Regulation of Pyruvate Oxidation in Isolated Rabbit Heart Mitochondria*

SHELDON M. SCHUSTER AND MERLE S. OLSON

From the Department of Biochemistry, College of Medicine, University of Arizona, Tucson, Arizona 85724

SUMMARY

Evidence is presented in support of the hypothesis that the pyruvate dehydrogenase multienzyme complex of intact rabbit heart mitochondria may be regulated by a phosphorylation-dephosphorylation mechanism. Mitochondria incubated in the presence of pyruvate plus L-malate and either ADP or uncoupler exhibit nearly identical, rapid rates of pyruvate oxidation but possess markedly different ATP levels. It was shown that under metabolic conditions which lead to a release or mobilization of intramitochondrial magnesium and which also have a high intramitochondrial ATP level, pyruvate oxidation was nearly completely inhibited after a brief lag phase. It was shown that the addition of exogenous magnesium to uncoupled mitochondria supplemented with ADP caused a more rapid inhibition of pyruvate oxidation. The observed inhibition of pyruvate oxidation was dependent upon the time of preliminary incubation with uncoupler and ADP and was atractyloside-sensitive. Evidence was obtained indicating that the inhibition of pyruvate oxidation was specific for the substrate, pyruvate, i.e. the oxidation of L(-)-palmitoylcarnitine or α-ketoglutarate was unaffected under conditions leading to an inhibition of pyruvate oxidation.

In addition, it was demonstrated that the inhibition of pyruvate oxidation was not caused by an accumulation of either NADH or acetyl-CoA, both inhibitory products of the pyruvate dehydrogenase reaction, during the course of these experiments. The experiments reported in this communication indicate that the control of the availability of both ATP and magnesium are crucial for the regulation of the pyruvate dehydrogenase multienzyme complex. These studies are consistent with the possibility that the pyruvate dehydrogenase-linked protein kinase may be an effective means of regulating the conversion of pyruvate to acetyl-CoA in intact metabolic systems such as the isolated mitochondrion.

Two different types of mechanisms have been suggested for the regulation of the activity of the pyruvate dehydrogenase

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oxidized pyruvate in the presence of L-malate at a rapid, linear rate (290 nmoles of oxygen per min per mg of protein). Also shown in Fig. 1, Curve B, coupled mitochondria in the presence of ADP, glucose and hexokinase, which had a relatively high ATP level, oxidized pyruvate in the presence of L-malate at a nearly identical rate (224 nmoles of oxygen per min per mg of protein). As can be seen in Fig. 2, the ATP level of the mitochondria incubated in the presence of ADP plus glucose-hexokinase increased during the experiment to about 2.5 μmoles of ATP per mg of protein while the ATP level of the uncoupled mitochondria was constant and approximately 3 nmoles of ATP per mg of protein, nearly three orders of magnitude lower than observed in the coupled mitochondria.

This experiment implies that the ATP level of the isolated heart mitochondria per se was not a primary regulatory factor influencing the rate of pyruvate oxidation. An investigation of the effect of adding ADP to uncoupled mitochondria indicated that pyruvate oxidation was drastically inhibited in the presence of between 3 and 5 mM ADP. Upon initiation of respiration with ADP plus FCCP, the rate of oxygen consumption using pyruvate plus L-malate as substrates was linear and rapid (292 nmoles of oxygen per min per mg of protein) for approximately 1 min, after which an abrupt and nearly complete inhibition of pyruvate oxidation was observed (Fig. 3, Curve C). When ADP (5 mM) (Fig. 3, Curve B) or FCCP (1.0 μm) (Fig. 3, Curve A) were used separately to initiate respiration, nearly equivalent, rapid, linear rates of oxygen consumption were observed and an inhibition of pyruvate oxidation was not observed. That the inhibitory effect of ADP plus uncoupler on pyruvate oxidation was not due solely to an effect of ATP on the pyruvate oxidase system is shown in Fig. 4. As was the case in the experiment described in Figs. 1 and 2, the coupled mitochondria with a high ATP level and the uncoupled mitochondria with a very low intramitochondrial ATP level exhibited rapid and nearly equivalent rates of pyruvate oxidation. On the other hand, in the incubation to which ADP was added to uncoupled mitochondria,
the rate of pyruvate oxidation was nearly completely inhibited. Although it may not be apparent from the ordinate scale of Fig. 4, the ATP level during this incubation decreased from approximately 100 nmoles of ATP per mg of protein to 230 nmoles of ATP per mg of protein, which was between 90 and 10 times the ATP level of the uncoupled mitochondria, during the establishment of the inhibition of pyruvate oxidation.

