Activation of Fatty Acids by a Guanosine Triphosphate-specific Thiokinase from Liver Mitochondria*

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In the initial studies of Lehninger (1) on the oxidation of octanoate by rat liver mitochondria, it was noted that \( \alpha \)-ketoglutarate possessed a unique property in regard to its role as a co-oxidant. Whereas all other intermediates of the tricarboxylic acid cycle tested for sparking activity required the simultaneous addition of adenosine monophosphate, \( \alpha \)-ketoglutarate functioned efficiently by itself. It was suggested that \( \alpha \)-ketoglutarate may give rise to a nonadenylate intermediate that could activate fatty acids.

Pursuing this point, Rossi and Sacchetto (2) showed that succinate or acetate could block the sparking effect of \( \alpha \)-ketoglutarate in the oxidation of octanoate by guinea pig liver mitochondria. Under the conditions employed, these agents did not diminish the oxidation of \( \alpha \)-ketoglutarate in the absence of octanoate, nor did they impair the utilization of adenosine triphosphate (in place of \( \alpha \)-ketoglutarate) as an activator of octanoate oxidation. These results were interpreted in the framework of preceding studies of Sanadi, Ayengar, and Gibson (3), who showed that guanosine triphosphate is the primary product of substrate phosphorylation accompanying \( \alpha \)-ketoglutarate oxidation in mitochondria of mammalian tissues (Equations 1 and 2).

\[ \text{\( \alpha \)-Ketoglutarate + DPN}^+ + \text{CoA-SH} \rightarrow \text{succinyl-S-CoA + DPNH + H}^+ + \text{CO}_2 \] (1)

\[ \text{succinyl-S-CoA + Pi + GDP =} \]

\[ \text{GTP + succinyl + CoA-SH} \] (2)

Since arsenolysis of succinyl-CoA had been demonstrated with the succinic kinase system (Equation 2) (4), Rossi postulated that the inhibitory effect of arsenate was related to a failure of GTP formation from \( \alpha \)-ketoglutarate. Consequently it seemed possible that GTP, like ATP, could initiate the oxidation of fatty acids. Rossi and Sacchetto (5, 6) later showed that GTP did replace ATP as a requirement for fatty acid oxidation by the liver mitochondrial system. Indeed, GTP operated in certain situations in which ATP was inert. It was suggested that GTP may function in the nucleoside diphosphate kinase system (Equation 3) (7).

\[ \text{GTP + ADP } \rightleftharpoons \text{GDP + ATP} \] (3)

In the present investigation, a search was made for a GTP-linked fatty acyl kinase in liver mitochondria. A GTP-specific enzyme system was found that catalyzes the following reaction (Equation 4) (8).

\[ \text{GTP + RCOOH + CoA-SH = RCO-S-CoA + GDP + P}_1 \] (4)

This reaction is formally similar to succinic kinase (Equation 2) (4), in contrast to the classical fatty acyl thiokinase enzyme systems (Equation 5) (9).

\[ \text{ATP + RCOOH + CoA-SH =} \]

\[ \text{RCO-S-CoA + AMP + PP}_1 \] (5)

**EXPERIMENTAL PROCEDURE**

Extracts of beef liver mitochondrial acetone powder were prepared after the method of Mahler, Wakil, and Bock (9). Protein precipitates obtained with ammonium sulfate were ordinarily dissolved in 0.02 m KHCO\(_3\) dialyzed for 3 to 6 hours against the same buffer, and clarified by centrifugation. Chromatography of protein solutions on DEAE-cellulose columns was conducted according to Sober et al. (10) (see Fig. 1). Protein concentration was determined by the biuret reaction (11) or by measuring the absorbance of the solution at 280 m\(_\text{u}\).

Fatty acid and succinic kinase activities were assayed by measuring the rate of change in concentration of CoA sulphydryl by the nitroprusside reaction (12) (Table I) and acylhydroxamate formation (13) (Table II).

