The Activation of Fatty Acids in Castor Bean Endosperm*

(Received for publication, January 22, 1971)

T. G. Cooper

From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

SUMMARY

The demonstration that the reactions of β oxidation and the glyoxylate cycle occur in the glyoxysome, a cellular organelle isolated from castor bean endosperm, has raised the question of the intracellular location of the enzymatic activation of free fatty acids in this tissue. Glyoxysomes, isolated on sucrose gradients, have been shown to contain a thiokinase which, in the presence of CoA, MgCl₂, and ATP (GTP is ineffective), activates free fatty acids to their cognate fatty acyl-CoA derivatives. The thiokinase is specific for fatty acids of chain length greater than C-10. An acetate thiokinase was also found in glyoxysomes. It is specific for ATP and is more tightly bound to the organelles than the long chain thiokinase. Although the long chain thiokinase is easily solubilized, the data show that the intact glyoxysomes can convert free fatty acids to their CoA derivatives and oxidize them to acetyl-CoA.

The oxidation of fatty acids has long been known to be the principal source of energy and metabolites in germinating fatty seeds such as peanuts and castor beans. Recently the glyoxysome (1-3), an organelle containing the enzymes of the glyoxylate cycle, was shown (4) to be the sole location of β oxidation activity. In addition, Ory, Yatsu, and Kircher (5) have presented data suggesting that the triglyceride, triricinolein, composing 70% of the dry weight of the ungerminated bean, is stored and hydrolyzed to glycerol and ricinoleic acid in an organelle designated as the lysosome. These data yield a reasonable picture of the intracellular location of the reactions in the metabolism of triricinolein to the level of phosphoenolpyruvate. One of the enzymes yet to be investigated is the long chain fatty acid thiokinase. The studies of Hutton and Stumpf (6) demonstrating that free fatty acids are oxidized by particulate fractions of castor bean endosperm were isolated by previously established techniques and assayed for their ability to activate acetate and long chain fatty acids. It was known from earlier work (7) that this tissue lacked a carnitine transport system comparable to that found in animal tissues. The data presented here indicate that glyoxysomes contain long chain fatty acid thiokinase activity in an amount capable of accounting for 60% of the in vivo flux of metabolites. In contrast to the substrate specificity observed for the β oxidation sequence which utilized the CoA derivatives of all even numbered fatty acids to the same extent, this thiokinase has a strict specificity for long chain (greater than C-10) fatty acids. The glyoxysomes are also the sole location of an acetate thiokinase. A preliminary report of this work has already appeared (8).

MATERIALS AND METHODS

Preparation of Cellular Organelles—Mitochondria, plasts, and glyoxysomes were prepared as described earlier (3). A supernatant free of membrane fragments was obtained by centrifuging the 9.5 K supernatant for 2 hours at 30,000 rpm in a Beckman SW-39 rotor. The supernatant of this centrifugation was designated as the 30 K supernatant.

Assay of Long Chain Fatty Acid Thiokinase—The reactions for the assay of fatty acid thiokinase are summarized below.

Fatty acid + CoA-SH + uc-ATP →

\[ \text{Fatty acyl-CoA} + \text{uc-AMP} + \text{PP}_i \] (1)

\[ \text{uc-AMP} \xrightarrow{5'-nucleotidase} \text{uc-adenosine} + \text{Pi} \] (2)

The reaction of the thiokinase was coupled to that of 5'-nucleotidase which converted the AMP produced in Reaction 1 to free adenosine. As shown in Fig. 1, adenosine can be easily separated from AMP, ADP, and ATP through the use of a Dowex 1-formate column. The various nucleotides were bound to the column while the neutral adenosine was eluted with water. In practice a large number of columns (made by placing 1 ml of Dowex 1 formate in a Pasteur pipette containing a glass wool plug) were run simultaneously. A typical reaction mixture contained, in a volume of 0.56 ml: 8.9 × 10⁻³ m HEPES buffer adjusted to pH 7.5, 8.9 × 10⁻³ m MgCl₂, 4.5 × 10⁻⁴ m dithiothreitol, 3.6 × 10⁻⁴ m coenzyme A, 150 µg of pyruvate kinase, 1.8 × 10⁻⁴ m uc-ATP (specific activity = 0.25 µCi per µmole), 50 µg of 5'-nucleotidase, 1.34 × 10⁻³ m palmitic acid, 750 µg of bovine serum albumin, 5.4 × 10⁻⁴ m phosphoenolpyruvate,

\[ \text{uc-AMP} \xrightarrow{5'-nucleotidase} \text{uc-adenosine} + \text{Pi} \] (2)

1 T. G. Cooper and H. Beevers, unpublished observations.

2 The abbreviation used is: HEPES, N-2-hydroxyethylpipera-

zine-N'-2-ethanesulfonic acid.

