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SYNTHESIS OF GLUTAMATE FROM α-OXOGLUTARATE AND AMMONIA IN RAT-LIVER MITOCHONDRIA

III. MALATE AS HYDROGEN DONOR

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SUMMARY

1. The synthesis of glutamate and aspartate from α-oxoglutarate and ammonia with malate as hydrogen donor was studied in rat-liver mitochondria in the presence of arsenite (to prevent the oxidation of oxo acids) and glutamate (to prevent the inhibition of malate oxidation by oxaloacetate).

2. In the presence of malate, arsenite, glutamate, α-oxoglutarate and oligomycin, the addition of ammonia causes an increase in the rate of oxygen uptake and a substantial synthesis of glutamate + aspartate.

3. The synthesis of glutamate + aspartate coupled with the aerobic oxidation of malate is slightly stimulated by oligomycin in the absence of phosphate acceptor and greatly stimulated in its presence.

4. The synthesis of glutamate + aspartate is inhibited by antimycin or Amytal. ATP can relieve this inhibition, but not if oligomycin is also present. The inhibition by Amytal can be relieved by adding succinate.

5. Dinitrophenol inhibits the oxidation of malate, disappearance of glutamate and formation of aspartate, both in the presence and absence of ammonia.

6. It is concluded that an oligomycin-insensitive, high-energy intermediate of oxidative phosphorylation is necessary for the transfer of hydrogens from malate to α-oxoglutarate (+ ammonia). Possible explanations for the role of the high-energy intermediate in this system are considered.

INTRODUCTION

When the flavin-linked substrate succinate is used as hydrogen donor for the synthesis of glutamate from α-oxoglutarate and NH_3 in rat-liver mitochondria, an energy-dependent reversal of the respiratory chain takes place[1-3]. In contrast, no energy is required for the synthesis of glutamate when isocitrate, a nicotinamide nucleotide-linked substrate, is the hydrogen donor, as shown by the fact that the process is little affected by dinitrophenol, Amytal, antimycin or oligomycin[4]. Since malate dehydrogenase (EC 1.1.1.37), like the isocitrate dehydrogenases (EC 1.1.1.41 and 1.1.1.42), is a nicotinamide nucleotide-linked enzyme, the finding[4] that the transfer of hydrogens from malate to α-oxoglutarate ($+NH_3$) is inhibited by Amytal and

antimycin and slightly stimulated by oligomycin was unexpected. In this paper, it is shown that high-energy intermediates of oxidative phosphorylation are necessary for the transfer of hydrogens from malate to α-oxoglutarate + NH_3 in rat-liver mitochondria (*cf.* ref. 3). A preliminary account of this investigation has appeared[5].

METHODS

The methods, materials and experimental procedure used are described in the first paper of this series[4]. The standard reaction mixture used contained 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris–HCl buffer, 0.1 mM ADP and 25 mM sucrose (derived from the mitochondrial suspension) in a final volume of 1.0 ml. Other additions are indicated in the legends to the tables and figure. The final pH of the reaction mixture was 7.5. The reaction temperature was 25°.

Oxaloacetate and pyruvate were determined spectrophotometrically with NADH using malate dehydrogenase and lactate dehydrogenase (EC 1.1.1.27), respectively. When the reaction mixture was analysed within 30 min after deproteinization and neutralization, no pyruvate was found. After 30 min, however, any oxaloacetate present was gradually decarboxylated to pyruvate. In practice, both oxaloacetate and pyruvate were determined, and it was assumed that the sum of the oxaloacetate and pyruvate found equalled the amount of oxaloacetate present at the end of the experiment.

RESULTS

The synthesis of glutamate and aspartate from α-oxoglutarate + NH_3 with malate as hydrogen donor was studied in a reaction mixture containing arsenite (to prevent the oxidation of α-oxoglutarate and of any pyruvate formed) and glutamate (to prevent the inhibition of malate oxidation by oxaloacetate). The results of a typical experiment carried out in the presence of oligomycin are presented in Table I. A substantial synthesis of aspartate occurred, a small amount of glutamate disappeared and some oxaloacetate was formed. The time course of the reaction is shown in Fig. 1. The rate of synthesis of glutamate + aspartate remained constant for 16 min and then decreased slightly. Oxygen uptake and oxaloacetate formation remained linear throughout the experimental period.

TABLE I

SYNTHESIS OF AMINO ACIDS COUPLED WITH THE AEROBIC OXIDATION OF MALATE

Reaction mixture contained, in addition to the basic components, 20 mM potassium phosphate buffer, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 20 mM malate, 10 mM glutamate, 1 mM arsenite, 1% ethanol, 10 μg oligomycin and 8.5 mg mitochondrial protein. Reaction time, 24 min. (Expt. 179).

