Relationship of the ATP/ADP Ratio to the Site of Octanoate Activation*

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While investigating the inhibitory action of 5-(tetradecyloxy)-2-furoic acid on the oxidation of palmitate (1), we observed a slight (15%) inhibition of octanoate oxidation by TOFA in hepatocytes. This effect was not consistent with our other observations which suggested that TOFA competed for cytosolic CoA, thus depressing extramitochondrial activation of fatty acids. Octanoate is generally thought to be preferentially activated in the matrix by the medium chain fatty acyl-CoA synthetase (EC 6.2.1.2) (2), even though overlapping enzyme specificity would allow its extramitochondrial activation (3). Because of this belief, octanoate is routinely used in comparison with long chain fatty acids to distinguish between metabolic effects occurring before or after the carnitine-dependent steps of fatty acid oxidation. To demon-

strate inhibition of palmitate oxidation by TOFA in mitochondrial studies (1) required the preincubation of mitochondria with the drug before the addition of fatty acid. Using this protocol we observed that carnitine-independent octanoate oxidation in the preincubated control (absence of TOFA) was depressed when compared to rates normally observed with nonpreincubated mitochondria. These low rates of octanoate oxidation were not inhibited by TOFA. The addition of carnitine to the incubation system with preincubated mitochondria restored the rates of octanoate oxidation to those usually observed without preincubation, but under these conditions, TOFA inhibited octanoate oxidation by 40%. Under similar conditions palmitate oxidation was inhibited 80%. These observations suggested that a significant amount of octanoate could be oxidized via a carnitine-dependent route in both mitochondria and hepatocytes and that a thorough study of this question was warranted.

EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

Effect of Mitochondrial Preincubation on the Oxidation of Octanoate and Butyrate—Following preincubation of mitochondria for 20 min in the absence of substrate, carnitine-independent octanoate oxidation, reflected by ketone body synthesis (acetoacetate plus 3-hydroxybutyrate), was inhibited 55% when compared to rates observed without preincubation (Table I). The depressed rates of octanoate oxidation were not observed with the addition of carnitine to the incubation system. That this effect of carnitine was due to a channeling of octanoate to a carnitine-dependent route of oxidation, was clear from the observation that both 2-tetradecyglycic acid (an inhibitor of carnitine palmitoyltransferase I (19)) and Zwittergent 3-08 (an inhibitor of carnitine-acylcarnitine translocase (20)) could completely block this effect. The effect of preincubation, therefore, was not on the fatty acid oxidation pathway per se, but rather it was at the site of intramitochondrial activation of the fatty acid. This being inhibited after preincubation, the addition of carnitine allowed octanoate, which could be activated outside the mitochondria (3), to bypass this block by entering through a carnitine-dependent path. Under these conditions carnitine-dependent oxidation accounted for about 60% of the total oxidation. The data of Table I also indicate that without preincubation, octanoate was oxidized almost entirely (ap-

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The abbreviations used are: TOFA, 5-(tetradecyloxy)-2-furoic acid; TDGA, 2-tetradecyglycic acid; Z-08, Zwittergent 3-08 or N-octyl-N,N-dimethyl-3-ammonio-1-propanesulfonate.
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proximately 90%) via a carnitine-independent route. Similar effects of preincubation were seen with butyrate (Table I), which is activated exclusively in the matrix (3); thus carnitine had no effect on the depressed rates (59% of control) of butyrate oxidation.

