Control of Pyruvate Dehydrogenase Activity in Intact Cardiac Mitochondria

REGULATION OF THE INACTIVATION AND ACTIVATION OF THE DEHYDROGENASE

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SUMMARY

The control of pyruvate dehydrogenase activity by inactivation and activation was studied in intact mitochondria isolated from rabbit heart. Pyruvate dehydrogenase could be completely inactivated by incubating mitochondria with ATP, oligomycin, and NaF. This loss in dehydrogenase activity was correlated with the incorporation of $^{32}$P from [γ-$^{32}$P]ATP into mitochondrial protein(s) and with a decrease in the mitochondrial oxidation of pyruvate. ATP may be supplied exogenously, generated from endogenous ADP during oxidative phosphorylation, or formed from exogenous ADP in carbonyl cyanide p-trifluoromethoxyphenylhydrazone-uncoupled mitochondria. With coupled mitochondria the concentration of added ATP required to half-inactivate the dehydrogenase was 0.24 mM. With uncoupled mitochondria the apparent $K_a$ was decreased to 60 μM ATP. Inactivation of pyruvate dehydrogenase by exogenous ATP was sensitive to atracyloside, suggesting that pyruvate dehydrogenase kinase acts internally to the atracyloside-sensitive barrier. The divalent cation ionophore, A23187, enhanced the loss of dehydrogenase activity. Pyruvate dehydrogenase activity is regulated additionally by pyruvate, inorganic phosphate, and ADP. Pyruvate, in the presence of rotenone, strongly inhibited inactivation. This suggests that pyruvate facilitates its own oxidation and that increases in pyruvate dehydrogenase activity by substrate may provide a modulating influence on the utilization of pyruvate via the tricarboxylate cycle. Inorganic phosphate protected the dehydrogenase from inactivation by ATP. ADP added to the incubation mixture together with ATP inhibited the inactivation of pyruvate dehydrogenase. This protection may result from a direct action on pyruvate dehydrogenase kinase, as ADP competes with ATP, and an indirect action, in that ADP competes with ATP for the translocase. It is suggested that the intramitochondrial [ATP]:[ADP] ratio effects the kinase activity directly, whereas the cytosolic [ATP]:[ADP] ratio acts indirectly.

Mg$^{2+}$ enhances the rate of reactivation of the inactivated pyruvate dehydrogenase presumably by accelerating the rate of dephosphorylation of the enzyme. Maximal activation is obtained with the addition of 0.5 mM Mg$^{2+}$. Inasmuch as exogenous Mg$^{2+}$ is not needed for maximal inactivation of pyruvate dehydrogenase, these findings suggest that changes in the concentration of free Mg$^{2+}$ in the intact mitochondria may alter the relative activities of the phosphatase and the kinase and, consequently, may modify the activity of pyruvate dehydrogenase. Free Ca$^{2+}$ at physiological concentrations of $10^{-6}$ to $10^{-7}$ also increases the rate of reactivation of the inactivated dehydrogenase, suggesting that Ca$^{2+}$ may be a significant effectors of pyruvate dehydrogenase in cardiac mitochondria. Reactivation of the dehydrogenase was sensitive to A23187. In intact mitochondria A23187 may decrease dehydrogenase activity by inhibiting both ATPase and pyruvate dehydrogenase phosphatase activities. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone and inorganic phosphate increase the rate of reactivation. The actions of phosphate in simultaneously enhancing the reactivation and inhibiting the inactivation of the dehydrogenase may suggest a role for phosphate in increasing pyruvate dehydrogenase activity.