The butyryl-CoA product of the reaction was identified in the reaction mixture by the paper chromatography system of Stadtmann (14), ethanol-0.1 m sodium acetate buffer, pH 4.5, 1:1. After localization of the CoA ester by ultraviolet illumination on the completed chromatogram, the paper was sprayed with alcoholic NaOH (3 g of NaOH per 100 ml of 95% methanol) to hydrolyze the butyryl-CoA, and finally the paper was sprayed with the nitroprusside reagent of Toennies and Kolb (15) to detect the released CoA sulphydryl. The product was also identified by paper chromatography in an isobutyric-ammonia-water solvent system (16).

The guanosine nucleotide reactants were separated from the reaction mixture by chromatography on Dowex-1-formate by the method of Hurlbert et al. (17), as employed by Ayengar et al. (18) (see Fig. 3).

Nucleotide reagents were obtained commercially. CoA was completely reduced before use with sodium amalgam (19). Butyryl-CoA was prepared by the method of either Wieland and Rueff (20) or Trams and Brady (21).
RESULTS

Separation of Kinase Activities—Preliminary testing of extracts from acetone powder or beef liver mitochondria, prepared by the method of Mahler, Wall, and Bock (9), revealed that GTP could replace ATP in the assay for fatty acid thio kinase. Consequently a series of subfractions of these extracts was prepared in an attempt to separate GTP-specific from ATP-specific kinase activities. The fraction obtained from the mitochondrial extract between 20 and 35 g of ammonium sulfate per 100 ml of extract (Table I, Fraction P 20-35) was quite active with both GTP and ATP when either butyrate or octanoate was the substrate. However, it was noted that the ratio of specific activities obtained with the two fatty acids was not the same for ATP and GTP. Succinic kinase activity (4, 22), which was also present in this fraction, was eluted only in the presence of GTP. Subfractionation of P 20-35 with ammonium sulfate gave rise to a fraction, P 31-35, which showed fatty acid kinase activity only with ATP (Table I; compare Fraction P 31-35 with P 24-28, both of which were derived from P 20-35). This result suggested the possibility of a GTP-specific kinase for butyrate and octanoate.

Additional fractions were obtained by treatment of dialyzed P 24-28 with alumina gel Cy (8 mg of gel per 10 mg of protein). The dilute supernatant remaining after removal of the gel was fractionated with ammonium sulfate. The protein precipitating between 33 and 40 g of ammonium sulfate per 100 ml of supernatant was enriched in all kinase activities. Fraction P 40-48 was active only with succinate as substrate, indicating that succinic kinase was not catalyzing fatty acid activation.

A protein fraction containing GTP-hexanoyl CoA activity, but free of GTP-succinyl CoA activity, was obtained in the following manner: (a) Fraction P 20-35 was reprecipitated twice with 29 g of ammonium sulfate per 100 ml of initial solution; (b) the final precipitate was redissolved with 0.02 M KHCO3 and dialyzed for 5 hours against the same salt solution (Fraction P 0-29 in Table I); (c) 2 mg of aged calcium phosphate gel were added per mg of protein; and (d) the protein in the supernatant remaining after removal of the gel was recovered with an ammonium sulfate concentration of 42 g per 100 ml ("gel fraction" in Table I).

The ATP-specific kinase was separated from the GTP-specific enzyme by DEAE-cellulose chromatography. In Fig. 1, the elution schedule of the two enzymes is presented. An ATP-specific kinase of high specific activity was obtained by elution through 0.05 M potassium phosphate buffer (through tube 132). GTP was inactive in the incubation systems employing these fractions. The GTP-specific enzyme was eluted with 0.2 M potassium phosphate buffer (right side of Fig. 1). There was no contamination with the ATP-reacting kinase in these latter fractions.