A possible explanation for the inhibitory effects of preincubation on octanoate (carnitine-independent) and butyrate oxidation in mitochondria was that preincubation exhausted the supply of exogenous substrate for the Krebs cycle, thus limiting both flux through this pathway and production of GTP. With the subsequent addition and intramitochondrial activation of octanoate or butyrate, there would be an excessive elevation of matrix AMP due to low GTP levels and a resulting inhibition of intramitochondrial fatty acid activation (see Miniprint). If this were correct it would be expected that with increasing incubation time, following preincubation in the absence of substrate, the rate of octanoate oxidation would decrease as the level of AMP increased to inhibitory levels. However, the complete opposite was observed in the experiment of Fig. 1A. The rate was initially depressed by 50% but gradually increased to a rate equal to that seen with nonpreincubated mitochondria. The results of this experiment with preincubated mitochondria were similar to a respiratory control cycle observed after the addition of ADP. Since it was reasonable that ADP might build up during preincubation in the absence of substrate due to a slow rate of reducing equivalent production, the ATP/ADP ratio of the total adenine nucleotide pool was determined at each time point (Fig. 1B). Replotting these data (not shown) indicated that there was a direct relationship between the ATP/ADP ratio and the rate of carnitine-independent oxidation of octanoate.

Causal Relationship between the ATP/ADP Ratio and the Rate of Carnitine-independent Octanoate Oxidation—From the data of Fig. 1, it cannot be determined whether the changes in the ATP/ADP ratio are related to the cause or the effect of the oxidation rates. That a depressed ATP/ADP ratio might be causally involved in the inhibition of intramitochondrial fatty acid activation was tested in the experiment of Fig. 2. The addition of 3 mM ADP (in place of ATP) to the incubation system of nonpreincubated mitochondria depressed the rate of carnitine-independent octanoate oxidation approximately 70% (Fig. 2A), which was even greater than that observed after preincubation (Table I and Fig. 1). If limiting amounts of ADP were added (2 mM ATP plus 1 mM ADP) at the beginning of the incubation, the rate started low but gradually increased to that seen with ATP alone. Also if 1 mM ADP was added at 5 min to a system containing 2 mM ATP, the rate was depressed for the remainder of the incubation period. The adenine nucleotide additions had the expected effects on the ATP/ADP ratios (Fig. 2B). Thus the results of this experiment were qualitatively identical with those of Fig. 1. Again, replotting these data (not shown) indicated there was a direct and causal relationship between the ATP/ADP ratio and the rate of carnitine-independent octanoate oxidation in mitochondria. Further proof that a build up of ADP during preincubation caused the depressed rates of octanoate (carnitine-independent) and butyrate oxidation is shown in Table II. These results were essentially the same as those shown in Table I with preincubated mitochondria, except again the magnitude of inhibition with ADP addition (70%) was greater than after preincubation.

Extra- and Intramitochondrial Adenine Nucleotides—An experiment was performed in which the extra- and intramitochondrial levels of adenine nucleotides were measured in the ATP and ADP incubation systems (Table III). Intramitochondrial AMP in both incubation systems was much greater than that in freshly isolated mitochondria, which would be expected with intramitochondrial activation of octanoate (no carnitine added). However, intramitochondrial AMP was significantly decreased by 34% in the system with ADP when compared to that with ATP. Thus, the data are not consistent with inhibition of the matrix acyl-CoA synthetase by an elevation of AMP. A slight but significant decrease in the intramitochondrial ATP/ADP ratio and a marked decrease in the extramitochondrial ratio were observed in the ADP versus the ATP incubation system.

From these experiments, the exact mechanism for the inhibition of intramitochondrial fatty acid activation by a depressed extra- or intramitochondrial ATP/ADP ratio cannot be determined. However, two alternative hypotheses are proposed. First, some metabolite or other unknown factor, the level of which is determined by the ATP/ADP ratio, might directly inhibit the matrix medium chain fatty acyl-CoA synthetase. Alternately, the inhibition of this enzyme may result from a decreased availability of the intramitochondrial ATP required for fatty acid activation, as a consequence of the reduced intramitochondrial ATP/ADP ratio and/or the rapid transport of extramitochondrial ADP in exchange for intramitochondrial ATP.