The activity of highly purified preparations of the multienzyme pyruvate dehydrogenase complex from mammalian tissues is regulated by enzymatic phosphorylation and dephosphorylation (1-5). Phosphorylation and concomitant inactivation are catalyzed by a MgATP$^-$-dependent kinase, and dephosphorylation and concomitant reactivation are catalyzed by a specific phosphatase. Studies in vivo and with isolated perfused organs indicate that active and inactive forms of the dehydrogenase are present in various tissues and that the ratio of the two forms depends on the nutritional and hormonal state of the animal (6-12). It was of importance to demonstrate that pyruvate dehydrogenase in isolated intact mitochondria undergoes comparable interconversions as shown for the purified enzyme complex. This has been reported recently for mitochondria from rat liver (13-15) and epididymal adipose tissue (16). In the present studies the control of pyruvate dehydrogenase activity in intact mitochondria isolated from the mammalian heart is demonstrated. Some of the regulatory factors and kinetic parameters...
in the inactivation and reactivation of pyruvate dehydrogenase are described. A preliminary account of part of this investigation has been reported (17).

**EXPERIMENTAL PROCEDURES**

Mitochondria were isolated from hearts of male, New Zealand white rabbits, weighing approximately 3 kg, by a modification of the procedure described by Chappell and Hansford (18). Each heart, approximately 5.5 g, was washed with 30 ml of ice-cold isolation medium comprised of 0.21 M mannitol, 0.07 M sucrose, 10 mM Hepes, and 1 mM EGTA, adjusted to pH 7.1 (MSHE medium). The heart was lightly chopped with a razor blade and washed again with 30 ml of the MSHE medium. The pieces of tissue were placed in a tube containing 0.5 ml of isolation medium comprised of 0.21 M mannitol, 0.07 M sucrose, 10 mM Hepes, 5 mM NAD, 5 mM FAD, and 10 mM MgCl₂, dissolved in 20 ml of isolation medium. After thoroughly mincing the heart with scissors for 5 min, the tissue was transferred to a glass homogenizer and the preparation was diluted to 50 ml with MSHE medium. The mixture was homogenized by five passes with a motor-driven Teflon pestle. The homogenate was incubated with the nagarse for an additional 10 min on the spectrophotometer to determine the rate of liberation of NADH. Intact mitochondria were incubated as will be described and at the times indicated a 40-μl aliquot of the incubation reaction was withdrawn and immediately placed in a cuvette containing a mixture of 10 nmol of rotenone in a volume of 160 μl and 4 μl of 50% Triton X-100. After 30 s of thorough mixing, 0.8 ml of the standard assay medium was added. The final concentrations of components in the assay medium were 50 mM Hepes, 1 mM MgCl₂, 0.2 mM thioridine phosphate, 0.13 mM CoA, 2.6 mM thiamine phosphate, 0.13 mM CaCl₂, 2.6 mM dithiothreitol, 2.5 mM NAD, 2 mM pyruvate, and 10 μM rotenone. The pH was 7.1 and the temperature was 30°C. Activity is expressed as micromoles of NADH per min and is based on the initial rate. With the use of the 40 μl of mitochondrial incubation mixture an A₅₅₀ of not more than 0.06/min was obtained. Control experiments demonstrated that neither lactate dehydrogenase nor NADH oxidase activity was detectable.

Measurements of the rate of inactivation of pyruvate dehydrogenase (i.e., pyruvate dehydrogenase kinase activity) were carried out as follows. Except as noted in the text, the intact mitochondria, approximately 1 mg of protein, were incubated at 30°C in a total volume of 0.5 ml of MSHE medium containing 0.5 mM ATP, 5 mM NaF, and 2 μg of oligomycin/mg of protein. At the indicated time, 40 μl of the incubation medium were transferred to the cuvette for assay of pyruvate dehydrogenase, as described above. The difference (i.e., decrease) in A₅₅₀/min between the zero time incubation sample and the incubated samples is a measure of kinase activity.

