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# Acetyl-Coenzyme A Can Regulate Activity of the Mitochondrial Pyruvate Dehydrogenase Complex *in Situ*[1]

**Raymond J. A. Budde, Tung K. Fang, Douglas D. Randall*, and Jan A. Miernyk**
*Department of Biochemistry, University of Missouri, Columbia, Missouri 65211*

## ABSTRACT

***In vitro*, the pyruvate dehydrogenase complex is sensitive to product inhibition by NADH and acetyl-coenzyme A (CoA). Based upon $K_m$ and $K_i$ relationships, it was suggested that NADH can play a primary role in control of pyruvate dehydrogenase complex activity *in vivo* (JA Miernyk, DD Randall [1987] Plant Physiol 83: 306–310). We have now extended the *in vitro* studies of product inhibition by assaying pyruvate dehydrogenase complex activity *in situ*, using purified intact mitochondria from green pea (*Pisum sativum*) seedlings. *In situ* activity of the pyruvate dehydrogenase complex is inhibited when mitochondria are incubated with malonate. In some instances, isolated mitochondria show an apparent lack of coupling during pyruvate oxidation. The inhibition by malonate, and the apparent lack of coupling, can both be explained by an accumulation of acetyl-CoA. Inhibition could be alleviated by addition of oxalacetate, high levels of malate, or L-carnitine. The CoA pool in nonrespiring mitochondria was approximately 150 micromolar, but doubled during pyruvate oxidation, when 60 to 95% of the total was in the form of acetyl-CoA. Our results indicate that *in situ* activity of the mitochondrial pyruvate dehydrogenase complex can be controlled in part by acetyl-CoA product inhibition.**

The mPDC[2] occupies a crucial branch point in intermediary metabolism, and activity is regulated by several mechanisms including compartmentation, product and metabolite inhibition, and reversible phosphorylation (reviewed in refs. 13, 17). Initial studies of the regulatory properties of plant mPDC have been conducted *in vitro* using preparations that had been purified 20- to 300-fold (18–21). Recently, we (2–4, 16) have developed a method for assaying mPDC activity *in situ* using purified intact pea seedling mitochondria. Using this method, several observations on the regulation by reversible phosphorylation have been clarified and extended (2–4). This report continues our investigation of the role of product inhibition in regulation of PDC activity within intact mitochondria.

Much like mammalian and microbial PDCs, plant mPDCs are quite sensitive *in vitro* to product inhibition (17, 18, 24). At a constant total CoA concentration, a linear decrease in CoA-SH/acetyl-CoA results in a linear decrease in PDC activity. In contrast, a linear decrease in NAD/NADH results in a logarithmic decrease in PDC activity. Additionally, the $K_i$ for NADH is smaller than the $K_m$ for NAD while the $K_i$ for acetyl-CoA is larger than the $K_m$ CoA-SH (*e.g.* ref. 14). These two observations led to the proposal that NADH would be the more important product inhibitor *in vivo*. Relatively little, however, is known about the size of the total CoA pool in plant mitochondria, or of CoA-SH/acetyl-CoA under respiratory conditions. We now present evidence that, while respiring, pea seedling mitochondria can actively accumulate CoA and that during respiration most CoA is present within the mitochondrial matrix as acetyl-CoA. We now believe that acetyl-CoA could play a greater role in the *in vivo* regulation of mPDC activity than previously recognized.

