Control of Phosphoenolpyruvate Synthesis by Substrate Level Phosphorylation in Guinea Pig Liver Mitochondria*

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JADWIGA BRYLA,** COLLEEN M. SMITH,** and JOHN R. WILLIAMSON††

From the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104

SUMMARY

Control of P-enolpyruvate synthesis has been studied in guinea pig liver mitochondria with respect to the relative rates of GTP generation directly from substrate level phosphorylation or indirectly from ATP via nucleoside diphosphate kinase. With malate or pyruvate plus malate as substrates, P-enolpyruvate production was greatly decreased in State 3 by addition of fluorocitrate (an inhibitor of aconitase) to prevent flux through substrate level phosphorylation. On the other hand, when ATP production by the electron transport chain was prevented by use of an uncoupling agent (together with oligomycin to inhibit ATPase activity), rates of P-enolpyruvate production from a-ketoglutarate were the same as in State 3, and were also unaffected by addition of fluorocitrate. In the absence of oxidative phosphorylation and with fluorocitrate present together with malate or pyruvate plus malate, addition of exogenous ATP was much less effective than a-ketoglutarate in promoting P-enolpyruvate synthesis. Intramitochondrial GTP levels and rates of P-enolpyruvate formation were consistently lower in State 4, State 3, and the uncoupled plus oligomycin state with pyruvate plus malate than with a-ketoglutarate plus malate as substrate when fluorocitrate was present, indicating a limitation in the rate of phosphorylation of GDP by intramitochondrial ATP for optimal rates of P-enolpyruvate synthesis. The maximal rate of conversion of ATP to GTP by nucleoside diphosphate kinase in the intact mitochondrion was estimated to be 2 to 3 nmoles per min per mg of protein compared with a maximal rate of P-enolpyruvate formation of about 22 nmoles per min per mg of protein obtained with a-ketoglutarate plus malate as substrate.

It is concluded that provision of GTP by substrate level phosphorylation is essential to support rates of P-enolpyruvate formation by guinea pig liver mitochondria greater than 3 nmoles per min per mg of protein.

In animal species such as the guinea pig, rabbit, and human, mitochondria may contain the major part of the total P-enolpyruvate carboxykinase (EC 4.1.1.32) activity of the liver. The exact proportion depends on the dietary status since only the cytosolic enzyme is adaptive (1–3). Theoretically, the GTP requirement for mitochondrial P-enolpyruvate formation may be provided either by substrate level phosphorylation of GDP associated with the conversion of succinyl-CoA to succinate, or be derived from ATP, either via ATP:phosphocreatine phosphotransferase (EC 2.7.4.6) or possibly by the combined actions of ATP:GMP phosphotransferase (EC 2.7.4.8) and GTP:AMP phosphotransferase (EC 2.7.4.10). Earlier studies have shown that with a-ketoglutarate as substrate, P-enolpyruvate production was stimulated both by addition of uncoupling agents and by addition of a phosphate acceptor system, and that exogenously added ATP was relatively ineffective (4–9). Ishihara and Kikuchi (9) noted that under conditions of maximal P-enolpyruvate synthesis in guinea pig liver mitochondria, an approximately equal amount of a-ketoglutarate was oxidized, and suggested that substrate level phosphorylation was preferred over ATP as an energy donor due to the low activity of nucleoside diphosphate kinase. However, Garber and Ballard (10) challenged this view on the basis of their data, also with guinea pig liver mitochondria, which showed that addition of 2,4-dinitrophenol to uncouple oxidative phosphorylation decreased the rate of P-enolpyruvate formation relative to the rate obtained with State 3 respiration when a variety of precursors were used. Furthermore, addition of a-ketoglutarate did not relieve the inhibition of P-enolpyruvate formation induced by the uncoupler. An apparent equilibration of the ATP and GTP pools was also observed, such that the ATP levels appeared to correlate well with both ATP formation and P-enolpyruvate synthesis. The main reason for the discrepancies in results obtained by the different workers may relate to the fact that uncoupling agents not only release respiratory control in the electron transport chain but also progressively stimulate a latent ATPase in isolated mitochondria before inhibitory effects are observed with increasing uncoupler concentrations (11–14). Thus, with the relatively high uncoupler concentration used by Garber and Ballard (10), utilization of GTP for P-enolpyruvate synthesis would compete with its hydrolysis by the combined actions of nucleoside diphosphate kinase and the activated ATPase.