Comparison of Properties of Kinase Enzymes—Fatty acyl kinase activity was measured readily either as thiohydroxamate appearance (12) or as acylhydroxamate formation (hydroxylamine present during incubation) (13). Fraction P 33-40 (described in Table I) was active for all three kinase activities (ATP-succinyl CoA, ATP-octanoyl CoA, and GTP-succinate) when assayed by either method. By employing this fraction, a study was made of the requirement for reduced CoA in acylhydroxamate formation (Table II). As previously observed by Jencks and Lipmann (23), the ATP-dependent acylhydroxamate formation did not require the presence of reduced CoA (Table II). On the other hand, GTP activation of octanoate in the presence of hydroxylamine required CoA. This same requirement for CoA was previously demonstrated for the GTP-specific formation of succinylhydroxamate in the succinic kinase system prepared from kidney cortex (4).

These results indicated a formal similarity between the succinic kinase and the new fatty acid-activating system. Consequently it was anticipated that GDP rather than GMP would be a product in the reaction (4).

The stoichiometry of the three kinase reactions was the same with respect to acylhydroxamate formation (hydroxylamine added after incubation) or CoA sulfhydryl disappearance (Table III). A product indistinguishable from butyryl-CoA was identified in the complete system after incubation with GTP, potassium butyrate, and CoA-SH (see conditions in Table I and "Experimental Procedure"). With purified enzyme fractions, activity could be measured by following the net increase in absorbance at 233 nm, which is indicative of thioster bond formation (24). Fatty acids of chain length from C14 to C12 were active substrates in the GTP-kinase reaction (11P, but not CTP or UTP, could replace GTP in this kinase system.

Identification of GDP as Product of Reaction—The enzyme fraction P 33-40 was employed to determine which of the three kinase activities could be measured in the pyruvic kinase-lactic dehydrogenase-coupled reaction system, wherein nucleotide diphosphate generation can be measured spectrophotometrically (25) (Equations 6, 7, and 8).
Fig. 1. The distribution of protein (absorbance at 280 nm indicated on the left ordinate) and the specific enzyme activities (right ordinate) for the enzymes reacting with ATP (open bars) and with GTP (black bars) in the fractions obtained during chromatography on DEAE-cellulose. Specific activities are expressed as micromoles of sulfhydryl per 60 minutes per mg of protein. Assay conditions are given in Table I. Each protein fraction was assayed separately with ATP and GTP. Butyrate was used as substrate throughout. Enzyme fraction P 20-35 (see Table I) was used for chromatography after dialysis for 8 hours against 0.002 M potassium phosphate, pH 7. Dialyzed fraction P 20-35, 640 mg, was added to a column 3 × 40 cm (flow rate, 100 ml per hour). Elution with potassium phosphate buffer, pH 7.0, was performed in the following steps: tubes 1 to 24, 0.002 M potassium phosphate buffer; tubes 25 to 57, 0.005 M; tubes 58 to 107, 0.01 M; tubes 108 to 154, 0.05 M; tubes 155 to 183, 0.10 M; and tubes 184 to 222, 0.20 M. The whole operation was performed at 5°C.

**Table II**

**Hydroxaminate formation**

Activity was measured as acylhydroxaminate formation in the presence and absence of CoA–SH. The incubation mixture contained, in 0.5 ml, Tris buffer, pH 8.0, 100 μmoles; HNO₂, pH 8, 260 μmoles; MgCl₂, 6 μmoles; CoA–SH, 0.12 μ mole; GSH, 6 μmoles; ATP or GTP, 2 μmoles; potassium octanoate, 10 μmoles, or potassium succinate, 25 μmoles; and 120 μg of Fraction P 33–40. After 10-minute incubations, the mixtures were analyzed for acylhydroxaminate by the method of Lipmann and Tuttle (13).

<table>
<thead>
<tr>
<th>Substrate added</th>
<th>Nucleotide added</th>
<th>Acylhydroxaminate formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CoA–SH added</td>
<td>CoA–SH omitted</td>
</tr>
<tr>
<td>Octanoic acid</td>
<td>ATP</td>
<td>0.82</td>
</tr>
<tr>
<td>Octanoic acid</td>
<td>GTP</td>
<td>0.71</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>GTP</td>
<td>0.96</td>
</tr>
</tbody>
</table>

RCOOH + CoA–SH + GTP ⇌ RCO–S–CoA + GDP + P₁ (6)