The correlation between the degree of inhibition of pyruvate oxidation and the ATP content of the mitochondrial incubation is illustrated in the data shown in Fig. 5. As the amount of ADP added to the uncoupled mitochondria was increased from 0 to 6 mM ADP, the amount of ATP which accumulated during the reaction period increased and corresponded to the extent of the inhibition of pyruvate oxidation. In another experiment (not shown) it was observed that addition of up to 10 mM ATP caused only slight inhibition of pyruvate oxidation in uncoupled mitochondria. Apparently ATP produced in the adenylate kinase reaction in uncoupled mitochondria was more effective for the inhibitory effect on pyruvate oxidation than exogenously added ATP. That ADP was not the inhibitory species was inferred from the data shown in Figs. 3 and 4 which indicated that the addition of ADP to pyruvate-oxidizing mitochondria resulted in little or no inhibition of the respiratory rate. Fig. 6 indicates that the inhibition of pyruvate oxidation could be largely prevented by including atractyloside in the incubation (Curve C). This experiment indicates that the ATP formed presumably in the intermembrane space from ADP in the adenylate kinase reaction (17) was not available to cause the inhibition of pyruvate oxidation in the presence of atractyloside. This implies that the point of inhibition of pyruvate oxidation was within the atractyloside barrier which seems reasonable due to the fact that the acknowledged locus of the pyruvate dehydrogenase complex is on the inner mitochondrial membrane (17).

It is our contention that the inhibition of pyruvate oxidation caused by incubation of the cardiac mitochondria with ADP and FCCP was due to the mobilization or release of intramitochondrial magnesium. The "released" magnesium would be available to form an ATP-magnesium chelate with ATP formed from 2 molecules of ADP in the adenylate kinase reaction. This ATP-magnesium complex is the substrate for the protein kinase reaction capable of regulating the activity of pyruvate dehydrogenase. That this is a reasonable suggestion may be supported by the observations of Kun et al. (18, 19) and of Bogucka and Wojtczak (20), who demonstrated that incubation of isolated rat liver mitochondria with uncouplers and ADP effected a release of intramitochondrial magnesium. In the experiments reported by Bogucka and Wojtczak (20) magnesium was released from the matrix and intermembrane compartments during incubation of rat liver mitochondria with uncoupler and ADP. That the mobilization or release of intramitochondrial magnesium was occurring in the present experiments using cardiac mitochondria is illustrated in Table I. As may be seen in Table I, both the addition of FCCP and FCCP plus ADP effected a release of magnesium from the mitochondrial pellet and an appearance of this magnesium in the supernatant after 4 min of incubation. This relationship may be illustrated by the difference in the ratio of the magnesium content of the pellet to that of the supernatant. This ratio decreased from a value of 1.15 to a value of 0.7 in the experiment to which uncoupler was added and a ratio of 0.6 obtained in the experiment containing uncoupler plus ADP. It was observed that the rate of pyruvate oxidation was not inhibited in the incubation to which uncoupler alone was added, presumably because there was a very low ATP

![Fig. 5](http://www.jbc.org/)

**Fig. 5.** The effect of varying amounts of ADP on the oxidation of pyruvate plus L-malate, and on the change in the ATP content of the mitochondrial incubation during the experiment. The reaction conditions were the same as those described in the legend for Fig. 1, with the exception that the ADP concentration was varied as noted in the figure. Samples were taken as indicated (S1 and S2) for ATP measurement.
level, i.e. between 2 and 3 nmol of ATP per mg of protein, even though sufficient magnesium was released by the action of the uncoupler. The ATP content of the experiment with uncoupler plus ADP was on the order of 200 to 300 nmol of ATP per mg of protein resulting in the inhibition of pyruvate oxidation. Hence, in uncoupled mitochondria in the absence of exogenous ADP, it is postulated that the ATP component of the protein kinase reaction was not present to effect an inhibition of pyruvate dehydrogenase.

Fig. 7 demonstrates that as the time of preliminary incubation with ADP and FCCP was increased from 0 to 4 min prior to the addition of the substrate-couple, pyruvate plus L-malate, the extent of the inhibition of pyruvate oxidation was markedly increased. Because of the inadequacy of rapid sampling and separation techniques, the kinetics of magnesium release during this period was not determined. Preliminary incubation of mitochondria with uncoupler alone prior to substrate addition resulted in no inhibition of pyruvate oxidation, presumably due to the virtual absence of sufficient ATP to cause the inhibitory effect.