* This work was supported by an Air Force Office of Scientific Research-National Research Council award, National Science Foundation Grants GB-13228 and GB-5322, and United States Public Health Service Grants GM-67440 and AM-13884.
Fig. 1. Elution pattern of adenosine and its derivatives from Dowex 1-formate. Ten micromoles each of adenosine, AMP, ADP, and ATP were placed on a column, 1 x 10 cm, prepared as described earlier (4). The adenosine was washed from the column and 20 to 40 μg of purified glyoxysomal protein. The reaction mixture was incubated for 9 min at 37° and terminated by the addition of 1.0 ml of ice-cold ethanol. Following absorption of the reaction mixture to the column the adenosine was eluted with five 1.0-ml washes of water (additional washing did not increase the recovery of adenosine). Although the above techniques have proven to be the only method of assay possessing the required sensitivity, they suffer from one severe disadvantage. Crude extracts from castor bean endosperm contain reasonable quantities of ATPase and myokinase. The result of the reactions catalyzed by these two enzymes is the production of AMP as shown in Reactions 3 and 4.

\[
\text{ATPase} \xrightarrow{\text{ATP}} \text{ADP} + P_i \tag{3}
\]

\[
2\text{ADP} \xrightarrow{\text{myokinase}} \text{ATP} + \text{AMP} \tag{4}
\]

Two steps were used to cope with this problem. (a) As noted above, a large excess of phosphoenolpyruvate, pyruvate kinase, and 5'-nucleotidase was added to the reaction mixture. The ATP generation system competed successfully with any myokinase that was present and channeled the ADP back to ATP. These reactions, however, should have little effect upon the thio­kinase reaction since in that case the product is AMP. 5'-Nucleotidase was also used in large excess in order to ensure that a minimum of the AMP produced in the thio­kinase reaction participated in the myokinase reaction (Reaction 4 in the reverse direction). The consequences of these side reactions in the first case would be to increase the apparent rates of the thio­kinase reaction while in the second case the apparent rates would be decreased. (b) As a further precaution only coenzyme A-dependent adenosine (AMP) production was considered relevant. Ninety-one and 79% of the observed adenosine production were coenzyme A-dependent when glyoxysomes and crude particles, respectively, were used as the source of enzyme protein. Coenzyme A was chosen as the best indicator of the reaction because it is possible to show, by a variety of means, the presence of reasonable levels of free fatty acids in the glyoxysomal and crude particulate preparations.

A second method of assaying the long chain fatty acid thio­kinase depends upon the ability of glyoxysomes to oxidize the CoA derivatives of fatty acids. In this instance the thio­kinase reaction was coupled to that of the β oxidation sequence and fatty acid activation was measured by the ensuing NAD reduction. The reaction mixture was identical with that described by Cooper and Beevers (4) except for the inclusion of 2.0 x 10^{-5} M ATP and the replacement of the fatty acyl-CoA by the cognate free fatty acid and coenzyme A.

Assay of Acetate Thio­kinase—The pertinent reactions used in the assay of acetate thio­kinase are:

\[
\text{Acetate} + \text{ATP} + \text{CoA} \xrightarrow{\text{acetate thio­kinase}} \text{acetyl-CoA} + \text{AMP} + P_i \tag{5}
\]

\[
\text{Acetyl-CoA} + \text{oxalacetate} \xrightarrow{\text{citrate synthetase}} \text{citrate} + \text{CoA} \tag{6}
\]

\[
\text{Malate} + \text{NAD} \xrightarrow{\text{malic dehydrogenase}} \text{oxalacetate} + \text{NADH} \tag{7}
\]
RESULTS