Measurements of the rate of reactivation of inactivated pyruvate dehydrogenase in the intact mitochondria were carried out at 30°C in incubation reactions as detailed in the text. At the indicated time, 40 μl of the incubation mixture were used for assay of pyruvate dehydrogenase activity. The difference (i.e., increase) in A₅₅₀/min between the zero time incubation sample and the incubated sample is a measure of phosphatase activity. To prepare the inactive dehydrogenase, the intact mitochondria, approximately 1 mg, were incubated with 0.5 mM ATP, 5 mM NaF, and 2 μg of oligomycin/mg of protein in buffered medium containing MSHE medium in a total volume of 0.5 ml. After approximately 30 min, at which time the mitochondrial pyruvate dehydrogenase was essentially completely inactivated, 10 ml of ice-cold MSHE medium were added to the incubation and the mixture was centrifuged at 8000 × g for 7 min. The mitochondrial pellet was resuspended in 2 ml of MSHE medium and centrifuged again at 8000 × g for 7 min. The resultant mitochondrial pellet was resuspended in MSHE medium and kept on ice.

In experiments to demonstrate the phosphorylation of mitochondrial protein(s) by γ-[32P]ATP concomitant with the inactivation of pyruvate dehydrogenase, the mitochondria were incubated in a total volume of 1 ml of MSHE medium containing 0.1 mM γ-[32P]ATP (32 μCi/mmol), 3 mM NaN₃, 5 μM FCCP, and oligomycin (2 μg/mg of protein). In stopping the reaction, a 0.1-ml aliquot of the incubation reaction was pipetted into an Eppendorf micro-centrifuge tube containing 0.2 ml of stopping reagent containing 0.1% trichloroacetic acid, 1 mM ATP, and 1 mM Pi. The tubes were centrifuged at 8000 × g for 3 min. The precipitated protein was dissolved in 0.2 ml of 1 N NaOH and heated at 40°C for 10 min. The protein was reprecipitated with the addition of 1 ml of 12% perchloric acid. The extract was adjusted to pH 6 with 0.2 ml of 0.5 N Mes and 2 N KOH. The precipitated potassium perchlorate was removed by centrifugation. Protein concentrations were estimated by the biuret reaction.

The divalent cation ionophore A23187 was a generous gift of Dr. David T. Wong of Eli Lilly and Co. Other reagents were of the highest commercially available grades.

**RESULTS**

When intact heart mitochondria were incubated with 0.5 mM ATP and oligomycin (2 μg/mg of protein) in MSHE medium containing 5 mM NaF, pyruvate dehydrogenase was completely inactivated in approximately 20 min (Fig. 1). Control incubations, i.e., in the absence of ATP and oligomycin or in the presence of both, but assay at zero time, showed no loss of activity. When oligomycin was omitted from the incubation reaction, the addition of 0.5 mM ATP effected a small transient decrease in pyruvate dehydrogenase activity, but the activity returned almost to the preincubation level on further incubation. When ATP was omitted, the addition of oligomycin caused a slow inactivation of the enzyme, reaching 50% in 20 min. Measurements of the endogenous ATP in these mitochondria, at zero time, indicated a content of approximately 2 μmol/g of mitochondrial protein. This suggests that when the level of ATP in the intact mitochondria was maintained by inhibition of ATPase, sufficient endogenous nucleotide was available to effect a decrease in pyruvate dehydrogenase activity.

Fig. 2 describes an experiment showing the relationship between the time courses of inactivation of pyruvate dehydrogenase in intact mitochondria and the incorporation of 32P from γ-[32P]ATP into mitochondrial protein(s). Dehydrogenase activity was minimal at the time that 32P incorporation was maximal. Although phosphorylation of mitochondrial protein(s) proceeded at a somewhat faster rate than did the inactivation of the dehydrogenase, these results provide evidence consistent with the

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1 The abbreviations used are: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; EGTA, ethanedioxybis(ethylamine)-tetraacetate; Mes, 2-(N-morpholino)-ethanesulfonic acid; MSHE medium, 0.21 M mannitol-0.07 M sucrose-10 mM Hepes-1 mM EGTA (adjusted to pH 7.1).
FIG. 1. The decrease in pyruvate dehydrogenase activity in intact heart mitochondria incubated with ATP and oligomycin. Mitochondria, 1 mg of protein, were incubated at 30° in 0.25 M mannitol, 0.07 M sucrose, 10 mM Hepes, and 1 mM EGTA (MSHE medium), pH 7.1, containing 5 mM NaF and 0.5 mM ATP and oligomycin (2 μg/mg of protein), where indicated, in a final volume of 0.5 ml. Control incubations were carried out in the absence of added ATP and oligomycin. Pyruvate dehydrogenase activity was measured as described in the text. The upper and lower portions of the figure describe experiments carried out with different mitochondrial preparations on different days.

