

Additions	Phospho*enol*-pyruvate formation (nmol/min per mg protein)	Intramitochondrial phospho*enol*-pyruvate level (nmol/mg protein)
None	7.0	0.8
n-Butylmalonate	4.0	7.1
n-Butylmalonate and atractyloside	1.5	10.1

loacetate precursor can occur in exchange for malate which is released from the mitochondria in exchange for extramitochondrial phosphate via the dicarboxylate-transporting system. In the presence of atractyloside together with *n*-butylmalonate, the inhibition of phospho*enol*pyruvate formation was increased to about 80% while the intramitochondrial phospho*enol*pyruvate level was almost 13-fold higher than that measured in the absence of transport inhibitors, suggesting contribution of both the tricarboxylate carrier and adenine nucleotide translocase to the efflux of phospho*enol*pyruvate from kidney cortex mitochondria of rabbit. In order to check this hypothesis we have studied the displacement of phospho*enol*pyruvate from mitochondria to the suspending medium.

Displacement of phosphoenolpyruvate from mitochondria to the suspending medium

Fig. 1 shows the time course of the accumulation of phospho*enol*pyruvate induced by either 1,2,3-benzenetricarboxylate or atractyloside in uncoupled mitochondria which exhibit high rates of phospho*enol*pyruvate production [9]. The highest level of intramitochondrial phospho*enol*pyruvate were observed after approx. 9 min of incubation of renal mitochondria with inhibitor. The rate of phospho*enol*pyruvate accumulation in the extramitochondrial space was linear with time over the period studied

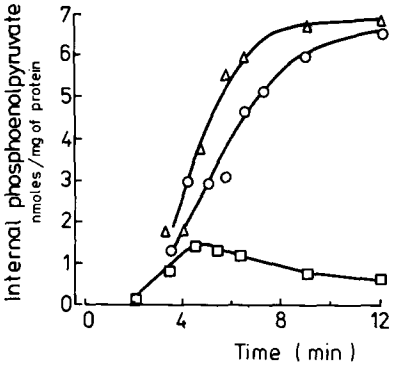

Fig. 1. Effect of 1,2,3-benzenetricarboxylate and atractyloside on the time course of accumulation of phospho*enol*pyruvate in uncoupled mitochondria. The incubation mixture contained 3.1 mg of mitochondrial protein/ml. (□———□) No inhibitor, (○———○) + atractyloside, (△———△) + 1,2,3-benzenetricarboxylate.

(not shown). This may indicate that trace amounts of endogenous malate in the presence of 1,2,3-benzenetricarboxylate, which are released from mitochondria in the presence of atractyloside or ATP, may exchange with intramitochondrial phospho*enol*pyruvate and prevent the complete inhibition of its production.

The addition of either 5 mM citrate, 5 mM malate or 5 mM 2-phosphoglycerate to the mitochondrial suspension incubated with atractyloside caused a fast displacement of phospho*enol*pyruvate from mitochondria to the suspending medium (Fig. 2). In the presence of either 5 mM citrate or 5 mM malate, the rates of phospho*enol*pyruvate production (14.5 and 10.0 nmol/min per mg protein, respectively) were higher than those determined in the absence of transport inhibitors (5.9 nmol/min per mg protein; cf. Table I). Thus, it seems likely that the addition of these metabolites for the exchange with intramitochondrially generated phospho*enol*pyruvate resulted in a stimulation of phospho*enol*pyruvate production. However, the possibility that these compounds might act through providing further oxaloacetate for phospho*enol*pyruvate synthesis could not be discounted. When lower concentrations of citrate and malate were added to the reaction medium, the rates of release of

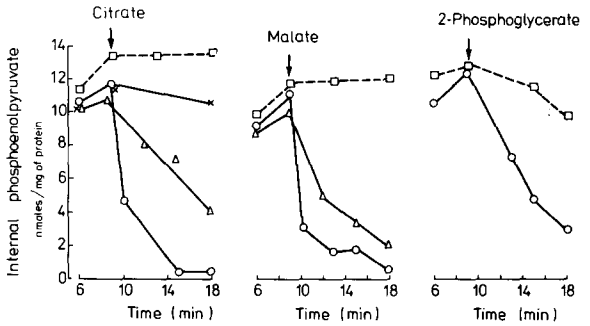

Fig. 2. Effect of citrate, malate, 2-phosphoglycerate, and 1,2,3-benzenetricarboxylate on the phospho*enol*pyruvate levels in mitochondria incubated with atractyloside. Mitochondria (3.2 mg protein/ml) were incubated under uncoupled conditions with either 1 mM atractyloside alone (———) or with both 1 mM atractyloside and 50 mM 1,2,3-benzenetricarboxylate (- - - - -). Following 9 min of incubation various concentrations of citrate (○, □, 5 mM; △, 2 mM; X, 1 mM), malate (○, □, 5 mM; △, 2 mM) or 2-phosphoglycerate (□, ○, 5 mM) were added, where indicated by the arrows.