## MATERIALS AND METHODS

Growth of pea (*Pisum sativum*) seedlings (2) and isolation of intact mitochondria by rate-zonal sedimentation through consecutive discontinuous Percoll gradients (8) have been described previously. The methods used for measurement of oxygen uptake are described by Budde *et al.* (3). Reaction mixtures contained 0.5 to 0.7 mg of mitochondrial protein. Activity of the mPDC was measured spectrophotometrically as previously described (2), or by using the INT-coupled assay of Hinman and Blass (9). In the latter cases, 1 mL assays contained the following components at the indicated final concentrations: 65 mM Tes-NaOH (pH 7.5), 0.1% (v/v) Triton X-100, 0.5 mM $MgCl_2$, 2 mM NAD, 0.2 mM thiamine-pyrophosphate, 0.12 mM CoA-SH, 1 mM cysteine, 1 mM sodium pyruvate, 0.6 mM INT, and 0.1 mg porcine lipoamide dehydrogenase. Reactions were initiated with mitochondria to minimize the time-dependent detergent inactivation of the mPDC, and the increase in absorbance at 500 nm was recorded. Carnitine:acetyl transferase was assayed according to Markwell *et al.* (12). Protein was determined by the method of Bradford (1), using BSA as the standard.

For the determination of CoA concentrations, 0.6 mL samples of intact mitochondria were layered over 0.6 mL of silicone oil (Wacker-Chemie: Ar 200:20 in a 7:3 ratio (v/v), which was in turn layered over 0.2 mL of 1.6 M $HClO_4$. Disposable 1.5 mL microcentrifuge tubes containing the $HClO_4$ and silicone oil layers were prepared ahead of time and stored at 4°C. Mitochondria were centrifuged in an Eppendorf 5415 microfuge (1 min, 15,000*g*, 4°C), through the silicone

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[2] Abbreviations: mPDC, mitochondrial pyruvate dehydrogenase complex; OAA, oxalacetic acid; PDC, pyruvate dehydrogenase complex; INT, 2(*p*-iodophenyl)-3-*p*-nitrophenyl-5-phenyl-tetrazolium chloride; Cof, cofactors.

oil layer and into the acid. The upper layer of sample loaded and silicone oil layers were removed and, to minimize cross-contamination, the walls and inner cap of the tube were carefully cleaned with a cotton swab. The acid layer was suspended with a microfuge tube homogenizer (Research Products International) and then vortexed. The sample was partially neutralized with saturated $KHCO_3$ to give a slightly acidic pH (4–7) at which CoA and its esters are most stable. Samples were vortexed, centrifuged, and the supernatant removed, frozen-thawed, and centrifuged once again. The final supernatant (150 $\mu$L) was injected onto a Microsorb $C_{18}$ reverse-phase HPLC column (Rainin) and eluted isocratically with 220 mM potassium phosphate buffer, pH 4.0, containing 12% methanol (10). CoA-SH and its esters were detected at 254 nm. Recoveries of greater than 95% were obtained when CoA-SH and acetyl-CoA standards were treated in an identical manner. Control samples of CoA-SH alone layered over the silicone oil and centrifuged resulted in no carryover into the acid layer. An internal volume for Percoll-purified plant mitochondria of 3.5 $\mu$L per mg mitochondrial protein was used to calculate the concentrations of CoA and acetyl-CoA (6).

## RESULTS AND DISCUSSION

Pyruvate oxidation by purified pea seedling mitochondria was inhibited by the addition of 2 mM malonate (Table I). Malonate inhibits the succinate dehydrogenase reaction that will prevent the formation of the acetyl-CoA acceptor, OAA. Consequently, acetyl-CoA will accumulate and pyruvate oxidation will be inhibited as seen in Table I. Inhibition by malonate was abrogated by the addition of 1 mM L-carnitine. This is the result of carnitine:acetyl transferase, present in these mitochondria at a specific activity of 31 nmol·$min^{-1}mg^{-1}$ protein, converting acetyl-CoA to CoA-SH plus acetylcarnitine.