Phosphoenolpyruvate + GDP ⇌ pyruvate + GTP (pyruvate kinase) (7)

Pyruvate + DPNH + H⁺ = lactate + DPN⁺ (lactic dehydrogenase) (8)

In Fig. 2 it is seen that additions of octanoate, GTP, and CoA bring about rapid DPNH oxidation. Octanoate, ATP, and CoA do not give rise to any change above the blank in which nucleoside triphosphate is omitted, since the product, AMP, does not enter into the reaction sequence. Addition of succinate, like that of octanoate, in the presence of GTP and CoA is rapidly followed by sustained DPNH oxidation. If GMP were a product of the reaction, it could still be detected in this system provided that a nucleoside monophosphate kinase were present, permitting GDP generation from the interaction of the GTP initially present and GMP (Equation 9).

GTP + GMP ⇌ 2 GDP (9)

A reaction of this sort can be demonstrated with added GTP and GMP, although the rate is considerably less than that of the complete system (Fig. 2).

If GDP is the product in the acyl kinase reaction, the quantity...
TIME IN MINUTES

FIG. 2. DPNH oxidation was measured (absorbance at 340 nm, ordinate) as a function of time in the GTP-specific fatty acyl kinase reaction coupled to the pyruvic kinase and lactic dehydrogenase enzyme systems. The line designated GTP + octanoate is the complete system. Succinate activation was similarly measured (GTP + succinate line). Blanks containing only GTP, or GTP + GMP, are shown for comparison. The reaction contained, in 0.5 ml, Tris buffer, pH 8, 30 μmoles; KCl, 10 μmoles; MgCl₂, 2 μmoles; DPNH, 0.1 μmole; phosphoenolpyruvate, 1.0 μmole; CoA-SH, 0.3 μmole; GTP, 2.0 μmoles; pyruvic kinase and lactic dehydrogenase, 10 μg; and (where indicated) potassium octanoate, 10 μmoles; potassium succinate, 10 μmoles; and GMP, 0.1 μmole. Fraction P 33-40, 60 μg, was added to initiate the reaction. Incubations were carried out in the spectrophotometer at 38°.

TABLE IV

Stoichiometry (DPNH and —SH)

GTP specific kinase activity with octanoate or succinate as substrate was measured in the coupled pyruvic kinase-lactic dehydrogenase system. CoA-SH utilization and DPNH oxidation were measured in each sample after 5 minutes of incubation at 38°. (See Fig. 2 for conditions of incubation.)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Decrease in DPNH</th>
<th>Decrease in —SH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octanoic acid</td>
<td>0.081 μmole</td>
<td>0.087 μmole</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>0.074 μmole</td>
<td>0.070 μmole</td>
</tr>
</tbody>
</table>

FIG. 3. The chromatographic separation of GDP and GTP on a Dowex 1-formate column. The initial reaction mixture contained, in 0.4 ml, glycylglycine buffer, pH 8, 50 μmoles; MgCl₂, 5 μmoles; GSH, 5 μmoles; CoA-SH, 0.24 μmole; NH₂OH, 50 μmoles; GTP, 2.2 μmoles; potassium octanoate, 10 μmoles; and 350 μg of enzyme. Identical incubation mixtures were also employed from which octanoate was omitted, or to which succinate (25 μmoles) was added in place of octanoate. After incubation for 10 minutes at 38°, 0.02 ml of 30% HClO₄ was added at 0° to each sample. Each was then neutralized with KOH. An aliquot of 0.6 ml of the supernatant obtained after removal of protein was added to a small Dowex 1-formate column. Eluent fractions of 5 ml were collected, and the optical density at 252 nm of each sample was measured (indicated on the ordinate). Elution was carried out stepwise with 1.5 M formic acid (tubes 0 to 32), 4 M formic acid and 0.2 M ammonium formate (tubes 32 to 64), and 4 M formic acid and 0.8 M ammonium formate (from tube 64). The nucleotide products were identified by comparison with the established elution patterns of nucleotides (17, 18) and with known nucleotide mixtures.
of DPNH oxidized should be the same as CoA-SH consumed. Equation 10 gives the summation of the over-all reaction (sum of Equations 6, 7, and 8).

\[
\text{RCOOH} + \text{CoA-SH} + \text{phosphoenolpyruvate} + \text{DPNH} + \text{H}^+ \rightarrow \text{RCO-S-CoA} + \text{lactate} + \text{DPN}^+ + \text{Pi}
\]

A 1:1 ratio is observed for both succinate and octanoate in the coupled system (Table IV). If the product were GMP, then a stoichiometry of 2 DPNH per CoA-SH would obtain (see Equation 9).

