THE INACTIVATION OF PYRUVATE DEHYDROGENASE BY FATTY ACID IN ISOLATED RAT LIVER MITOCHONDRIA*

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Summary

The influence of fatty acid on the interconversion of the pyruvate dehydrogenase complex (PDH) between its active (dephospho-) and inactive (phospho-) forms and on the intramitochondrial ATP/ADP, NADH/NAD+ and acetyl-CoA/CoASH ratios was studied in isolated rat liver mitochondria. Conditions were found in which the PDH activity was inversely correlated only with the NADH/NAD+ ratio. Under other conditions the PDH activity was inversely correlated solely with the acetyl-CoA/CoASH ratio. These experiments suggest that the activity of the regulatory enzymes involved in the inactivation and reactivation of the pyruvate dehydrogenase multienzyme complex may be controlled by both the intramitochondrial NADH/NAD+ and acetyl-CoA/CoASH ratios.

Since the discovery of the phosphorylation-dephosphorylation mechanism for the regulation of the activity of PDH+ by Reed and his colleagues (1-3) the delineation of factors which may regulate the PDH kinase and phosphatase reactions involved in this mechanism has become a crucial consideration. Various reports have indicated that adenine nucleotides (4-6) and di- and monovalent cations (7-9) may be involved in the differential regulation of the inactivation (kinase) and activation (phosphatase) of the multienzyme complex. Among the agents which have been shown to lead to an inactivation of the PDH activity in intact tissues (10-12) and more recently in isolated mitochondria (4,13,14) are fatty acids and ketone bodies. Little, if any, perception into the mechanism of this fatty acid-mediated inactivation of the mitochondrial PDH has been provided until very recently. Taylor et al. (14) suggested that the fatty acid effect may be mediated by an alteration in

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+Abbreviation: PDH, pyruvate dehydrogenase complex

oxidation-reduction state of the mitochondria but presented little experimental evidence to substantiate this suggestion. The present experiments were performed to attempt to delineate a possible mechanism for the fatty acid-mediated inactivation of the mitochondrial PDH.

METHODS AND MATERIALS

Rat liver mitochondria were prepared using a slight modification of the procedure of Schneider and Hogeboom (15). The homogenization medium contained 225 mM mannitol, 75 mM sucrose and 0.1 mM EGTA. The washing medium contained 225 mM mannitol and 75 mM sucrose. Mitochondrial protein was estimated using a biuret procedure (16).

Mitochondria (7-10 mg protein) were incubated for 10 min at 25°C in 2 ml of a medium containing as standard components: 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris-Cl (pH 7.5), 10 mM potassium phosphate, 10 mM succinate, 30 mM glucose, 0.2 mM ADP, 20 U hexokinase (EC 2.7.1.1), 22.5 mM mannitol, 32.5 mM sucrose and 0.16 g Dextran T40. The incubations were performed in 25 ml Erlenmeyer flasks in a shaking water bath.

When PDH activity was to be assayed, the incubation was stopped with a rapid freezing technique essentially as described by Taylor et al. (14). The incubation mixture (0.5 ml) was pipetted into a tube containing 0.5 ml ice-cold 2 mM dithiothreitol in 20% ethanol. This mixture was immediately plunged into a dry ice-acetone bath and kept in this bath for at least 10 min. After thawing in chipped ice the contents of the tube (or 0.25 ml thereof; see legend to Table II) were transferred to 25 ml Erlenmeyers kept at 0°C. PDH activity was assayed by measuring the formation of $^{14}CO_2$ from [1-^{14}C] pyruvate. The assay was initiated by adding 1 ml of an assay mixture containing: 5 mM [1-^{14}C] pyruvate (spec.act. 20 mC/mole), 1.11 mM NAD^+, 0.37 mM CoASH, 0.8 mM thiamine pyrophosphate, 5.2 mM cysteine, 5 mM oxaloacetate and 0.02% Lubrol WX. The flasks were closed with rubber serum stoppers equipped with plastic center wells and were shaken at 25°C in a water bath. After 10 min the incubation was terminated by injection of 1 ml of 18% $HClO_4$ into the flasks. $^{14}CO_2$ was measured as described previously (8).