Relationship between the Cellular ATP/ADP Ratio and the Carnitine Dependence of Octanoate Oxidation—The data of Table II indicated that the inhibition of carnitine-independent oxidation of octanoate by a depressed ATP/ADP ratio could be overridden by addition of carnitine. Therefore, it was of interest to determine whether a change in the ATP/ADP ratio in a more physiological system, such as hepatocytes, could shift the oxidation of octanoate from a carnitine-independent to a carnitine-dependent route. Thus the experiment of Tables IV and V was performed. Oxidation of [1-14C] octanoate by hepatocytes (Table IV) was followed by measuring the production of its products, 14CO2 and 14C-labeled ketone bodies. The cellular ATP/ADP ratio was decreased by the addition of either fructose or glycerol (Table V), both of which are known to decrease the cellular ATP content, as well as the total cellular adenine nucleotide pool (32-40). Carnitine-dependent oxidation was determined by the addition of TDGA. In the control, carnitine-dependent oxidation of octanoate accounted for only 10% of the total oxidation (Table IV). With a decrease in the ATP/ADP ratio from 2.24 to approximately 1.0 in the presence of fructose or glycerol (Table V), there was a net increase (1.7-fold) in carnitine-dependent oxidation, representing approximately 20% of the total oxidation (Table IV). Thus, the data indicate that, with carnitine addition to mitochondria or with hepatocytes, a limitation on medium chain fatty acid oxidation imposed at the site of intramitochondrial activation may be compensated for by shifting to extramitochondrial activation and subsequent oxidation via a carnitine-dependent route. The mechanism for this shift is not apparent. The data also suggest that the ATP/ADP ratio is involved in determining the distribution of octanoate oxidation between carnitine-independent and carnitine-dependent routes.

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**EXPERIMENTAL PROCEDURES**

Isolation and isolation of liver mitochondria - Adult maleistar rats weighing 200-250 g were anesthetized with diethyl ether. The rats were decapitated, and the liver was homogenized in 10 volumes of 0.25 M sucrose containing 5 mM Tris-HCl, pH 7.4, and then centrifuged at 1000 x g for 10 min. The supernatant was then layered on a Folin-phenol solution containing 0.15 M sucrose at 4°C to provide a concentration of approximately 10 mg of mitochondrial protein per ml. The mitochondrial preparations in these experiments had a respiratory control ratio (R) above 2.0 and a specific activity of 50-100 mg of submaximal phosphate incorporation per mg of protein to 5 mg protein.

Mitochondria were isolated from the liver of eight male rats and incubated for 30 and 60 min with 0.4 mM ATP and 0.4 mM ADP, respectively. The proteins were then isolated and subjected to electrophoresis in a 5-15% SDS-PAGE gel. The gels were stained with Coomassie Blue and the bands were analyzed using a gel imaging system. The relative intensity of the bands was quantified using ImageJ software.

**Table I**

| Substrate | Additions | with 20 min preincubation | without preincubation |
|-----------|-----------|--------------------------|-----------------------|
| Osmatane | None | 17.8 ± 1.2 | 31.1 ± 0.6 |
|          | 0.4 mM | 31.3 ± 1.4 | 37.3 ± 0.2 |
| TICA, 10 µM | None | 18.1 ± 0.5 | 33.6 ± 0.4 |
|          | 25-50, 20 µM | 35.0 ± 0.4 | 38.2 ± 0.7 |
| Butyrate, 10 µM | None | 15.7 ± 0.5 | 18.6 ± 0.3 |

**Table II**

| Substrate | Additions | with 1 µM ATP | without ATP |
|-----------|-----------|---------------|-------------|
| Osmatane, 0.4 µM | None | 12.3 ± 0.4 | 37.4 ± 0.4 |
| TICA, 10 µM | None | 11.6 ± 0.4 | 37.6 ± 0.4 |
| Butyrate, 10 µM | None | 8.7 ± 0.3 | 31.8 ± 0.1 |

**Figure 1.** Effect of preincubation on the rate of QOXR-dependent oxidation of octanoyl-CoA in mitochondria. (A) The association ATP/ADP ratio of the total extractable octanoyl-CoA pool. Freshly isolated mitochondria were incubated in the presence of 0.4 mM ATP at 37°C for the respective times as described under "Experimental Procedures." ATP (2 nM and 2 µM) added at 0 min; ATP (20 min preincubation) - (B), without preincubation. The reaction was stopped by the addition of 20 µM ADP.