After incubation of the complete fatty acyl kinase system, GDP can be directly separated from the reaction mixture by chromatography on Dowex 1-formate by the procedure of Hurlbert et al. (17) and Ayengar et al. (18). The nucleotides obtained after incubation of GTP, octanoate, and CoA-SH are shown in Fig. 3. GDP (shown in black, tubes 45 to 60) was absent at zero time. It appeared only after incubation of the complete system. The initial quantity of GTP was lowered correspondingly. Incubation with succinate gave the same results as with octanoate. No change was observed after incubation in the absence of substrate. The material eluted ahead of GDP (tubes 18 to 45) was present before incubation and probably represents CoA-SH.

These results established directly that GDP is the nucleotide product of this reaction.

By employing the same incubation conditions and isolation procedures, it was possible to determine the stoichiometry among the acylhydroxamate formed, the GTP consumed, and the GDP released (Table V). A 1:1:1 ratio was observed.

**Table V**

Stoichiometry (acylhydroxamate, GDP, and GTP)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Acylhydroxamate</th>
<th>GDP</th>
<th>GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octanoic acid</td>
<td>+0.720</td>
<td>+0.670</td>
<td>-0.630</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>+0.543</td>
<td>+0.778</td>
<td>-0.940</td>
</tr>
</tbody>
</table>

**Table VI**

Reversibility of reaction

In this table the components needed for the reverse reaction are listed. Incubations were carried out with the complete system and with systems in which orthophosphate, GDP, or enzyme was omitted. Butyryl-CoA was the substrate throughout. The reaction mixture for the complete system contained, in 0.35 ml, Tris buffer, pH 8.0, 20 amoles; MgCl₂, 2.5 amoles; KBH₄, 1 amole; butyryl-CoA, 0.31 amole; GDP, 0.6 amole; K₂HPO₄, 2.5 amoles; and 30 μg of enzyme. Samples were incubated for 15 minutes at 38° under nitrogen. Aliquots were then removed for sulfhydryl and acylhydroxamate determination.

<table>
<thead>
<tr>
<th>System</th>
<th>Net change in</th>
<th>Acylhydroxamate</th>
<th>μmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>+0.14</td>
<td>-0.15</td>
<td></td>
</tr>
<tr>
<td>No phosphate</td>
<td>+0.04</td>
<td>-0.04</td>
<td></td>
</tr>
<tr>
<td>No GDP</td>
<td>+0.04</td>
<td>-0.04</td>
<td></td>
</tr>
<tr>
<td>No enzyme</td>
<td>+0.04</td>
<td>-0.08</td>
<td></td>
</tr>
</tbody>
</table>