Δ O (μatoms)	*Δ Glutamate (μmole)*	*Δ Aspartate (μmoles)*	*Δ (Glutamate + aspartate) (μmoles)*	*Δ Oxaloacetate (μmole)*
2.35	−0.46	12.08	11.62	0.77

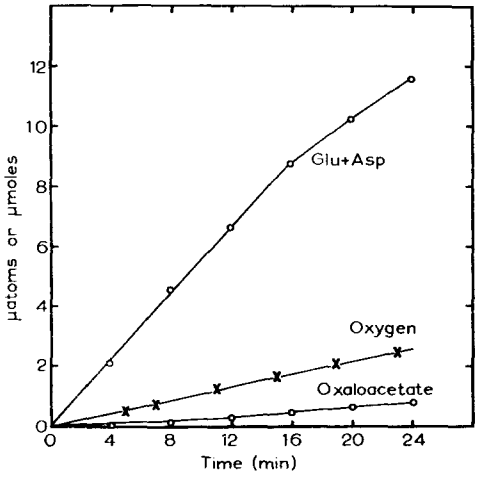

Fig. 1. Time course of oxygen uptake, synthesis of amino acids and formation of oxaloacetate coupled with the aerobic oxidation of malate. Conditions indicated in Table I. (Expt. 179).

The effect of NH_3 on the oxidation of malate in the presence of α-oxoglutarate and phosphate acceptor is shown in Table II. NH_3 brought about a decrease in the amount of glutamate that disappeared and an increase in the amount of aspartate formed, resulting in a net synthesis of 3.2 μmoles amino acids. In the presence of oligomycin, NH_3 had no effect on the amount of glutamate that disappeared and stimulated aspartate synthesis greatly, so that there was a net synthesis of 9.6 μmoles amino acids.

The addition of NH_3 inhibited oxygen uptake in the presence of phosphate acceptor, and stimulated it when oligomycin was present. This "uncoupling" by α-oxoglutarate + NH_3 of malate oxidation is also shown in Table III, where the effects on the oxidation of both malate and succinate are compared. Oxygen uptake was stimulated 2.4-fold in the case of succinate (*cf.* ref. 1) and 1.4-fold in the case of malate (*cf.* Table II).

TABLE II

EFFECT OF NH_3, OLIGOMYCIN AND DINITROPHENOL ON OXYGEN UPTAKE AND THE SYNTHESIS OF AMINO ACIDS COUPLED WITH THE AEROBIC OXIDATION OF MALATE

Reaction mixture contained, in addition to the basic components, 20 mM malate, 10 mM α-oxoglutarate, 1 mM arsenite, 5 mM glutamate, 20 mM glucose, 150 units hexokinase, 20 mM potassium phosphate buffer and 8.8 mg mitochondrial protein. Reaction time, 20 min. (Expt. 157).

Additions	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*	*Δ (Glutamate + aspartate) (μmoles)*
None	7.0	−4.69	5.02	0.33
Dinitrophenol (50 μM)	2.9	−2.44	1.91	−0.53
Oligomycin (1.1 μg/mg protein)	1.8	−1.47	1.69	0.22
NH_4Cl (20 mM)	5.5	−3.89	7.12	3.23
NH_4Cl + dinitrophenol	1.6	−1.07	2.12	1.05
NH_4Cl + oligomycin	2.6	−1.31	10.91	9.61

TABLE III

STIMULATION BY α-OXOGLUTARATE + NH_3 OF THE AEROBIC OXIDATION OF SUCCINATE AND OF MALATE IN THE PRESENCE OF OLIGOMYCIN

Reaction mixture contained, in addition to the basic components, 20 mM potassium phosphate buffer, 1 mM arsenite, 10 μg oligomycin and 4.7 mg mitochondrial protein. Reaction time, 24 min (Expt. 214).

Additions	*Δ O (μatoms)*
Succinate (60 mM)	3.27
Succinate + α-oxoglutarate (20 mM) + NH_4Cl (20 mM)	8.05
Malate (20 mM) + glutamate (10 mM)	1.07
Malate + glutamate + α-oxoglutarate + NH_4Cl	1.48

TABLE IV

OLIGOMYCIN-SENSITIVE STIMULATION BY ATP OF THE SYNTHESIS OF GLUTAMATE + ASPARTATE COUPLED WITH THE OXIDATION OF MALATE IN THE PRESENCE OF ANTIMYCIN

Reaction mixture contained, in addition to the basic components, 10 mM α-oxoglutarate, 20 mM NH_4Cl, 20 mM malate, 5 mM glutamate, 1 mM arsenite, 2 mM potassium phosphate buffer, 2% ethanol and 7.2 mg mitochondrial protein. Reaction time, 20 min. (Expt. 163).