For the assay of intramitochondrial ATP, ADP and NAD^+, 1.0 ml of the incubation mixture was equally divided in 2 Eppendorf centrifuge tubes (1.5 ml), each containing 0.2 ml 15% $HClO_4$ and 0.5 ml silicone oil (spec.gr. 1.054). After centrifugation for 1 min at maximum speed in an Eppendorf microcentrifuge, the top layers were removed. Of the combined $HClO_4$ extracts 0.3 ml was neutralized with KOH. ATP, ADP and NAD^+ were assayed fluorometrically (17) in the neutralized extract. Intramitochondrial ATP and ADP concentrations were corrected for extramitochondrial adenine nucleotides retained with the mitochondria during the centrifugation. The volume of extramitochondrial fluid adhering to the mitochondria during the centrifugation was obtained from parallel incubations with [U-^{14}C] sucrose. As the incubation mixture contained hexokinase, which could exert its action during the centrifugation, it was considered useless to determine ATP and ADP in the upper layer after the centrifugation. Extramitochondrial adenine nucleotides were determined by difference, i.e. adenine nucleotide in total extract minus intramitochondrial adenine nucleotide. The total mitochondrial extract was obtained by adding 1 ml of ice-cold 1.2 N $HClO_4$ to the incubation mixture; the protein was removed by centrifugation; neutralization was accomplished with KOH.

For determination of NADH in the mitochondrial incubation 0.5 ml of incubation mixture was added to a centrifuge tube containing 0.25 ml of 1N KOH in 100% ethanol (17). After neutralization with $HClO_4$ to pH 8.5 and removing the protein and $KClO_4$ by centrifugation, NADH was determined immediately.

For assay of CoASH and acetyl-CoA, total extracts were obtained by adding 1 ml of ice-cold 1.2 N $HClO_4$ to the mitochondrial incubations; the protein was removed by centrifugation; neutralization was accomplished with KOH. To the extract 1 mM dithiothreitol was added and CoASH was measured fluorometrically with α-ketoglutarate dehydrogenase (18). Acetyl-CoA was measured in the same cuvette by adding phosphotransacetylase (EC 2.3.1.8) (18). All intermediate determinations and PDH assays were performed in triplicate and the results reported represent the average of the three values.

α-Ketoglutarate dehydrogenase was isolated from pig heart according to Sanadi et al. (19). Hexokinase (type V) was obtained from Sigma, lactate dehydrogenase (EC 1.1.1.27) from Worthington and the other enzymes from Boehringer. Lithium acetoacetate was prepared according to Hall (20). Dextran T40 was supplied by Pharmacia. [1-^{14}C]pyruvate, [U-^{14}C]sucrose, Protosol and Omnifluor were obtained from New England Nuclear. Serum stoppers and center wells were supplied by Kontes.

RESULTS AND DISCUSSION

Addition of fatty acids to metabolic preparations metabolizing pyruvate has been shown to lead to an inhibition of PDH (4,10-14). The early explanation for this effect centered entirely around the fact that both acetyl-CoA and NADH (both products of pyruvate oxidation and β-oxidation) are competitive inhibitors of PDH (21-23). Recently it has been shown that fatty acid addition to intact systems leads to a conversion of the active to the inactive form of PDH (4,10-14).

It is an interesting possibility that the known allosteric inhibitors of the PDH reaction (NADH and acetyl-CoA) may be involved in the kinase-phosphatase mediated regulation of the multienzyme complex. However, Linn et al. (2), Wieland and von Jagow-Westermann (24) and Hucho et al. (25) were unable to demonstrate such effects on the enzymes regulating PDH.

Taylor et al. (14) recently demonstrated a significant inactivation of PDH in rat liver mitochondria upon the addition of fatty acid and under incubation conditions in which a significant change in the oxidation-reduction state could be expected. Although these authors suggested that the fatty acid mediated inactivation of PDH involved an oxidation-reduction effect, they were

unable to distinguish between effects of the NADH/NAD^+ ratio and the intramitochondrial acyl-CoA/CoASH ratio on the inactivation of PDH. Effects of the ATP/ADP ratio were deemed unimportant as this ratio remained unchanged in their experiments.

Utilizing mitochondrial incubation conditions which would resist changes in the intramitochondrial ATP/ADP ratio, e.g. state 3 in the presence of succinate as the substrate, the activity of PDH was measured following the addition of fatty acid and/or acetoacetate. Additionally, the intramitochondrial levels of various nucleotides were determined in order to correlate changes in PDH activity with alterations in the levels of these intermediates. It was observed that the addition of octanoate caused a 4-fold inactivation of the PDH, without a change in the ATP/ADP ratio. This finding is in agreement with the data of Taylor et al. (14) indicating that the inactivation of PDH upon fatty acid addition did not involve an effect of the ATP/ADP ratio on the regulatory enzymes. Addition of octanoate caused a 2-fold increase in the NADH/NAD^+ ratio and a large increase in the acetyl-CoA/CoASH ratio. Because there occurred significant changes in both of these ratios, a distinction between possible individual effects of the NADH/NAD^+ ratio or the acetyl-CoA/CoASH ratio on the inactivation of PDH could not be made.

Upon inclusion of acetoacetate in the incubation medium there occurred a 4-fold increase in the PDH activity. This activation of PDH was likely not due to effects of the adenine nucleotides on the PDH kinase as the ratio of ATP/ADP changed in a direction opposite to that which would be consistent with activation of the PDH. Also, in this experiment the acetyl-CoA level remained at an undetectable level and there was no change in the acetyl-CoA/CoASH ratio while there was a significant decrease in the NADH/NAD^+ ratio. Hence, upon addition of acetoacetate to the mitochondria there was a positive correlation between the oxidation of mitochondrial pyridine nucleotide and the activation of the PDH.