**Table VII**

Arsenolysis of butyryl-CoA

Butyryl-CoA was incubated in the absence of orthophosphate and GDP under conditions identical with those described in Table VI, except for the duration of incubation (30 minutes) and the substitution of glycylglycine buffer for Tris. In one series, 5 μmoles of potassium arsenate were added.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Net change in</th>
<th>Acylhydroxamate</th>
<th>μmole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without arsenate</td>
<td>+0.054</td>
<td>-0.073</td>
<td></td>
</tr>
<tr>
<td>With arsenate</td>
<td>+0.117</td>
<td>-0.133</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. Enzyme activity (ordinate) plotted as a function of enzyme concentration. The middle plot (●) was obtained from a series incubated in the presence of 0.04 M K⁺; the upper plot (○), from a series identical with the first, to which KCl was added to give a final concentration of 0.073 M K⁺; and the lower plot (△), from a series identical with the first, to which NaCl was added to give a final concentration of 0.033 M Na⁺. The incubation mixture contained, in 0.3 ml, glycylglycine buffer, pH 8.0, 5 μmoles; MgCl₂, 2.5 μmoles; KBH₄, 1 μmole; potassium octanoate, 5 μmoles; CoA-SH, 0.3 μmole; and GTP, 1 μmole. Samples were incubated for 10 minutes at 38° under nitrogen. CoA sulfhydryl disappearance was followed in the nitroprusside reaction.
Reversibility of Kinase Reaction—The components needed for the reverse reaction are given in Table VI. With butyryl-CoA as substrate, both inorganic orthophosphate and GDP were necessary for sulfhydryl generation and acyl ester consumption. In the absence of phosphate and GDP, an arsenolysis of butyryl-CoA could be demonstrated (Table VII).

DISCUSSION

A new route for fatty acid activation has been identified. It represents an addition to the list of GTP-specific enzymes (26). The distribution of the enzyme in cell fractions and among tissues is under investigation. Preliminary results indicate that the kinase is not confined to liver mitochondria, although the latter remain the best source material. Similarly, the specificity and the $K_v$ values of various substrates have not been mapped out sufficiently well to decide whether or not this kinase is confined to the activation of fatty acids. Indeed, the function of the enzyme in the cell may be pictured as a phosphorylation step starting with an acyl-CoA, analogous to the succinic kinase system.

The participation of guanosine nucleotides in fatty acyl-CoA formation should permit tight coupling of fatty acid activation and $\alpha$-ketoglutarate oxidation. That such could be done was demonstrated by Lehninger (1) in liver mitochondrial preparations incubated with $\alpha$-ketoglutarate, AMP, and octanoate. Omitting AMP diminished but did not abolish octanoate oxidation. In view of the present information on the GTP-fatty acid-activating system, the coupling would occur as follows.

\[
\begin{align*}
\text{\textit{a-Ketoglutarate} + DPN+ + CoA-SH + acetate} & \\
\text{was not activated by any of the mitochondrial fractions} & \\
\text{seems unlikely in view of the following evidence: (a) direct} & \\
\text{single enzyme. Although the over-all reaction could be ex-} & \\
\text{plained by a coupling of succinic kinase and a thiophorase (in} & \\
\text{the case of butyrate (27) and octanoate (28). In the present study, aceto-} & \\
\text{acetate was not activated by any of the mitochondrial fractions} & \\
\text{containing the GTP kinase activity.} & \\
\text{The GTP-fatty acid-activating system appears to involve a} & \\
\text{single enzyme. Although the over-all reaction could be ex-} & \\
\text{plained by a coupling of succinic kinase and a thiophorase (in} & \\
\text{the presence of a catalytic concentration of succinic acid), this} & \\
\text{would not be expected in view of the following evidence: (a) direct} & \\
\text{acylhydroxamate formation (preliminary succinyl-CoA forma-} & \\
\text{tion would be blocked; see Table II), and (b) separation of a} & \\
\text{fraction containing GTP-linked acyl kinase activity which is} & \\
\text{virtually free of succinic kinase activity (gel fraction in Table I).} & \\
\end{align*}
\]

The results of Harel, Mendoza, and LeBreton (29) show that the ATP-specific thio kinase may be activated by a phosphorylation of the enzyme itself. Thus the existence of two forms of the enzyme, one more active than the other, would suggest that a change can be imposed in the affinity of the enzyme for some of the reactants in the system. Whether or not this interconversion may include a shift in the specificity for the nucleotide substrate is unknown.

SUMMARY

A new acyl thio kinase which is specific for the nucleotide guanosine triphosphate has been identified in soluble fractions of beef liver mitochondria. The enzyme catalyzes the esterification of fatty acids in a manner similar to succinic kinase, with the release of guanosine diphosphate and orthophosphate as products.

REFERENCES

Activation of Fatty Acids by a Guanosine Triphosphate-specific Thiokinase from Liver Mitochondria
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