Additions	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*	*Δ (Glutamate + aspartate) (μmoles)*	*Δ Oxalo-acetate (μmole)*
None	3.2	−1.35	9.39	8.04	0.89
Oligomycin (10 μg)	2.3	−0.87	9.47	8.60	0.71
Antimycin (5 μg)	1.0	0.48	2.73	3.21	0.75
Antimycin + oligomycin	1.0	0.34	2.68	3.02	0.78
Antimycin + ATP (10 mM)	0.9	1.12	7.39	8.51	0.69
Antimycin + ATP + oligomycin	1.0	0.90	3.33	4.23	0.77

TABLE V

OLIGOMYCIN-SENSITIVE STIMULATION BY ATP OF THE SYNTHESIS OF GLUTAMATE + ASPARTATE COUPLED WITH THE OXIDATION OF MALATE IN THE PRESENCE OF AMYTAL

Reaction mixture contained, in addition to the basic components, 10 mM α-oxoglutarate, 20 mM NH_4Cl, 20 mM malate, 5 mM glutamate, 1 mM arsenite, 2 mM potassium phosphate buffer, 1% ethanol and 6.2 mg mitochondrial protein. Reaction time, 20 min. (Expt. 162B).

Additions	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*	*Δ (Glutamate + aspartate) (μmoles)*	*Δ Oxalo-acetate (μmole)*
None	2.4	−1.39	8.61	7.22	0.39
Oligomycin (10 μg)	2.1	−0.96	8.65	7.69	0.44
Amytal (2 mM)	0.2	0.75	0.85	1.60	0.46
Amytal + oligomycin	0.2	0.88	0.81	1.69	0.44
Amytal + ATP (10 mM)	0.3	0.75	5.77	6.52	0.44
Amytal + ATP + oligomycin	0.4	0.51	1.43	1.94	0.10

TABLE VI

UTILIZATION OF HIGH-ENERGY INTERMEDIATES FORMED DURING THE AEROBIC OXIDATION OF SUCCINATE FOR AMINO ACID SYNTHESIS WITH MALATE AS HYDROGEN DONOR

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 20 mM potassium phosphate buffer, 1 mM arsenite, 10 mM glutamate, 10 μg oligomycin and 7.2 mg mitochondrial protein. Reaction time, 20 min. (Expt. 212).

Additions	*Δ O (μatoms)*	*Δ Glutamate (μmoles)*	*Δ Aspartate (μmoles)*	*Δ (Glutamate + aspartate) (μmoles)*	*Δ Oxalo-acetate (μmole)*
Succinate (60 mM)	7.7	5.86	6.15	12.01	0.36
Malate (20 mM)	2.0	−0.73	10.75	10.02	0.73
Succinate + Amytal (2 mM)	5.1	1.04	3.95	4.99	0.38
Malate + Amytal	0.1	1.21	0.58	1.79	0.38
Succinate + malate + Amytal	6.6	0.98	11.06	12.04	0.73

Table II shows that dinitrophenol markedly inhibited the oxidation of malate, disappearance of glutamate and synthesis of aspartate, both in the presence and absence of NH_3.

The synthesis of amino acids coupled with the oxidation of malate was inhibited by antimycin or Amytal (Tables IV and V; *cf.* Table IX of ref. 4). This inhibition could be relieved by ATP, and the stimulation by ATP was prevented by oligomycin

TABLE VII

EFFECT OF OLIGOMYCIN ON THE SYNTHESIS OF AMINO ACIDS COUPLED WITH THE OXIDATION OF MALATE IN THE ABSENCE OF PHOSPHATE ACCEPTOR

Reaction mixture contained, in addition of the basic components, 20 mM malate, 5 or 10 mM glutamate, 10 or 20 mM α-oxoglutarate, 20 mM NH_4Cl, 1 mM arsenite, 2 or 20 mM potassium phosphate buffer and 4.3–8.5 mg mitochondrial protein. Reaction time, 20 min.

Oligomycin (μg/ml)	*Glutamate + Aspartate (μmoles/mg protein/20 min)*		
	Mean	*Range*	*No. of measurements*
0	0.89	0.79–1.27	8
10	1.18	0.89–1.39	9

(Tables IV and V). The inhibition by Amytal of the malate-induced synthesis of amino acids could also be overcome by coupling the reaction with the aerobic oxidation of succinate (Table VI).