In the presence of acetoacetate the inclusion of octanoate in the incu-

Table I

The Effect of Fatty Acid and Acetoacetate on the PDH Activity and the Levels of Various Nucleotides of Rat Liver Mitochondria

Additions	ATP	ADP	$\frac{ATP}{ADP}$	NADH	NAD^+	$\frac{NADH}{NAD^+}$	Acetyl-CoA	CoASH	$\frac{Acetyl\text{-}CoA}{CoASH}$	PDH Activity
	nmoles/mg protein			nmoles/mg protein			nmoles/mg protein			nmoles/min. mg protein
None	2.52	3.87	0.65	1.94	1.55	1.25	0	1.62	0	5.68
Octanoate	1.78	2.64	0.67	2.85	1.13	2.52	0.352	1.05	0.34	1.75
Acetoacetate	4.01	3.72	1.08	1.39	2.32	0.60	0	1.65	0	23.72
Acetoacetate + octanoate	2.63	3.30	0.80	1.60	2.11	0.76	0.304	1.26	0.24	3.40

To the standard incubation medium 2 mM octanoate and 10 mM lithium acetoacetate were added as indicated.

bation medium resulted in a 7-fold inactivation of PDH. Again, this effect was probably not due to an effect of the ATP/ADP ratio. There was only a minimal change in the NADH/NAD^+ ratio upon octanoate addition while there occurred a large increase in the acetyl-CoA/CoASH ratio. It is unlikely that this slight increase in NADH/NAD^+ had much effect on the inactivation as a 2-fold increase in NADH/NAD^+ discussed above was necessary to convert the PDH activity from 23.7 to 5.7 nmoles/min.mg protein. Hence, it is concluded that the extensive inactivation of PDH upon fatty acid addition in the presence of acetoacetate was correlated with the large increase in the acetyl-CoA/CoASH ratio.

It is interesting to compare the control incubation with the incubation to which octanoate and acetoacetate were added. In this case there was a small change in the ATP/ADP ratio, the NADH/NAD^+ ratio decreased with a magnitude which should have caused a significant activation of PDH, and the acetyl-CoA/CoASH ratio increased by an amount consistent with an extensive inactivation of the enzyme complex. The resultant effect on the enzyme with these opposing changes in the NADH/NAD^+ and acetyl-CoA/CoASH ratios was only a slight inactivation of the enzyme.

An alternative explanation for the effects of NADH/NAD^+ and acetyl-CoA/CoASH on the measured activity of PDH might be that NADH and acetyl-CoA exert direct effects on the active form of PDH during the assay procedure. If this were the case, dilution of the mitochondrial suspensions preincubated under the various conditions prior to assay should have minimized the observed changes in PDH activity. That this was not the case, may be seen in the experiment described in Table II. Although dilution caused a consistent increase in the measured specific activity of PDH, the ratios of PDH activity under the different incubation conditions remained unchanged even though the mitochondrial sample was diluted 4-fold prior to assay.

The observed correlation of the ratios of NADH/NAD^+ and acetyl-CoA/CoASH and the PDH activity in our experiments suggests that the fatty acid mediated

Table II

Effect of Dilution on the Measured Activation and Inactivation of Mitochondrial PDH

Incubation Condition	Additions	Measured undiluted				Measured 4 times diluted			
			PDH activity ratios				PDH activity ratios		
		PDH Activity	B:A D:C	C:A	D:A	PDH Activity	B:A D:C	C:A	D:A
		nmoles/min. mg protein				nmoles/min. mg protein			
A	None	4.40				6.08			
B	Octanoate	1.53	0.348			2.10	0.345		
C	Acetoacetate	11.49		2.61		17.65		2.91	
D	Acetoacetate + octanoate	2.63	0.229		0.598	3.65	0.207		0.600

To the standard incubation medium 2 mM octanoate and 10 mM lithium acetoacetate were added as indicated. For measurement of PDH activity undiluted the total amount of each rapidly frozen incubation mixture (1 ml) was transferred to the appropriate incubation flask. For measurement of the activity 4 times diluted 0.25 ml of each rapidly frozen incubation mixture was diluted with 0.75 ml of a solution containing the same ingredients except mitochondria, octanoate and acetoacetate.

inactivation of PDH involves changes in both of the above ratios. Supportive of this suggestion, Pettit et al. (26) have recently found that the PDH kinase is stimulated by NADH and acetyl-CoA and is inhibited by NAD^+ and CoASH. The PDH phosphatase is inhibited by NADH and this effect is reversed by NAD^+.

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