To investigate further the effect of acetyl-CoA on pyruvate oxidation, we took advantage of the fact that our purified mitochondrial preparations occasionally show an apparent lack of coupling with pyruvate as the substrate. One parameter by which the quality of mitochondrial preparations is judged is their coupling, or their ability to link aerobic respiration to ATP synthesis. This is determined indirectly by the enhanced rate of substrate oxidation following the addition of ADP (6). Isolated pea seedling mitochondria usually (>90% of the time) show good coupling, *i.e.* a two- to five-fold increase in the rate of pyruvate oxidation upon addition of ADP (Fig. 1A). Occasionally, we find preparations that show an apparent lack

**Table I.** *Effects of Malonate and L-Carnitine upon Pyruvate Oxidation by Mitochondria Purified from 14 d Light-Grown Pea Seedlings*

Reactions contained 1 mM pyruvate plus 0.1 mM malate.

| $O_2$ Uptake (State 3) | Plus 1 mM L-carnitine? | Plus 2 mM Malonate | Inhibition |
|---|---|---|---|
| *nmol·min$^{-1}$ mg protein$^{-1}$* | | *nmol·min$^{-1}$ mg protein$^{-1}$* | % |
| 36.4 | No | 17.4 | 52.2 |
| 34.4 | Yes | 29.2 | 15.1 |

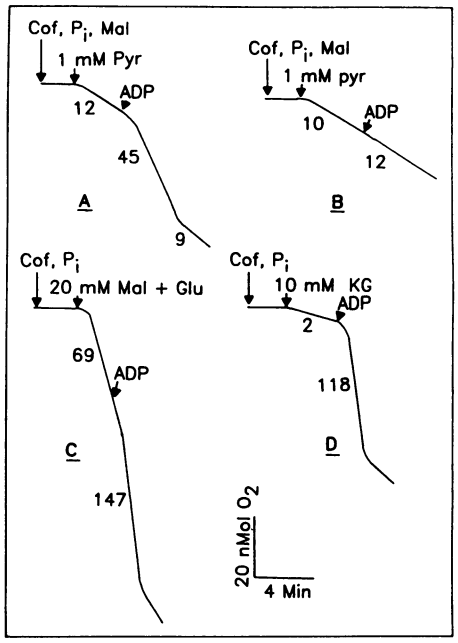


**Figure 1.** The apparent lack of coupling by pea mitochondria preparations oxidizing pyruvate. (A) Typical enhancement of pyruvate oxidation upon addition of ADP, (B) a typical lack of enhancement of pyruvate oxidation, (C and D) enhanced malate (20 mM) and 2-oxoglutarate (2 mM) oxidation. B, C, and D are from the same preparation of mitochondria. Numbers below traces are nmol $O_2$ consumed $min^{-1}$ mg mitochondrial $protein^{-1}$, calculated from the slopes of $O_2$-electrode traces. Additions were Cof: 1 mM $\beta$-NAD, 0.1 mM CoA, and 0.1 mM thiamine pyrophosphate (cofactors required for pyruvate oxidation); 5 mM $KPO_4$, 0.1 mM sparker malate, 1 mM pyruvate, 0.2 mM ADP, and 20 mM glutamate.

of coupling when pyruvate is used as the substrate (Fig. 1B) but exhibit normal coupling when malate or 2-oxoglutarate are used as substrates (Fig. 1, C and D). Analysis of the PDC activity from these apparently uncoupled mitochondria indicated that PDC was very active (specific activity of 0.2 unit·mg $protein^{-1}$). Thus, the lack of ADP-dependent enhancement of respiratory rates (measurement of coupling) was not due to the phosphorylation (inactivation) of PDC (3). Increasing the concentration of pyruvate to 10 mM and ADP to 0.5 mM, or preincubating with the cofactors, did not eliminate this apparent lack of coupling. The NADH produced during the oxidation of pyruvate, along with that produced from the oxidation of malate and 2-oxoglutarate, is reoxidized by the same NADH dehydrogenase (complex I; ref. 6). Therefore, the mitochondria which exhibited enhanced oxidation rates upon addition of ADP, except with pyruvate as the substrate (Fig. 1, B *versus* C and D), were indeed coupled. Because activity of the 2-oxoglutarate dehydrogenase complex is also regulated by NADH inhibition (6) it seems unlikely that NADH accumulation is responsible for inhibiting the oxidation of pyruvate. Potential alternatives are acetyl-CoA product inhibition and/or CoA-SH availability.