Occurrence and Distribution of Long Chain Fatty Acid Thiokinase—As shown earlier (4) and in Fig. 2, glyoxysomes can oxidize palmitoyl-CoA. This oxidation is manifest as NAD reduction in this particular experiment. NAD reduction, however, was shown in the absence of palmitoyl-CoA (line designated COMPLETE) if the normal reaction mixture was supplemented with palmitic acid and ATP. The balance of the cofactors necessary for fatty acid activation is already present in the reaction mixture. A typical reaction mixture contained in a volume of 0.76 ml: 1.3 × 10^{-3} M HEPES buffer adjusted to pH 7.5, 1.3 × 10^{-4} M MgCl₂, 2.6 × 10^{-3} M dithiothreitol, 2.6 × 10^{-4} M coenzyme A, 1.7 × 10^{-4} M acetylpyridine NAD, 2.6 × 10^{-5} M ATP, 3.3 × 10^{-3} M malic acid, 6 μg of citrate synthetase, 26 μg of malic dehydrogenase, 4.0 × 10^{-3} M potassium acetate, and 20 to 40 μg of purified glyoxysomal protein. Following equilibration of the reaction mixture, the dehydrogenase reaction acetyl-CoA production was added and monitored.

The occurrence of multiple fatty acid thiokinases is well documented in animal systems. In these organisms there exist examples of long and short chain specific enzymes that require either ATP or GTP. In order to ascertain whether such multiple forms also exist in the glyoxysomes, the nucleotide specificity of the activation was determined. The data in Table III attest to a single activation system which is specific for ATP, GTP being minimally effective.

The above CoA-dependent AMP production assay techniques were applied to the various subcellular fractions obtained from castor bean endosperm. The direct AMP assay was used, because the coupling of activation to the β oxidation of the CoA derivatives is only possible when glyoxysomes are the source of enzyme protein. As shown in Table IV, 66% of the thiokinase activity was solubilized and 33% remained in the crude particulate fraction. Of that which remained with the particles, 58% was found in the glyoxysomes. The 29% found in the proplastid area is most likely due to glyoxysomal contamination of this area of the gradient in a fashion similar to that noted in the earlier enzyme distribution studies. The mitochondria, however, were conspicuously free of any activation activity. The gradient

![Fig. 2. Palmitoyl-CoA and palmitate-dependent NAD reduction in glyoxysomes from castor bean endosperm. The reaction conditions used were those described under "Materials and Methods" of the present work as well as that appearing earlier (4).](image-url)
Table III

Nucleotide specificity of long chain fatty acid thiokinase from castor bean glyoxysomes

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Nucleotide</th>
<th>NAD reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitate</td>
<td>ATP</td>
<td>12.0 nmoles/min</td>
</tr>
<tr>
<td></td>
<td>GTP</td>
<td>1.8</td>
</tr>
<tr>
<td>Caprate</td>
<td>ATP</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>GTP</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Table IV

Distribution of long chain fatty acid thiokinase in castor bean endosperm

<table>
<thead>
<tr>
<th>Fraction</th>
<th>14C-AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>umoles/g (fresh wt)/hr</td>
</tr>
<tr>
<td>9.5 K supernatant</td>
<td>9.8</td>
</tr>
<tr>
<td>30 K supernatant</td>
<td>4.2</td>
</tr>
<tr>
<td>Crude particles</td>
<td>3.3</td>
</tr>
<tr>
<td>Gradient supernatant</td>
<td>1.1</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0</td>
</tr>
<tr>
<td>Proplastids</td>
<td>2.5</td>
</tr>
<tr>
<td>Glyoxysomes</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Table V

Cofactor requirements of acetate thiokinase from castor bean glyoxysomes

<table>
<thead>
<tr>
<th>Deletion</th>
<th>NAD reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>umoles/min</td>
</tr>
<tr>
<td>None</td>
<td>7.8</td>
</tr>
<tr>
<td>Protein</td>
<td>0</td>
</tr>
<tr>
<td>ATP</td>
<td>0.4</td>
</tr>
<tr>
<td>Coenzyme A</td>
<td>0.5</td>
</tr>
<tr>
<td>MgCl2</td>
<td>0</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>2.4</td>
</tr>
</tbody>
</table>

supernatant contained 13% of the activity present on the gradient. It may be significant that only 43% of the activity present in the 9.5 K supernatant was in fact soluble. The remainder of the thio kinase could be removed by centrifugation methods that are known to remove membranes and membrane fragments. Therefore, the origin of this significant portion of the total long chain fatty acid thiokinase is still in question.