That magnesium was an essential component of the inhibition of pyruvate oxidation in this isolated mitochondrial system may be noted in the data shown in Fig. 8. The addition of exogenous magnesium to uncoupled mitochondria supplemented with ADP considerably enhanced the inhibition of pyruvate oxidation. In other experiments (not shown) the addition of exogenous magnesium at the same concentrations shown in Fig. 8 to mitochondria incubated with either FCCP or ADP did not produce an inhibition of pyruvate oxidation. These observations were not unexpected in the case of the uncoupler plus magnesium addition, but the addition of magnesium in the presence of ADP and, as a result, significant concentrations of ATP, might have been expected to produce an inhibition of pyruvate dehydrogenase as both components of the protein kinase system would have been present.

Of crucial importance in the consideration of this proposed regulation of pyruvate dehydrogenase in isolated heart mitochondria is the substrate specificity of this effect. It has been shown that under conditions which mobilize or release intramitochondrial magnesium and which also contain a high ATP concentration, pyruvate oxidation was markedly inhibited after a brief lag phase (Figs. 3 and 4). As shown in Fig. 9, when two other substrate systems which have a reasonably high affinity for heart mitochondria were employed, i.e. L(-)-palmitylcarnitine or a-ketoglutarate, there was no apparent inhibition of the oxidation of these substrates. The rate of oxidation of either of these substrates was identical in the mitochondrial incubation with either uncoupler or uncoupler plus ADP. In the series of experiments shown in Fig. 9, the rates of oxidation of pyruvate, L(-)-palmitylcarnitine, and a-ketoglutarate in uncoupled mitochondria in the presence of L-malate were 209, 47, and 75 nmol of oxygen per min per mg of protein, respectively. When ADP was included in the incubation in the presence of the uncoupler, pyruvate oxidation was significantly inhibited (see Fig. 3), while the rate of oxidation of either L(-)-palmitylcarnitine or a-ketoglutarate was the same as was obtained in the absence of ADP. Furthermore, the addition of either L(-)-palmitylcarnitine or a-ketoglutarate following the establishment of nearly complete inhibition of pyruvate oxidation resulted in an uninhibited rate of oxidation of either of these additional substrates. These observations suggest that the inhibition of pyruvate oxidation under metabolic conditions leading to a mobilization of intramitochondrial magnesium and high ATP content was specific for the pyruvate oxidase system.

At this point it was considered essential to demonstrate that the observed inhibition of pyruvate oxidation in uncoupled, ADP-supplemented mitochondria was not due to the accumulation of either inhibitory product of the pyruvate dehydrogenase reaction, i.e. NADH or acetyl-CoA. Fig. 10 demonstrates that the acetyl-CoA level decreased from approximately 1.3 nmol to about 0.3 nmol of acetyl-CoA per mg of protein when either FCCP or FCCP plus ADP were added to mitochondria which were oxidizing pyruvate plus L-malate. The nearly identical, low acetyl-CoA levels in the inhibited and uninhibited incubations suggest that acetyl-CoA was not causing an inhibition of the pyruvate dehydrogenase reaction.

In a similar fashion, the experiments shown in Fig. 11 indicate that NADH accumulation did not occur in the experiment to which FCCP plus ADP were added to inhibit pyruvate oxida-
bodies on pyruvate oxidation in rat muscle may be mediated by fatty acids and ketone bodies on pyruvate oxidation in rat muscle may be mediated by fatty acids and ketone bodies. Garland (1) and Garland and Randle (2) have shown that pyruvate dehydrogenase from pig heart may be regulated by simple feedback inhibition by two of the products of the reaction, acetyl-CoA and NADH. These authors suggested that the inhibitory effects of fatty acids and ketone bodies on pyruvate oxidation in rat muscle may be mediated by increased levels of acetyl-CoA and NADH which obtain under these conditions.