The apparent lack of coupling with pyruvate as the substrate was reversed by adding OAA or L-carnitine, or increasing the concentration of sparker malate (Fig. 2). All three methods

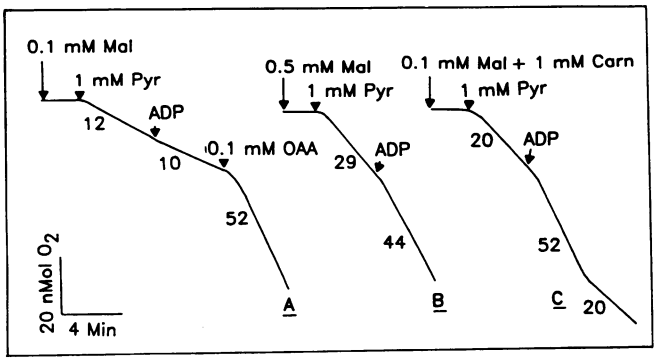


**Figure 2.** Reversal of the apparent lack of coupling by addition of (A) 0.1 mM OAA, (B) increased concentration of sparker malate to 0.5 mM, or (C) addition of 1 mM L-carnitine. Other additions as in the legend for Figure 1.

will decrease the amount of acetyl-CoA (or increase the CoA-SH) by providing an acceptor for the acetyl group. OAA is condensed with acetyl-CoA by citrate synthase; malate dehydrogenase generates OAA from malate for citrate synthase, and carnitine is the acetyl acceptor for carnitine:acetyltransferase. The effect seen with carnitine was not observed when it was replaced with acetylcarnitine (data not shown), indicating that the method by which carnitine relieved the apparent lack of coupling was by reacting with acetyl-CoA to generate CoA-SH. Acetylcarnitine cannot serve as an alternate substrate for carnitine in the reaction to form CoA-SH but will in fact increase the acetyl-CoA fraction of the CoA pool.

The results of *in vitro* kinetic analyses (14, 18, 23) of the partially purified PDC have indicated that NADH should potentially play a greater role in the regulation of PDC activity than acetyl-CoA because the $K_i$ (NADH, 20 $\mu$M) is smaller than the $K_m$ (NAD, 120 $\mu$M). To test this we developed assay procedures in which both products, only one, or neither was allowed to accumulate during the spectrophotometric determination of PDC activity (Table II). The initial rates from all four assays were equivalent. Calculating the amount of product formed at the point where the rate deviates from linearity, as determined by a linear least squares calculation conducted by the Gilford Response spectrophotometer, enables one to determine that 88% of the product inhibition was due to NADH and 12% to acetyl-CoA. The effect of these two products was additive, not synergistic. This is consistent with the kinetic constants (14). The discrepancy between what *in vitro* kinetic data predict concerning NADH inhibition *versus* acetyl-CoA inhibition and that which was observed when there was an apparent lack of coupling with pyruvate is understandable when the *in situ* events are considered. The product NADH may not necessarily reach inhibitory concentrations *in situ* while acetyl-CoA can become inhibitory, because these two products are recycled by different mechanisms which may operate at different rates under different metabolic conditions. In addition, the pool sizes of the substrates and products may be very different.

Since the concentration of sparker malate was important in pyruvate oxidation (Fig. 2, B and C), we examined in more detail the effect of different concentrations of sparker malate on pyruvate oxidation, coupling, and CoA-SH concentrations in intact mitochondria which showed good coupling with pyruvate. Sparker malate was required for pyruvate oxidation to provide OAA (via malate dehydrogenase) to regenerate CoA-SH from acetyl-CoA through the citrate synthase catalyzed reaction. A malate concentration of 0.5 mM gave the best results in that respiration due to malate alone was negligible, there was no observable coupling with malate alone, and maximal levels of coupling and pyruvate oxidation were obtained (Fig. 3).