**Figure 2.** Effect of ATP or ADP addition on (A) the rate of QOXR-dependent oxidation of octanoyl-CoA and (B) the association ATP/ADP ratio of the total extractable octanoyl-CoA pool. Freshly isolated mitochondria were incubated in the presence of 0.4 mM ATP at 37°C for the respective times as described under "Experimental Procedures." ATP (2 nM and 2 µM) added at 0 min; ATP (20 min preincubation) - (B), without preincubation. The reaction was stopped by the addition of 20 µM ADP.

**Figure 3.** The ATP/ADP ratio of the total extractable octanoyl-CoA pool. The reaction was stopped by the addition of 20 µM ADP. ATP (2 nM and 2 µM) added at 0 min; ATP (20 min preincubation) - (B), without preincubation.
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Table III: Extra- and Intramitochondrial Adenine Nucleotide Levels with the Addition of ATP or ADP to Nitrocellulose-Adsorbed Octanoate

|          | Freshly Isolated Mitochondria | 3 mM ATP | 3 mM ADP |
|----------|-------------------------------|----------|----------|
| lnct       | nmol/mg protein               | nmol/mg protein | nmol/mg protein |
| ATP       | 5.0 ± 0.5                     | 5.14 ± 0.16 | 3.3 ± 0.26 |
| ADP       | 6.0 ± 0.2                     | 6.2 ± 0.20 | 7.1 ± 0.45 |
| ATP+ADP   | 14.0 ± 0.3                    | 17.6 ± 0.27 | 7.3 ± 0.41 |

ATP/ADP 1.00 ± 0.02 0.46 ± 0.11 0.30 ± 0.01 0.29 ± 0.11

**Significantly different from the corresponding value in the 3 mM ATP system; p<0.05.**

**Significantly different from the corresponding value in freshly isolated mitochondria; p<0.05.**

Table IV: Inhibition of [1-14C]Octanoate Oxidation by 3-ERPentenylpyruvate and the Presence or Absence of Fructose or Glycerol: Measurement of Oxidation Rates

|          | TCA- | Glucose- | Fructose- |
|----------|------|----------|-----------|
| Octanoate | mol/l | cells    | mol/l     |
| Control  | 0    | 0        | 0         |
| 20 mM Fructose | 0    | 100 ± 10  | 100 ± 10   |
| 10 mM Glycerol   | 0    | 100 ± 10  | 100 ± 10   |

Oxidation-dependent oxidation calculated as the difference in total oxidation (TCA + glucose + fructose) between groups with and without TCA addition.

Table V: Effects of 10 mM or 100 mM Fructose on the Oxidation of [1-14C]Octanoate by Freshly Isolated Mitochondria

|          | ATP, mol/l                 | ADP, mol/l                 | ATP, mol/l                 |
|----------|----------------------------|----------------------------|----------------------------|
| Control  | 2.35 ± 0.02                | 1.02 ± 0.02                | 4.86 ± 0.02                |
| 10 mM Fructose | 2.07 ± 0.02 | 1.02 ± 0.02 | 4.66 ± 0.02 |
| 10 mM Glycerol   | 2.69 ± 0.02 | 0.60 ± 0.02 | 1.20 ± 0.02 |

**Significantly different from the corresponding value in the 10 mM Fructose condition; p<0.05.**

Table VI: Adenosine Monophosphate Content of Mitochondrial Suspensions

|          | Freshly Isolated Mitochondria | After Incubation for 10 min with 10 mM Fructose |
|----------|-------------------------------|-----------------------------------------------|
| ATP, mol/l | 2.35 ± 0.02                  | 2.07 ± 0.02                                  |
| ADP, mol/l | 1.02 ± 0.02                  | 0.60 ± 0.02                                  |
| ATP/ADP  | 2.17 ± 0.02                  | 1.26 ± 0.06                                  |

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