In the present experiments, use of an uncoupling agent to prevent ATP formation by the electron transport chain and induce a high respiratory activity and an oxidized state of the intramitochondrial pyridine nucleotide has been combined with the simultaneous addition of oligomycin. This causes an inhibition...
of respiratory chain-linked ATPase activity (14), and hence eliminates any possible drain on GTP via ATP formation through nucleoside diphosphate kinase. In addition, metabolism of substrates through specific portions of the citric acid cycle has been defined by use of fluorocitrate to inhibit aconitate (15) and 2-thienoyltrifluoroacetone to inhibit succinate dehydrogenase (16). Thus, in the presence of fluorocitrate and with malate or pyruvate plus malate as substrates, effects of externally added ATP can be investigated without the complication of GTP formation at the substrate level phosphorylation step. Likewise, when succinate dehydrogenase is inhibited by 2-thienoyltrifluoroacetone, α-ketoglutarate can be used to generate GTP without providing a source of intramitochondrial malate. Measurements of flux through P-enolpyruvate carboxykinase have been combined with direct assays for intramitochondrial ATP, GTP, CoA, succinyl-CoA, glutarate can be used to generate GTP without providing a source of ATP and GTP (19). A reinvestigation of the nucleotide specificities under conditions of the assays showed that at concentrations of 5 μM, ATP and GTP but not GTP or ATP reacted with hexokinase, while ATP and GTP but not UTP reacted with P-glycerate kinase. Thus, ATP in the sample was first reacted by following the increase of NADPH fluorescence in a reaction system containing NADP+, glucose, hexokinase, and glucose-6-P dehydrogenase. Subsequently, NADH and 3-P-glycerate were added and GTP was assayed by following the decrease of NADH fluorescence upon addition of glyceraldehyde-3-P dehydrogenase and P-glycerate kinase. Succinyl-CoA was assayed by following the fluorescence decrease of NADH upon addition of succinyl-CoA: acecoacetate transferase (EC 2.8.3.5) in a reaction medium containing acecoacetate and β-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.35) (20). α-Ketoglutarate was assayed fluorometrically in the presence of excess aspartate and NADH by coupling aspartate aminotransferase with male dehydrogenase in an analogous manner to the method described for the aspartate assay (19). P-Enolpyruvate was assayed by coupling pyruvate kinase with lactate dehydrogenase (10). Protein was determined by the biuret method as described by Cleland and Slater (21).

RESULTS AND DISCUSSION

Table I shows a comparison of rates of P-enolpyruvate synthesis and respiration by guinea pig liver mitochondria incubated with different substrates in the presence and absence of fluorocitrate under conditions of State 3 and uncoupled plus oligomycin respiration. These data illustrate the restriction imposed on the rate of P-enolpyruvate formation when GTP can only be generated from ATP, and in contrast to the results of Garber and Ballard (10) reveal the importance of direct GTP formation by substrate level phosphorylation for optimal rates of P-enolpyruvate synthesis from malate. With malate as sole substrate, P-enolpyruvate formation was inhibited about 70% relative to the State 3 rate of 6.7 nmoles per min per mg of protein by addition of either uncoupler plus oligomycin or fluorocitrate. Similar effects were observed with pyruvate plus malate as substrates.