In the absence of phosphate acceptor, oligomycin stimulated the malate-induced synthesis of amino acids slightly (Table VII).

DISCUSSION

In the experiments described in this paper, the only source of reducing equivalents for amino acid synthesis was malate. Endogenous substrate contributes very little

to amino acid synthesis (*cf.* ref. 1) and, since arsenite was present, α-oxoglutarate could not be oxidized. In the absence of respiratory-chain inhibitors, some glutamate disappeared, the amount depending on the extent of the aerobic oxidation of malate. Thus, more glutamate disappeared in the absence of oligomycin than in its presence (Table II, *cf.* lines 4 and 6). When the oxidation of NADH by oxygen was blocked by Amytal or antimycin, no glutamate disappeared (Tables IV and V). At the end of the reaction, the only products detected besides aspartate were oxaloacetate and α-oxoglutarate. Thus, the overall reactions occurring in the absence of respiratory-chain inhibitors are the following (Reactions 1–4),

$$(n+x+y)\text{malate}+(n+x+y)\text{NAD}^+ \rightleftharpoons (n+x+y)\text{oxaloacetate}+(n+x+y)\text{NADH}+(n+x+y)\text{H}^+ \quad (1)$$

$$n\ \text{NADH} + n\ \text{NH}_4^+ + n\ \alpha\text{-oxoglutarate} \rightleftharpoons n\ \text{glutamate} + n\ \text{NAD}^+ \quad (2)$$

$$(x+y)\text{NADH} + (x+y)\text{H}^+ + (x+y)\text{O} \rightarrow (x+y)\text{NAD}^+ + (x+y)\text{H}_2\text{O} \quad (3)$$

$$(n+x)\text{glutamate} + (n+x)\text{oxaloacetate} \rightleftharpoons (n+x)\ \text{aspartate} + (n+x)\alpha\text{-oxoglutarate} \quad (4)$$

the sum of which is Reaction 5

$$(n+x+y)\text{malate} + n\ \text{NH}_3 + (x+y)\text{O} + x\ \text{glutamate} \rightarrow$$
$$\rightarrow (n+x)\text{aspartate} + y\ \text{oxaloacetate} + (x+y)\text{H}_2\text{O} + x\ \alpha\text{-oxoglutarate} \quad (5)^*$$

Reaction 5 explains the inhibition of oxygen uptake when ammonia is added to the system in the presence of phosphate acceptor, since some of the NADH is oxidized by α-oxoglutarate (+ ammonia) instead of by oxygen.

Reaction 5 emphasizes that there is a net synthesis of aspartate when malate is used as hydrogen donor for the reduction of α-oxoglutarate + NH_3. The following are the principal properties of this system: (a) When α-oxoglutarate + NH_3 are added to mitochondria in the presence of malate, glutamate, arsenite and oligomycin, there is a stimulation of the oxygen uptake, and, concomitantly a net synthesis of amino acids occurs. (b) When the aerobic oxidation of malate is inhibited by antimycin or Amytal, the synthesis of amino acids is also inhibited. (c) The inhibition of amino acid synthesis by antimycin or Amytal can be overcome by adding ATP. However, ATP is without effect if oligomycin is also present. (d) The inhibition of amino acid synthesis by antimycin or Amytal can be overcome by coupling the reaction with the aerobic oxidation of tetramethyl-*p*-phenylenediamine[6] or, in the case of Amytal, with the aerobic oxidation of succinate. (e) In the absence of antimycin, Amytal or phosphate acceptor, oligomycin stimulates the synthesis of amino acids slightly.

These results show that high-energy intermediates of oxidative phosphorylation that are formed before the oligomycin block play a role in the synthesis of amino acids coupled with the oxidation of malate. The intermediates can be generated during the aerobic oxidation of malate, succinate or tetramethyl-*p*-phenylenediamine and can also be formed from ATP by an oligomycin-sensitive pathway.

When the hydrogen donor for amino acid synthesis is succinate, high-energy

* Some endogenous substrate must have been oxidized in addition to malate, since the oxygen uptake was actually greater than the sum of the glutamate disappearing and the oxaloacetate formed (see Table I).

intermediates are required in stoicheiometric amounts to drive an endergonic reaction[2]. In contrast, the energy requirement for amino acid synthesis in the malate system is not stoicheiometric, several molecules of amino acids being synthesized for each high-energy bond consumed[2]. Thus a high-energy bond does not enter any of Reactions 1–4, but is necessary for one or more of them to proceed.

