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 exhibited rapid rates of pyruvate oxidation but possessed 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 preincubation 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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The protein kinase capable of phosphorylating and inactivating purified pyruvate dehydrogenase requires ATP and magnesium for its activity (4, 5). For this reason, the rate of pyruvate oxidation in isolated mitochondria should be sensitive to alterations of the intramitochondrial concentrations of both ATP and magnesium. An apparent contradiction of this concept that the intramitochondrial ATP level alone may regulate the rate of pyruvate oxidation in isolated mitochondria derives from the fact that at least two metabolic conditions which vary markedly in their energetic state are compatible with maximal rates of ATP-dependent pyruvate oxidation in rabbit heart mitochondria (4, 5).

The abbreviation used is: FCCP, p-trifluoromethoxyphenylhydrazone of carbonyl cyanide.
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 increased from approximately 100 nmoles of ATP per mg of protein to 230 nmoles of ATP per mg of protein, which was between 50 and 70 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 (Curves 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, 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

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**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 ($S_1$ and $S_2$) for ATP measurement.
level, i.e. between 2 and 3 nmoles 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 nmoles 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. \( \alpha \)-ketoglutarate or \( \alpha \)-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, \( \alpha \)-ketoglutarate, and \( \alpha \)-ketoglutarate in uncoupled mitochondria in the presence of L-malate were 209, 47, and 75 nmoles 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 \( \alpha \)-ketoglutarate or \( \alpha \)-ketoglutarate was the same as was obtained in the absence of ADP. Furthermore, the addition of either \( \alpha \)-ketoglutarate or \( \alpha \)-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 nmoles to about 0.3 nmoles 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-
Two mechanisms for the regulation of the pyruvate dehydrogenase multi-enzyme 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 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 phosphorlysylative 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 an 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).

Relatively few data are presently available regarding the occurrence of the kinase-phosphatase regulation of pyruvate dehydrogenase in intact metabolic systems. In this regard, Wieland et al. (24) have postulated that the supply of long chain free fatty acids as well as ketone bodies may be responsible for an active-inactive transition of pyruvate dehydrogenase by an, as yet, unknown mechanism in perfused rat heart. Jungas (22), Weiss et al. (12), and Coore et al. (23) have reported substantial conversion of pyruvate dehydrogenase to the active form in adipose tissue upon administration of insulin. Thus far, studies performed using isolated pyruvate dehydrogenase might suggest that the pyruvate to acetyl-CoA conversion is inhibited under high energy conditions, i.e. high ATP. In addition, the pyruvate dehydrogenase reaction may be stimulated by the magnesium-activated phosphatase reaction. Also, since ATP is a potent chelator of magnesium, free intramitochondrial magnesium would be chelated upon elevation of the ATP level. The increase in ATP and subsequent decrease in free magnesium would have the effect of stimulating the kinase reaction and inhibiting the phosphatase reaction, and net effect would be a significant inactivation of pyruvate dehydrogenase. In this consideration an elevated energy charge as defined by Atkinson (25) would
also be characterized by a decreased free Mg\(^{++}\) to bound Mg\(^{++}\) 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 ADP 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 \(\alpha\)-ketoglutarate nor \(t\)-(\(-\))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 Randel 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 Mg\(^{++}\) 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 the 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 I). 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 on 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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