FIG. 2. The relationship between the time courses of the decrease in pyruvate dehydrogenase activity in intact heart mitochondria and the incorporation of 32P from [γ-32P]ATP into mitochondrial protein. Incubation mixture contained mitochondria, 6 mg of protein, MSHE medium, 5 μM FCCP, 0.1 mM [γ-32P]ATP (32 μCi/mmol), and oligomycin (2 μg/mg of protein) in a final volume of 1 ml. Incorporation of 32P and pyruvate dehydrogenase activity were estimated as described in the text. The pyruvate dehydrogenase activity of the mitochondria at zero time incubation was considered as 100% maximal.

view, but not proof, that the decrease in pyruvate dehydrogenase activity was mediated by the action of pyruvate dehydrogenase kinase. The possibility that acid-precipitable mitochondrial constituents other than pyruvate dehydrogenase were also phosphorylated during the incubation was not ruled out.

As shown in Fig. 3, the inactivation of pyruvate dehydrogenase was concomitant with a decrease in the rate at which the mitochondria oxidized pyruvate. Mitochondria, 1.5 mg of protein, were preincubated for 5 min at 30° in 0.25 ml of MSHE medium containing 1 mM ATP and 5 μg of oligomycin, as indicated. At the end of the preincubation period, pyruvate dehydrogenase activities (PDH) in aliquots were determined as described in the text and the relative activities are shown. Other 0.2-ml aliquots of the mixtures were taken at the end of the preincubation period and the respiratory rates of the mitochondria (RHM) were measured with an oxygen electrode at 30°. The respiratory medium contained MSHE medium, 5 mM pyruvate, 0.1 mM malate, 5 mM phosphate, 5 mM NaF, and 0.5% bovine serum albumin in a final volume of 2 ml. Respiration was initiated with the addition of 1 μM FCCP.

5 min in MSHE medium containing either ATP + oligomycin or oligomycin without nucleotide, and then aliquots were taken for measurements of pyruvate dehydrogenase activity and respiratory activity with 5 mM pyruvate + 0.1 mM malate as substrate. In a control experiment, in which the mitochondria were preincubated in only MSHE medium, pyruvate dehydrogenase was considered fully active (100%) and the FCCP uncoupled rate of oxidation was 206 ng atoms of oxygen/min. The pyruvate dehydrogenase activity of mitochondria preincubated with ATP and oligomycin was decreased to 20% of the control value. As shown by the oxygen electrode traces the respiratory rate of these mitochondria was markedly reduced; initially the rate was 125 ng atoms of oxygen/min which decreased further to 83, 3 min after starting respiration with FCCP. Mitochondria preincubated with oligomycin, but no ATP, had a pyruvate dehydrogenase activity 90% that of the control level. The rate of respiration of these mitochondria was lowered to 167 and 104 ng atoms of oxygen/min, initially and 3 min later, respectively. It is apparent from these results that the relationship between inhibition of pyruvate dehydrogenase activity and decrease in pyruvate oxidation was not in quantitative agreement. This, however, could be due to the
Fig. 4. Pyruvate dehydrogenase kinase activity in intact mitochondria as a function of the concentration of ATP, in the presence and absence of FCCP. The incubation medium was the same as described in Fig. 1. Mitochondrial protein was 1.2 mg and 2 μg of oligomycin/mg of protein were used. The incubation periods were 5 min when FCCP was present and 10 min when FCCP was omitted. The low rates obtained with oligomycin alone, in the absence of added ATP, were subtracted from the values with ATP. Pyruvate dehydrogenase kinase activity was estimated by the decrease in pyruvate dehydrogenase activity between the zero time incubation sample and the incubated sample. Maximal kinase activity was attained when the pyruvate dehydrogenase activity of the incubated sample was not detectable.