Recently, Linn et al. (4, 5) have demonstrated that purified pyruvate dehydrogenase from a variety of tissues may be phosphorylated and nearly completely inhibited by a specific protein kinase. As is the case with most kinase reactions, this enzyme required ATP and magnesium for its phosphoregulatory activity. These authors also described a magnesium-dependent pyruvate dehydrogenase phosphatase capable of reactivating the inactive phosphoenzyme by cleaving the seryl phosphate. Wieland et al. (8) have confirmed this kinase-phosphatase-mediated inactivation-activation regulation of purified pyruvate dehydrogenase isolated from pig heart. Wieland and Sies (9) also suggested that the pyruvate dehydrogenase-linked phosphatase from pig heart was regulated by adenosine 3',5'-monophosphate cyclic sensitive protein kinase. Cyclic adenosine 3',5'-monophosphate effects on pyruvate dehydrogenase or its regulatory enzymes have not been confirmed by other workers (5, 22, 23).

FIG. 9 (left). A comparison of the rates of oxidation of L-(-)-palmitoylscarnitine and a-ketoglutarate in rabbit heart mitochondria treated with FCCP or FCCP plus ADP. The reaction conditions were the same as those described in the legend for Fig. 3 except that the substrates were L-(-)-palmitoylscarnitine (40 µM) plus L-malate (1.0 mM) and a-ketoglutarate (1.0 mM) plus L-malate (1.0 mM) as noted in the figure. The mitochondrial protein concentration for Experiments A and B was 0.32 mg per ml and for Experiments C and D was 0.42 mg per ml.

FIG. 10 (center). A comparison of the effect of either FCCP or FCCP plus ADP on the acetyl-CoA content of rabbit heart mitochondria oxidizing pyruvate plus L-malate. Reaction conditions were the same as those described in the legend for Fig. 3.

FIG. 11 (right). A comparison of the effect of FCCP, ADP, or ADP plus FCCP on the absorption of intramitochondrial reduced pyridine nucleotides in rabbit heart mitochondria oxidizing pyruvate plus L-malate. Reaction conditions were the same as those described in the legend for Fig. 3. Absorption measurements were made as described under "Materials and Methods."

FIG. 9 (center). A comparison of the rates of oxidation of L-(-)-palmitoylscarnitine and L-(-)-palmitoylscarnitine and a-ketoglutarate in rabbit heart mitochondria treated with FCCP or FCCP plus ADP. The reaction conditions were the same as those described in the legend for Fig. 3 except that the substrates were L-(-)-palmitoylscarnitine (40 µM) plus L-malate (1.0 mM) and a-ketoglutarate (1.0 mM) plus L-malate (1.0 mM) as noted in the figure. The mitochondrial protein concentration for Experiments A and B was 0.32 mg per ml and for Experiments C and D was 0.42 mg per ml.

**DISCUSSION**

Catabolic energy production as well as the biosynthetic capacities of various tissues depend upon the regulation of both production and utilization of acetyl-CoA. Primary consideration of the regulation of the fate of acetyl-CoA has centered on the regulation of the initial enzyme in the citric acid cycle, citrate synthase (21). By regulating this enzyme, acetyl units may enter the citric acid cycle for energy production, may be diverted into the ketogenic pathway, or may be directed into a biosynthetic pathways. Studies of the regulation of the production of acetyl-CoA have been concerned with (a) the transport and β oxidation of fatty acids, and (b) the oxidative decarboxylation of pyruvate which may arise from either the glycolytic pathway or from the deamination or transamination of various amino acids. The studies reported in this communication were concerned with the regulation of the pyruvate dehydrogenase reaction in mitochondrial systems.

Two mechanisms for the regulation of the pyruvate dehydrogenase multienzyme complex have been postulated largely on the basis of experiments using the purified enzyme from various sources. Garland (1) and Garland and Randle (2) have shown that pyruvate dehydrogenase from pig heart may be regulated by simple feedback inhibition by two of the products of the reaction, acetyl-CoA and NADH. These authors suggested that the inhibitory effects of fatty acids and ketone bodies on pyruvate oxidation in rat muscle may be mediated by...
also be characterized by a decreased free Mg²⁺ to bound Mg²⁺ ratio.