Table I

| Substrate  | Fluorocitrate, 20 μM | Oxygen uptake        | P-enolpyruvate formation |
|------------|---------------------|----------------------|-------------------------|
|            | State 3 | Uncoupled + oligomycin | State 3 | Uncoupled + oligomycin |

|                      | mmoles/min/mg protein | nmoles/min/mg protein |
|----------------------|-----------------------|-----------------------|
| 5 mm malate          | 18 ± 1 | 12 ± 1 | 6.7 ± 0.2 | 2.3 ± 1.3 |
| 2 mm pyruvate +      | 34 ± 2 | 26 ± 2 | 46.90 ± 0.653 | 8.6 ± 0.30 |
| 1.5 mm malate        | 26 ± 1 | 21 ± 1 | 47.49 ± 0.20 | 4.3 ± 0.05 |
| 2 mm α-ketoglutarate | 23 ± 1 | 20 ± 1 | 5.4 ± 1.0 | 9.3 ± 0.3 |
| 1 mm α-ketoglutarate | 22 ± 1 | 20 ± 1 | 5.2 ± 0.2 | 9.3 ± 0.3 |

Metabolites were determined by fluorometric enzyme procedures as described by Williamson and Corkey (19). ATP and GTP were assayed in the same cuvette by taking advantage of the specificities of hexokinase and P-glycerate kinase for ATP and GTP (19). A reinvestigation of the nucleotide

1 Kindly supplied by Dr. Peter Heytler of the DuPont Chemical Company.
FIG. 1. P-enolpyruvate formation and intramitochondrial levels of ATP and GTP in guinea pig liver mitochondria incubated under different respiratory states in the presence of 20 μM fluorocitrate. Solid lines refer to conditions with 1 mM α-ketoglutarate and 1.5 mM malate as substrates, and the dotted lines refer to conditions with 2 mM pyruvate and 1.5 mM malate. Values shown are the means of 2 to 3 separate experiments. (Table I). In the presence of uncoupler plus oligomycin, ATP formation by oxidative phosphorylation is prevented, while fluorocitrate addition stops the generation of GTP by substrate level phosphorylation in the citric acid cycle. When the mitochondrial ATPase was allowed to remain active by addition of uncoupler alone, the rate of P-enolpyruvate synthesis from malate fell to 0.6 nmoles per min per mg of protein compared with the rate of 2.3 nmoles per min per mg of protein observed in the uncoupled plus oligomycin state. The occurrence of flux into the citric acid cycle and subsequent substrate level phosphorylation with malate as sole substrate may be inferred from the high ratios of oxygen consumption to P-enolpyruvate formation shown in Table I, and the accumulation of citrate in the medium upon fluorocitrate addition. Presumably acetyl-CoA required for citrate formation is provided by the oxidation of endogenous fatty acids (22). Since addition of fluorocitrate in State 3 diminished P-enolpyruvate formation from 6.7 to 2.2 nmoles per min per mg of protein without affecting the intramitochondrial ATP levels, the latter value would appear to represent the maximal rate of GTP formation from ATP via nucleoside diphosphate kinase. The decreased rates of P-enolpyruvate formation observed in the uncoupled and uncoupled plus oligomycin states relative to State 3 may be accounted for partly by the absence of GTP formation from ATP and partly by a decreased flux through substrate level phosphorylation induced by ATP depletion (cf. Fig. 1) which diminishes activation of endogenous fatty acids to the CoA esters (22). With fluorocitrate present in addition to uncoupler and oligomycin, with malate or pyruvate plus malate as substrates, the generation of both ATP and GTP is prevented, and the rate of P-enolpyruvate synthesis falls essentially to zero.