Occurrence and Distribution of Acetate Thiokinase—The early in vivo studies of Carvin and Beevers (7) show that castor bean endosperm possesses an active acetate thiokinase. Since the present work shows that the long chain fatty acid thiokinase is not capable of functioning in this capacity, the various cellular fractions were assayed for this activity. With the coupled assay techniques described under "Materials and Methods," the thiokinase reaction was shown (Table V) to possess an absolute requirement for acetate, CoA, ATP, and MgCl2. The incomplete requirement for MgCl2 is again presumed to be the result of preparing the organelles in a magnesium containing buffer.

The distribution of acetate thiokinase activity among the various cellular fractions is quite distinct from that of the long chain fatty acid thiokinase. As shown in Table VI, 100% of the activity was particulate. In addition, all of the particulate activity is located in the glyoxysomal and proplastid portions of the gradient. It is noteworthy that this enzyme is very tightly bound to the organelles with virtually none being detected in either the 9.5 K or gradient supernatants. To ascertain the presence of multiple forms of the thio kinase, the nucleotide specificity of the reaction was determined and as shown in Table VII ATP was exclusively effective in supporting this reaction. Neither ATP nor GTP was effective in eliciting a response when mitochondria were used as the source of protein. The above data are consistent with suggesting that glyoxysomes are the sole source of acetate thiokinase activity.

There is, however, a significant loss of activity when comparing the total activity in the crude particles to that obtained from the sucrose gradients. This apparent loss in activity has been shown to be an artifact of the assay procedures by the following two experiments. If each of the subcellular organelles is assayed individually, only the glyoxysomes possess acetate thiokinase activity with a stoichiometry of NAD reduction to acetate utilization of 1:1. If, however, glyoxysomes and mitochondria are incubated together the observed stoichiometry is increased to 2:1. Dowex 1-formate chromatography of the reaction products produced under the above conditions showed that citrate was the sole product in the former case and alpha-ketoglutarate in the latter. These data suggest that in the crude particulate fraction the apparent activity is 2-fold greater than it should be, because the citrate produced in the acetate thiokinase assay is being subse-
quently oxidized to the level of \( \alpha \)-ketoglutarate by the mitochondrial Kreb cycle enzymes.

**DISCUSSION**

Physiological studies of castor bean endosperm indicated that the tissue was capable of producing hexose from fat at a rate of 25 \( \mu \)moles per hour per g fresh weight. This flux of metabolites is possible only if the long chain fatty acid thiokinase is capable of supporting the activation of 12.5 \( \mu \)moles per hour per g. The above data show that glyoxysomes contain 60\% of the necessary amount. To designate glyoxysomes as the specific location of this enzyme, however, is inappropriate in view of the large amount of activity that is solubilized. While this may be the result of partial destruction of the glyoxysomes during preparation, the data do not exclude the endoplasmic reticulum as the site of activation. If indeed the endoplasmic reticulum is the in situ location of the activation reaction, then the activity observed in the glyoxysomes could be accounted for by the observations of Frederick et al. (9), who noted that microbodies were often observed with a piece of rough endoplasmic reticulum attached to their membranes. Although the former conclusion is felt to be the more probable, the question must be left open until higher quality preparations are attainable.

The distribution of the acetate thiokinase suggests a clear explanation of Canvin and Beevers’ observations that \(^{14}\)C-acetate is channeled exclusively through the glyoxylate cycle with a negligible amount being metabolized through the tricarboxylic acid cycle.

**Acknowledgments**—This work is a continuation of that begun at Purdue with Dr. Harry Beevers. The author wishes to express his sincere gratitude to Professor Beevers for his advice and to Professor Boris Magasanik, in whose laboratory this work was done, for his support and encouragement.

**REFERENCES**

The Activation of Fatty Acids in Castor Bean Endosperm

T. G. Cooper


Access the most updated version of this article at http://www.jbc.org/content/246/11/3451

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/246/11/3451.full.html#ref-list-1