phospho*enol*pyruvate from mitochondria were lower than those observed in the presence of citrate and malate at 5 mM. In contrast to observations reported by Robinson [5], the presence of 50 mM 1,2,3-benzenetricarboxylate prevented the displacement of intramitochondrially accumulated phospho*enol*pyruvate on the addition of either citrate or malate. The discrepancies between our results and those reported by Robinson [5] could be due to the higher concentration of 1,2,3-benzenetricarboxylate used in our experiments.

The addition of 5 mM 2-phosphoglycerate to the mitochondrial suspension incubated with atractyloside resulted also in the displacement of phospho*enol*pyruvate from mitochondria to the suspending medium (Fig. 2) accompanied by an increase in phospho*enol*pyruvate formation (not shown). However, the rates of both phospho*enol*pyruvate release from mitochondria as well as its appearance in the extramitochondrial space were lower (about 5.5 nmol/min per mg protein) than those measured in the presence of either citrate or malate at 5 mM. When 1,2,3-benzenetricarboxylate was included in the reaction medium, the release of phospho*enol*pyruvate accumulated in the renal mitochondria was largely prevented. These observations are consistent with a low specificity of the tricarboxylate-transporting system in kidney cortex mitochondria, postulated by Kleineke et al. [3] for rat liver mitochondria.

When the mitochondrial accumulation of phospho*enol*pyruvate was induced by 1,2,3-benzenetricarboxylate, the addition of 5 mM ATP but not ADP resulted in the displacement of phospho*enol*pyruvate from the renal mitochondria to the suspending medium (Fig. 3). However, the rates of both the release of intramitochondrial phospho*enol*pyruvate as well as phospho*enol*pyruvate formation (6.0 nmol/min per mg protein) were lower than those caused by 5 mM citrate or 5 mM malate (cf. Fig. 2). The addition of lower concentrations of ATP did not cause displacement of phospho*enol*pyruvate from mitochondria to the suspending medium (results not shown). These observations suggest that in uncoupled mitochondria phospho*enol*pyruvate is preferentially transported by the tricarboxylate-carrier system. This conclusion is consistent with that of Shug and Shrago [4] for rat liver mitochondria. The presence of atractyloside together with 1,2,3-benzenetricarboxylate in the reaction medium prevented release of phospho*enol*pyruvate from mitochondria on the addition of ATP.

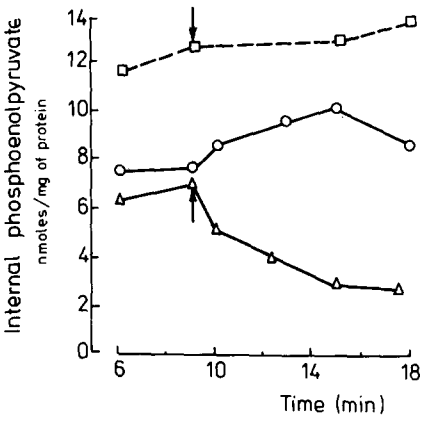

Fig. 3. Effect of ATP, ADP, and atractyloside on the accumulation of phospho*enol*pyruvate in uncoupled mitochondria incubated with 1,2,3-benzenetricarboxylate. The mitochondria (2.9 mg protein/ml) were incubated with either 50 mM 1,2,3-benzenetricarboxylate alone (———) or with both 50 mM 1,2,3-benzenetricarboxylate and 1 mM atractyloside (- - - - -). Following 9 min of incubation, 5 mM ATP (□, △) or 5 mM ADP (○) was added where indicated by the arrows.

As shown in Fig. 4, despite the presence of both 1,2,3-benzenetricarboxylate and atractyloside, the addition of either arsenite or rotenone plus antimycin to uncoupled mitochondria to inhibit phospho*enol*pyruvate formation resulted in leakage of this metabolite from the mitochondria, probably via a carrier-independent mechanism. The latter mechanism was also postulated by Pande and Parvin [24] to account for pyruvate transport into rat heart mitochondria at high concentrations of this compound. Release of phospho*enol*pyruvate was also observed when rotenone plus antimycin was added to 1,2,3-benzenetricarboxylate- and atractyloside-inhibited mitochondria incubated without uncoupler (not shown). The provision of energy from the oxidation of ascorbate plus TMPD did not prevent the release of phospho*enol*pyruvate from mitochondria. All these observations suggest that like citrulline [25], intramitochondrially generated phospho*enol*pyruvate is found to be accumulated in mitochondria while it is being produced.