In contrast to the above data, when α-ketoglutarate was used as substrate, neither fluorocitrate nor uncoupler plus oligomycin produced a significant inhibition of P-enolpyruvate formation (Table I). However, although not shown in Table I, the addition of uncoupler alone decreased P-enolpyruvate formation to 2.7 nmoles per min per mg of protein relative to the State 3 rate of 5.4 nmoles per min per mg of protein. Inhibition of the ATPase by oligomycin increased the intramitochondrial ATP levels to 4 nmoles per mg of protein (cf. Fig. 2) compared with values of 0.9 nmoles per mg of protein obtained with uncoupler alone. That the increased ATP level is the result of an altered balance between its rate of production from GTP and its hydrolysis by ATPase, rather than due to residual oxidative phosphorylation, may be deduced from the very low rates of glucose-6-P formation obtained when the glucose-hexokinase trapping system was included in the uncoupled and uncoupled plus oligomycin states. This interpretation is also supported by the observation of

Fig. 2. Production of P-enolpyruvate, malate, and succinate, and intramitochondrial levels of GTP, ATP, and succinyl-CoA in guinea pig liver mitochondria incubated with 2 mM α-ketoglutarate and 20 μM fluorocitrate under conditions of uncoupler plus oligomycin respiration.
The present data are in close agreement with those of Ishihara and Kikuchi (9) and confirm their conclusion that the production of oxalacetate from the added a-ketoglutarate, the stimulatory effects of both ATP and a-ketoglutarate addition were diminished. However, a-ketoglutarate remained far more effective than ATP in promoting P-enolpyruvate synthesis, again suggesting that the rate of production of GTP from ATP via nucleoside diphosphate kinase in guinea pig liver mitochondria is unable to support a rate of P-enolpyruvate formation greater than 2 to 3 nmoles per min per mg of protein, which is 10 to 15% of the observed maximal rate.

In order to substantiate the correlation between substrate level phosphorylation and flux through P-enolpyruvate carboxykinase, ATP and GTP levels were determined in mitochondria incubated with either pyruvate plus malate or a-ketoglutarate plus malate as substrates under conditions of State 4, State 3, and uncoupled plus oligomycin respiration (Fig. 1). With fluorocitrate included in the reaction medium, generation of GTP in the presence of pyruvate plus malate could only occur from intramitochondrial ATP via nucleoside diphosphate kinase. In State 4, when rates of P-enolpyruvate production were low because of oxalacetate limitation resulting from the high NADH:NAD ratio characteristic of this state, ATP levels remained high (5 to 7 nmoles per mg of protein) with both substrates, but P-enolpyruvate production and GTP levels were considerably lower in the absence of flux through the substrate level phosphorylation step. However, in State 4 with pyruvate plus malate as substrate, limited oxalacetate as well as limited GTP availability may contribute toward restricting the rate of P-enolpyruvate formation to the observed low rate of 1 n mole per min per mg of protein. This is because NADH levels were higher in the presence of pyruvate plus malate (1.26 nmoles per mg of protein) than with a-ketoglutarate plus malate (0.78 nmoles per mg of protein). The different effectiveness of pyruvate plus malate, and a-ketoglutarate plus malate as precursors for P-enolpyruvate synthesis was even more marked under conditions of State 3 and uncoupled plus oligomycin respiration. The levels of NADH remained relatively low and constant (0.3 to 0.5 nmoles per mg of protein), suggesting that differences of oxalacetate availability did not provide an uncon.