When comparing the effect of 0.1 *versus* 0.5 mM sparker malate on pyruvate oxidation, we consistently observed that with 0.1 mM malate the decline in respiration rate (state 4) following the initial ADP-dependent enhancement of pyruvate oxidation could not be reversed by subsequent additions of ADP (Fig. 4A). In contrast, the use of 0.5 mM malate did result in the recovery from state 4 upon further addition of ADP. Fluorometric analyses have demonstrated that ADP stimulates respiration by recycling NADH (6). Thus, with

**Table II.** *Inhibition of PDC Activity by NADH and Acetyl-CoA*

Assays: 1, PDC activity was determined by NADH formation: both NADH and acetyl-CoA accumulate in 1:1 ratio; 2, carnitine and carnitine acetyltransferase were added to PDC reaction mixture to keep the acetyl-CoA from accumulating while NADH accumulates; 3, PDC was assayed using the INT assay in which NADH is reoxidized with only acetyl-CoA accumulation; 4, PDC was assayed using the INT procedure in the presence of carnitine and carnitine acetyltransferase to recycle CoA-SH and NAD.

| Assay | Additions | Accumulated Product | Concn of Each Product at Deviation from Linearity | Specific Activity[a] |
|---|---|---|---|---|
| | | | $\mu M$ | |
| 1 | None | NADH + acetyl-CoA | 4.2 | 0.22 |
| 2 | Carn/CAT[b] | NADH | 5.0 | 0.21 |
| 3 | None | Acetyl-CoA | 10.5 | 0.22 |
| 4 | Carn/CAT[b] | | 11.4[c] | 0.21 |

[a] $\mu$mol·min$^{-1}$ mg mitochondrial protein$^{-1}$. [b] Carn/CAT = 1 mM carnitine plus 5 units carnitine:acetyltransferase. [c] Same deviation from linearity also occurred in the absence of either product due to time-dependent inactivation of PDC by Triton X-100.

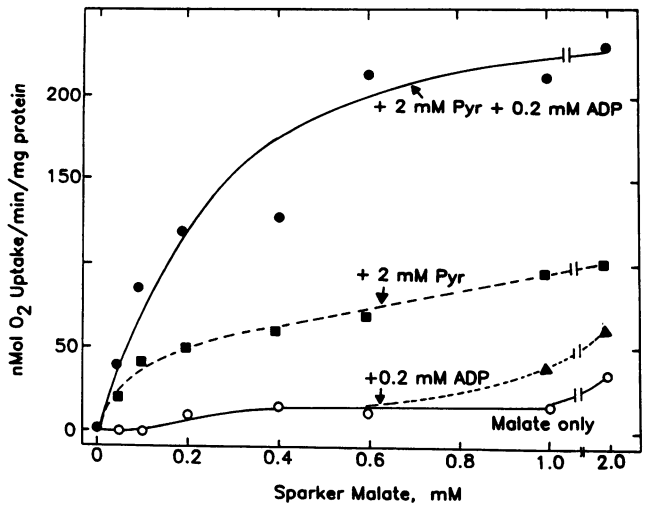


**Figure 3.** Effect of increasing concentrations of sparker malate on the oxidation of pyruvate. Rates were calculated from the slopes of $O_2$-electrode traces. Malate = 0 to 2 mM, pyruvate = 2, mM and ADP = 0.2 mM.