Based on the present results, it is possible to conclude that depending on the metabolic condition, the

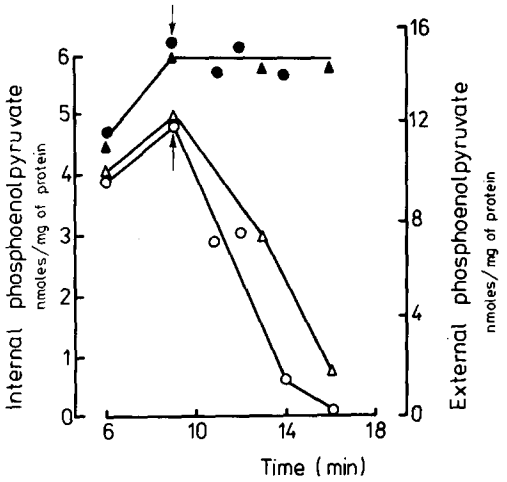

Fig. 4. Effect of rotenone plus antimycin and arsenite on the phospho*enol*pyruvate levels in uncoupled mitochondria incubated with 1,2,3-benzenetricarboxylate and atractyloside. After 9 min of incubation of mitochondria (3.6 mg protein/ml) in the presence of 50 mM 1,2,3-benzenetricarboxylate and 1 mM atractyloside, either 20 μM rotenone plus 5 μM antimycin (●, ○) or 2 mM arsenite (▲, △) was added to the reaction medium, where indicated by the arrows. (○, △) Internal and (●, ▲) external phospho*enol*pyruvate.

tricarboxylate-transporting system and adenine nucleotide translocase are operating to different extents in the efflux of phospho*enol*pyruvate from rabbit renal mitochondria to the surrounding medium. Since 1,2,3-benzenetricarboxylate did not cause marked accumulation of intramitochondrial phospho*enol*pyruvate in State 3, it seems that under these conditions transport of intramitochondrially generated phospho*enol*pyruvate is carried out via the adenine nucleotide translocase. However, since under these conditions a significant increase in the internal adenine nucleotide pool was not observed, this mechanism of phospho*enol*pyruvate transport from renal mitochondria does not seem to be important, especially in vivo. Thus, the efflux of phospho*enol*pyruvate from kidney cortex mitochondria incubated in State 3 in the presence of 1,2,3-benzenetricarboxylate might occur either via adenine nucleotide translocase according to a mechanism not specified as yet, or via a carrier-independent mechanism. In uncoupled mitochondria the phospho*enol*pyruvate efflux appears to occur preferentially via the tricarboxylate-transporting system. This can be concluded from the much higher rate of either citrate- or malate-induced release of phospho*enol*pyruvate from atractyloside-inhibited mitochondria to the surrounding medium (cf. Fig. 2) in comparison with that induced by ATP in 1,2,3-benzenetricarboxylate-inhibited mitochondria (cf. Fig. 3). Moreover, in contrast to rat liver mitochondria [4,26,27], the experiments showing both the citrate- and malate-induced efflux of phospho*enol*pyruvate from atractyloside-inhibited renal mitochondria (cf. Fig. 2) suggest that in rabbit kidney cortex mitochondria, atractyloside does not inhibit the tricarboxylate carrier. Thus, it seems possible that in these mitochondria the tricarboxylate-transporting system and adenine nucleotide translocase operate independently in the inner membrane.

Agaric acid at concentrations as high as 100 μM does not inhibit the phospho*enol*pyruvate efflux from renal mitochondria [14]. This observation is difficult to explain. However, since a higher K_i value of 1,2,3-benzenetricarboxylate was found in renal mitochondria [14] in comparison with that reported for rat liver mitochondria [5], the possibility is not excluded that concentrations of agaric acid greater than 100 μM are required to inhibit phospho*enol*pyruvate efflux from renal mitochondria of rabbit. It is not possible to check this phenomenon due to a detergent action of this compound at high concentration. Thus, it seems that in contrast to rat liver mitochondria, agaric acid at 100 μM is not an inhibitor of the tricarboxylate-transport system in rabbit kidney cortex mitochondria.

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