2 J. Bryla, M. H. Fukami, and J. H. Williamson, in preparation.
In the uncoupled plus oligomycin state, GTP levels were hardly detectable with pyruvate plus malate as substrate, and P-enolpyruvate production was negligible. With State 3 respiration, the steady state level of intramitochondrial ATP was lower than in State 4, but despite a high rate of ATP synthesis, the rate of P-enolpyruvate formation was 10 times lower with pyruvate plus malate as substrate than with α-ketoglutarate plus malate. Thus, with no direct generation of GTP by substrate level phosphorylation, the rate of P-enolpyruvate formation by guinea pig liver mitochondria appears to be limited to about 0.2 nmoles per mg of protein. On the other hand, the intramitochondrial levels of ATP and succinyl-CoA decreased appreciably after an initial rise over the first 6 min of incubation. These data illustrate the lack of equilibration between GTP and ATP when there is an energy drain on the system via P-enolpyruvate formation. The levels of acetyl-CoA remained negligible, and the sum of CoA and succinyl-CoA was constant within experimental error, so that the fall of succinyl-CoA between 6 and 10 min shown in Fig. 2 represents a decrease of the succinyl CoA:CoA ratio from 0.53 to 0.37. The accumulation of malate and succinate in the medium on addition of P-enolpyruvate indicates that flux through P-enolpyruvate carboxykinase was less than through α-ketoglutarate dehydrogenase, as also shown directly from measurements of α-ketoglutarate uptake. Since the calculated rate of production of GTP was greater than its rate of utilization for P-enolpyruvate synthesis, a flux of 2 to 3 nmoles per min per mg of protein in the direction of GTP to ATP via nucleoside diphosphate kinase must have occurred, thereby accounting for the rise of intramitochondrial ATP. This flux is about equal to the maximal flux through nucleoside diphosphate kinase previously estimated for the reverse direction from ATP to GTP (cf. Table I).

Upon addition of α-ketoglutarate to the mitochondria, the intramitochondrial levels of malate and P-enolpyruvate fell rapidly, presumably as a result of α-ketoglutarate entry, but subsequently increased again during the course of the incubation period (Table III). The stimulation of P-enolpyruvate formation with time, therefore, can be accounted for by an increase of the intramitochondrial concentration of oxaloacetate. This follows from the fact that the NADH levels remained approximately constant between 0.1 and 0.2 nmoles per mg of protein so that an increased intramitochondrial malate concentration would be expected to yield a proportional increase of oxaloacetate concentration. Although this could not be detected by direct assay, an estimate can be made assuming equilibration of malate dehydrogenase, a constant pH, and an NADH/NAD ratio of 200 characteristic of a highly oxidized state of the mitochondrial pyridine nucleotide system (23). For an intramitochondrial malate concentration of 340 μM (Table III), the estimated oxaloacetate concentration is 2 μM, which is below the lowest reported value of 9 μM for the Kₘ of mitochondrial P-enolpyruvate carboxykinase for oxalacetate (24). The observed rate of P-enolpyruvate formation was 8 nmoles per min per mg of protein, which is about one-third the maximal rate observed with α-ketoglutarate as substrate at saturating extramitochondrial malate concentration, in accordance with the postulate that when GTP formation is not rate-limiting, P-enolpyruvate formation is a function of the oxaloacetate concentration.

The sequence of events resulting from the increased availability of oxaloacetate to P-enolpyruvate carboxykinase may be interpreted as follows. Increased demand for GTP by P-enolpyruvate synthesis presumably causes a fall of the guanine nucleotide phosphate potential, thereby resulting in the observed fall of succinyl-CoA levels (Fig. 2), which was reproducible in a number of experiments. The fall of succinyl-CoA was accentuated at higher malate concentrations, since at 1 mM extramitochondrial malate, succinyl-CoA levels of 0.2 nmoles per mg of protein were obtained under the same incubation conditions with α-ketoglutarate as substrate. Since succinyl-CoA is an inhibitor of isolated α-ketoglutarate dehydrogenase (25, 26), the fall of the succinyl-CoA:CoA ratio produces an increased flux through this step in the intact mitochondria, so that an increased rate of GTP production is able to keep pace with the new rate of P-enolpyruvate formation. Deinhibition of α-ketoglutarate dehydrogenase probably results in a fall of the intramitochondrial α-ketoglutarate concentration, thus accounting for the increased rate of α-ketoglutarate influx observed with a rise of the extramitochondrial malate concentration. Intramitochondrial α-ketoglutarate concentrations could not be measured directly in these experiments because of the high concentration in the medium, but assuming a similar concentration gradient across the mitochondrial membrane as that of malate because of their similar charge (27), a fall of intramitochondrial α-ketoglutarate concentration with time is predicted from the data in Table III.