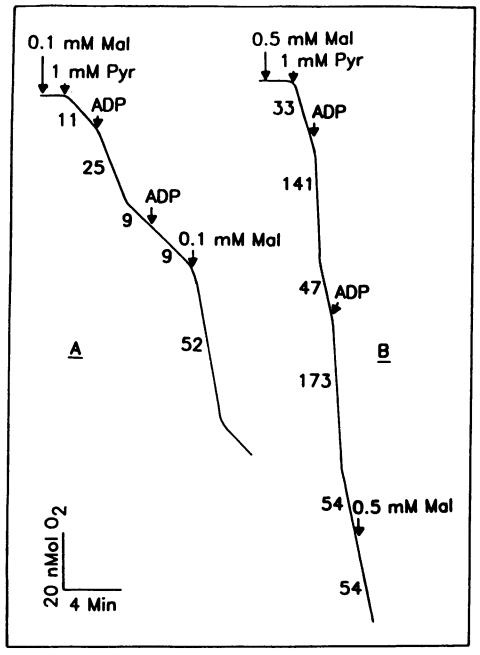


**Figure 4.** Effect of 0.1 *versus* 0.5 mM sparker malate on pyruvate oxidation, and the ability of the respiration rate to recover from state 4 (the decline in substrate oxidation after enhancement by first addition of ADP) by subsequent addition of ADP. Additions as described in the legend for Figure 1.

lower concentrations of sparker malate one would expect product inhibition by acetyl-CoA to be potentially more extreme, and with high concentrations of malate NADH inhibition may predominate.

We used these two concentrations of sparker malate to study the effect of pyruvate respiration on the CoA pool and percent of CoA-SH in the mitochondrial matrix. Samples were withdrawn from the $O_2$-electrode chamber during different states of respiration, and centrifuged through a layer of silicone oil to separate the exogenous CoA from that which accumulated within the matrix. Samples were analyzed by a reverse phase-HPLC system in which CoA-SH, acetyl-CoA and succinyl-CoA were well separated (Fig. 5A). A standard curve was made correlating peak height to pmol of CoA-SH and acetyl-CoA. For mitochondria analyzed as isolated, i.e. in a nonrespiring state, we always found that the entire CoA pool was in the nonesterified form (Fig. 5B). During respiration, however, most of the CoA pool was in the form of acetyl-CoA (Fig. 5C). The succinyl-CoA peak was either not visible or seen as a minor peak, too small for accurate quantification.

The CoA pool size was stable during the first 24 h after isolation and then began to decline by 25% each subsequent

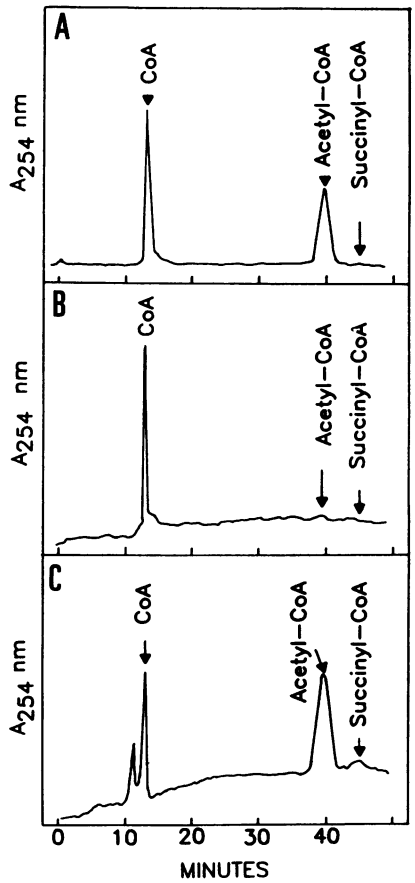


**Figure 5.** Determination of the CoA pool (free plus esterified CoA) and percent CoA-SH. (A) HPLC-separation of standards, samples from (B) nonrespiring, and (C) respiring mitochondria. Samples were removed from the $O_2$-electrode chamber and centrifuged through a silicone oil layer before HPLC analysis.