KLINGENBERG[3] has also demonstrated that the synthesis of amino acids coupled with the oxidation of malate is an energy-linked process. He found that the synthesis was inhibited by anaerobiosis and that the inhibition could be overcome by adding ATP. KLINGENBERG has suggested that, in the absence of ATP, aspartate accumulates within the mitochondria and inhibits any further synthesis, and that energy is necessary to remove aspartate from within the mitochondria. He showed, in fact, that the ratio of extramitochondrial to intramitochondrial aspartate was greatly increased by the addition of ATP. However, his data show that intramitochondrial aspartate was not decreased but slightly increased as a consequence of the addition of ATP, which is difficult to reconcile with his theory.

Tentatively, we should prefer one of the following two explanations:

1. Energy is necessary to remove oxaloacetate from malate dehydrogenase, which it strongly inhibits, so that it can react with aspartate transaminase (EC 2.6.1.1). PARDEE AND POTTER[7] have suggested that ATP reacts with oxaloacetate to form a compound which is not inhibitory to succinate dehydrogenase. However, the stoicheiometry mentioned above would require that one high-energy bond could promote the movement of more than one oxaloacetate molecule.

2. Energy is necessary for the transfer of NADH formed by the NAD^+-specific malate dehydrogenase to a "compartment" where it can react with glutamate dehydrogenase or into a form which reacts with this enzyme. KLINGENBERG[3,8,9] has suggested that glutamate dehydrogenase reacts with NADPH rather than with NADH in mitochondria (see also refs. 4, 10). This raises the possibility that energy might be necessary for the reduction of $NADP^+$ by NADH, for example as in the following mechanism:

$$\text{malate} + NAD^+ \rightleftharpoons \text{oxaloacetate} + NADH + H^+ \quad (1)$$

$$NADH + NADP \sim I \rightleftharpoons NADPH + NAD \sim I \quad (6)$$

$$NADPH + NH_4^+ + \alpha\text{-oxoglutarate} \rightleftharpoons \text{glutamate} + NADP^+ \quad (7)$$

$$NADP^+ + NAD \sim I \rightleftharpoons NADP \sim I + NAD^+ \quad (8)$$

$$\text{glutamate} + \text{oxaloacetate} \rightleftharpoons \text{aspartate} + \alpha\text{-oxoglutarate} \quad (4)$$

According to this mechanism, high-energy forms of NAD^+ and $NADP^+$ catalyze the transfer of hydrogens from NADH to $NADP^+$. Reactions 6 and 8 describe this transfer, which is a reversal of the NAD(P) transhydrogenase (EC 1.6.1.1) reaction. This mechanism for the transhydrogenase reaction was first proposed by PURVIS[11] in 1958. PURVIS[12] had previously demonstrated the existence of two new forms of nicotinamide nucleotide and suggested that these were high-energy forms, designated as $NAD \sim I$ and $NADP \sim I$. He obtained evidence[11] that NADH reacts with $NADP \sim I$ to form NADPH. Other evidence for a transhydrogenase reaction of this nature is provided by KLINGENBERG AND SCHOLLMEYER's[13] finding that in the presence of NAD-linked substrates, the level of NADPH is influenced by

the ATP level. In contrast, ATP has no effect on the extent of the reduction of $NADP^+$ by NADP-linked substrates. ESTABROOK AND NISSLEY[14] have also recently reported an oligomycin-sensitive ATP requirement for the reduction of $NADP^+$ by β-hydroxybutyrate.

Insufficient evidence is available to allow a decision as to whether either of these explanations of the energy requirement for aspartate synthesis from malate and NH_3, via glutamate dehydrogenase, is correct. The second explanation is in agreement with much that has been learned about the mechanism of glutamate synthesis. However, in assigning the energy requirement to the transfer of NADH formed in Reaction 1 to the glutamate dehydrogenase, it does not take account of the fact that dinitrophenol inhibits oxygen uptake, glutamate disappearance and aspartate formation. An inhibition by dinitrophenol of the transfer of NADH to Reaction 2 would lead to the replacement of Reaction 5 by Reaction 9

$$(x + y)\text{malate} + (x + y)O + x\ \text{glutamate} \rightarrow \rightarrow x\ \text{aspartate} + y\ \text{oxaloacetate} + (x + y)H_2O + x\ \alpha\text{-oxoglutarate} \quad (9)$$

i.e., there would be less aspartate formation, but the oxygen uptake and the glutamate disappearance would be unaffected. The inhibitions by dinitrophenol shown in Table II are more easily explained by an effect on oxaloacetate.

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