The observed fall of the succinyl-CoA levels without an apparent change of GTP content (Fig. 2) provides an unexpected

### Table III

| Time of incubation | P-enolpyruvate concentration | Malate concentration |
|--------------------|-----------------------------|---------------------|
|                    | Mitochondrial | Medium | Ratio of mitochondrial concentration to medium concentration | Mitochondrial | Medium | Ratio of mitochondrial concentration to medium concentration |
| min | μM | μM | μM | μM | μM | μM |
| 0 | 0.42 | 19.5 | 21.5 | 0.38 | 22.9 | 16.6 |
| 0.5 | 0.05 | 28.9 | 1.7 | 0.19 | 34.2 | 5.5 |
| 1 | 0.08 | 33.8 | 2.4 | 0.22 | 35.3 | 6.2 |
| 2 | 0.10 | 49.2 | 2.0 | 0.20 | 41.7 | 4.8 |
| 4 | 0.14 | 82.3 | 1.7 | 0.22 | 45.9 | 4.8 |
| 6 | 0.18 | 130.8 | 1.4 | 0.26 | 59.4 | 4.4 |
| 8 | 0.23 | 206.8 | 1.1 | 0.34 | 82.7 | 4.1 |

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of about 20 mM in the mitochondrial matrix, minimal values for liver mitochondria. With an inorganic phosphate concentration carnitine plus malate in State 4 (26), while values an order of magnitude lower were calculated for rat liver mitochondria under conditions of uncoupled plus oligomycin respiration with α-ketoglutarate as substrate. Differences between the two sets of values were caused primarily by a higher succinate content in the liver mitochondria. With an inorganic phosphate concentration of about 20 mM in the mitochondrial matrix, minimal values for liver mitochondria. With an inorganic phosphate concentration carnitine plus malate in State 4 (26), while values an order of magnitude lower were calculated for rat liver mitochondria under conditions of uncoupled plus oligomycin respiration with α-ketoglutarate as substrate. Differences between the two sets of values were caused primarily by a higher succinate content in the liver mitochondria.

With the intramitochondrial GDP concentration is very low and that even without changes of the phosphate concentration, large changes of the GTP phosphate potential could be produced without this being detected from analytical measurements of the GTP content. It would appear, therefore, that the succinyl-CoA:CoA ratio is a much more sensitive indicator of the GTP:GDP ratio than the GTP level. On the other hand, analytically determined ATP:ADP ratios provide values of about 5 or below, which makes the ATP level more responsive to the mitochondrial energy state than the GTP level. Since the equilibrium constant for nucleoside diphosphate kinase is about unity (28), it is clear that large differences between ATP:ADP and GTP:GDP ratios in the mitochondrial matrix are incompatible with the concept of rapid equilibration between ATP and GTP pools (10).

Results presented in this paper demonstrate the close interaction between the GTP requirements for mitochondrial P-enolpyruvate synthesis and its rate of production by substrate level phosphorylation. Flux through nucleoside diphosphate kinase from ATP to GTP in guinea pig liver mitochondria appears inadequate to support rates of P-enolpyruvate formation greater than 2 to 3 nmol per min per mg of protein, so that the possibility of rates of P-enolpyruvate formation significantly greater than flux through α-ketoglutarate dehydrogenase is precluded. A considerably lower rate of P-enolpyruvate formation than flux through α-ketoglutarate dehydrogenase is possible, however, because of independent control factors affecting the availability of intramitochondrial oxaloacetate. This is made possible in terms of GTP metabolism due to the conversion of GTP to ATP by nucleoside diphosphate kinase being kinetically favored over the reverse direction because of the different relative levels of the XTP and XDP nucleotides and the inhibitory effects of MgADP.

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