The studies reported in this communication were designed to demonstrate the presence of and to assess the effectiveness of an ATP-kinase-mediated regulation of pyruvate oxidation in isolated mitochondria. That the ATP level of the mitochondrion per se does not influence the rate of pyruvate oxidation was demonstrated in Figs. 1 and 2. However, using metabolic conditions leading to the release or mobilization of intramitochondrial magnesium (18-20), i.e. uncoupler plus ADP, nearly complete inhibition of pyruvate oxidation was effected (Fig. 3). It was shown that both the mobilization or release of intramitochondrial magnesium and the presence of ATP were essential to effect the inhibition of pyruvate oxidation. As will be shown in another paper the inhibition of the pyruvate dehydrogenase activity in this mitochondrial system can be prevented by adding magnesium chelators which presumably compete with the ATP for the free magnesium of the mitochondrion. In the absence of free magnesium even though high levels of ATP are present, the kinase is inactive and pyruvate oxidation occurs unimpeded. The stimulatory effect of chelator addition on pyruvate oxidation can be overcome by adding excess magnesium to the mitochondrial system. Exogenous magnesium accentuated the inhibitory effects on pyruvate oxidation only in the presence of both ADP and uncoupler. Added ATP gave much less inhibition than did the ATP produced presumably via the adenylate kinase reaction with the added ADP. That ADP was not the inhibitory species was shown by the fact that an inhibition of pyruvate oxidation was not observed using ADP and exogenous magnesium. The inhibitory effect of uncoupler plus ADP was shown to be atractyloside-sensitive. The inhibition of pyruvate oxidation in these experiments using cardiac mitochondria was specific for the substrate pyruvate as neither the oxidation of α-ketoglutarate nor β-(−)-palmitoylcarnitine was inhibited (see Fig. 9) under conditions leading to a complete inhibition of pyruvate oxidation. In addition, the inhibition of pyruvate oxidation in these experiments was shown not to be due to feedback inhibition of the pyruvate dehydrogenase by either NADH or acetyl-CoA as might be suggested by the work of Garland (1, 2) and Randle et al. (3).

The data presented in this paper suggest that the pyruvate dehydrogenase of isolated heart mitochondria exists in the active and presumably dephosphorylated form under most metabolic conditions. This may imply that the dehydrogenase phosphatase may be fully active while the kinase is relatively inactive under normal metabolic conditions. It is proposed that in the presence of elevated ATP levels and conditions which increase the availability of intramitochondrial magnesium, the kinase has access to its substrate, i.e. the Mg²⁺ ATP chelate, and an inactivation of the pyruvate dehydrogenase occurs. In addition, under high ATP conditions, magnesium is chelated by ATP making it unavailable for the activation of the dehydrogenase phosphatase. Hence, it is proposed that the primary regulator of pyruvate dehydrogenase in this mitochondrial system was the availability of the appropriate substrate for the dehydrogenase kinase. This proposal stands in contradiction to the suggestion of Wieland and Siess (9) that the natural form of the pyruvate dehydrogenase is the inactive, phosphorylated enzyme and that activation occurs upon making magnesium available to the dehydrogenase phosphatase. Magnesium availability to the mitochondrial system in the present studies seemed to accentuate the inactivation of the pyruvate oxidase function.

It may be appropriate to comment on the fact that these studies suggest that the “normal” state of intramitochondrial magnesium is not the ATP-magnesium complex. It has generally been assumed that most of the intramitochondrial magnesium occurs complexed to adenine nucleotides by virtue of the fact that intramitochondrial adenine nucleotide and magnesium levels are roughly equivalent. However, this notion has not been substantiated by experimental evidence other than this equivalence of the concentrations. We would predict that if intramitochondrial magnesium was normally chelated to ATP, pyruvate oxidation in our mitochondrial system would be inhibited. Uncouplers of oxidative phosphorylation have the ability to mobilize or release magnesium (Table 1). This effect could be due to the dissipation of intramitochondrial ATP, a strong chelator of magnesium, and the subsequent increase in ADP and AMP, weaker chelators of magnesium. This effect could also arise from an effect of uncouplers or low energy situations in the binding of magnesium to other species such as phospholipids or various proteins. This postulate is also not presently substantiated by experimental evidence but certainly deserves consideration. The fact remains that, when intramitochondrial magnesium was mobilized or released in the presence of ATP, pyruvate oxidation was strongly inhibited in our mitochondrial system.

It should be stressed that the experiments reported in this communication are consistent with but do not constitute absolute proof that the inhibition of mitochondrial pyruvate oxidation by ATP and magnesium occurs via a kinase-mediated phosphorylation of the pyruvate dehydrogenase multienzyme complex. Further studies are in progress to demonstrate that the pyruvate dehydrogenase complex is indeed in the inactivated, phosphorylated form under the conditions that pyruvate oxidation is totally inhibited in our mitochondrial experiments.

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