**Table III.** *Change in CoA Pool Size and Percent Free CoA during Respiration*

| Addition | PDC Activity | CoA-SH | Acetyl-CoA | Pool | CoA-SH |
|---|---|---|---|---|---|
| | %[a] | | μM | | % |
| 0.1 mM "Sparker Malate" | | | | | |
| Cof, Pi, 0.1 mM Malate | 100 | 0 | 120 | 0 | 120 | 100 |
| +1 mM Pyruvate | 97 | 30 | 46 | 245 | 290 | 16 |
| +0.2 mM ADP (state 3) | 95 | 48 | 110 | 268 | 380 | 29 |
| +0.2 mM ADP (state 4) | 104 | 11 | 38 | 284 | 320 | 12 |
| +0.1 mM Malate | 91 | 74 | 53 | 272 | 320 | 17 |
| 0.5 mM "Sparker Malate" | | | | | |
| Cof, Pi, 0.1 mM Malate | 100 | 0 | 170 | 0 | 170 | 100 |
| +1 mM Pyruvate and 0.4 mM Malate | 92 | 37 | 100 | 165 | 265 | 38 |
| +0.2 mM ADP (state 3) | 81 | 128 | 60 | 200 | 260 | 23 |
| +0.2 mM ADP (state 4) | 100 | 66 | 75 | 175 | 250 | 30 |
| +0.2 mM ADP | 85 | 132 | 85 | 245 | 320 | 26 |

[a] 100% = 0.2 $\mu$mol·min$^{-1}$ mg mitochondrial protein$^{-1}$, activity determined by monitoring NAD reduction. Aliquots removed from $O_2$ electrodes were used immediately to measure PDC activity in the presence of 0.1% Triton X-100 to rupture mitochondria.

day (data not shown). Table III shows the distribution of the primary components of the CoA pool under various conditions of pyruvate oxidation. The initial CoA pool in nonrespiring mitochondria was entirely in the form of CoA-SH and ranged from 120 to 190 μM. After addition of pyruvate the total pool-size increased to 200 to 450 μM, and most was in the form of acetyl-CoA. The net transport of CoA-SH into mitochondria is apparently an energy requiring process, since mitochondria incubated in the absence of pyruvate did not accumulate additional CoA. This is in agreement with Neuburger *et al.* (15) who have previously shown active uptake of CoA-SH by plant mitochondria. Unfortunately we were not able to correlate the CoA-SH:acetyl-CoA ratio with the respiration rate for each respiratory state. During respiration the concentration of CoA-SH varied from 30 to 120 μM and the concentration of acetyl-CoA ranged from 150 to 300 μM. With 0.1 mM sparker malate, CoA-SH was 5 to 30% of the total pool, whereas with 0.5 mM malate it was 20 to 40%. These values represent the ranges from four different preparations of mitochondria. A representative set of data is presented in Table III. Thompson *et al.* (24) and Randall *et al.* (18) determined the effect of changes in acetyl-CoA/CoA-SH on the activity of PDC. From the response curve of Thompson *et al.* (24) the data indicate that PDC would be inhibited by 33 to 50% due to acetyl-CoA inhibition alone. Note that the respiration rate (nmol $O_2$·min$^{-1}$ mg$^{-1}$) does not correlate with the activity of PDC when measured spectrophotometrically under the $V_{max}$ conditions to determine the phosphorylation state of the enzyme (3). Under these conditions respiration is not controlled by the steady-state phosphorylation of PDC (3), but rather by product inhibition. This is consistent with our model for regulation in which PDC remains fully active when mitochondria are incubated with 1 mM pyruvate (2, 3).

It has recently been reported (11) that most of the CoA in respiring mammalian mitochondria is in the form of acetyl-CoA. This could be reversed by addition of L-carnitine. Mammalian mitochondria also carry out the oxidation of fatty acids and are thought to contain separate pools of CoA for Krebs cycle activity and β-oxidation (22). The ability of plant mitochondria to oxidize fatty acids is controversial (26) and may be tissue specific; however, they do contain carnitine: acetyltransferase activity (5, 23, and this report). The role of carnitine and carnitine:acetyltransferase in modulating acetyl-CoA inhibition *in vivo* remains to be elucidated. Uziel *et al.* (25) report that carnitine stimulated PDC activity and pyruvate oxidation in isolated human muscle mitochondria along with an increase in acetylcarnitine formation. Dry and Wiskich (7) have suggested that the 2-oxoglutarate dehydrogenase complex is in part regulated by the availability of CoA-SH. Our data would clearly support this suggestion and also extend it to the regulation of PDC.

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