Fig. 5 (left). The rate of the decrease in pyruvate dehydrogenase activity in intact mitochondria by ATP and oligomycin in the presence and absence of uncoupling agent. Incubation medium was the same as described in Fig. 1. Mitochondrial protein was 1.2 mg. The ATP concentration was 0.5 mM and the content of oligomycin was 2 μg/mg of protein.

Fig. 6 (right). The decrease in pyruvate dehydrogenase activity in intact mitochondria with higher concentrations of ATP, in the absence of oligomycin and NaF. The concentrations of added ATP were as indicated. Other components of the incubation medium were as described in Fig. 1. Mitochondrial protein (1.1 mg) was used.

The rate of inactivation of pyruvate dehydrogenase with 0.5 mM ATP, in the presence of oligomycin, was enhanced by FCCP. As shown in Fig. 5, the rate of inactivation, when measured at 5 min, was accelerated 3-fold by 5 μM FCCP. The uncoupler seemingly markedly increased the affinity of the system for ATP (Fig. 4). The apparent K_m (S_0.5) was lowered to 60 μM and Michaelis-Menten type kinetics were evident. This effect of FCCP might be attributed, in part, to a collapse of the mitochondrial membrane potential, facilitating the movement of ATP into the mitochondria (21). FCCP also might mobilize intramitochondrial-bound Mg^2+ (22, 23), perhaps making the metal more accessible to the pyruvate dehydrogenase kinase. Interestingly, the apparent K_m value of 60 μM with intact mitochondria, in the presence of FCCP, oligomycin, and NaF, approached the value of 20 μM obtained with highly purified preparations of the pyruvate dehydrogenase complex from bovine heart and kidney (5).

As noted above, i.e. Fig. 1, when oligomycin was omitted from the incubation medium, the addition of 0.5 mM ATP caused a small transient inactivation of pyruvate dehydrogenase. Substantial inactivations of the enzyme could be achieved in the absence of the ATPase inhibitor, however, with higher concentrations of added nucleotide. Fig. 6 shows that 6 mM ATP decreased the activity of the dehydrogenase about 80%. With ATP added to 2 mM, inhibition was less pronounced and evidence for reactivation was seen after 15 min.

Pyruvate dehydrogenase was also inactivated by ATP generated from endogenous ADP during oxidative phosphorylation. As shown in Fig. 7, the activity of the enzyme was decreased 75% when measured 5 min after the mitochondria were incubated with succinate, in the presence of 10 μM rotenone. Further incubation to 20 min did not change the level of inactivation. Parallel experiments indicated that the ATP content of these mitochondria increased from 1.8 to 3.2 μmol/g of mitochondrial protein, reaching the maximal value at 1 min. Generation of additional ATP, thus further inactivation of pyruvate dehydrogenase, was precluded probably because the endogenous ADP was completely phosphorylated. NaF, an inhibitor of the phosphorylated pyruvate dehydrogenase phosphatase (5), was present in the incubation medium to prevent reactivation of the dehydrogenase. When
NaF was omitted from the incubation medium (Fig. 7), hence the phosphatase was presumably functional during the course of the incubation, at about 10 min reactivation of the inactivated dehydrogenase by the phosphatase became dominant relative to the inactivation of the enzyme by the kinase. At this time, however, oxidative phosphorylation should have been minimal because of depletion of ADP and oxygen.

Pyruvate inhibited the inactivation of pyruvate dehydrogenase in intact heart mitochondria. Fig. 8 describes the effect of different concentrations of pyruvate in the incubation medium on the rate of inactivation. In these experiments rotenone was added to the incubation to block oxidation of the pyruvate. A concentration of 1 mM pyruvate inhibited the decrease in dehydrogenase activity approximately 80% and half-maximal inhibition was found at 0.4 mM. These findings are in accord with the report that pyruvate was an inhibitor of the partially purified kinase, being noncompetitive with respect to ATP and oxygen.

As shown in Fig. 9, inorganic phosphate protected the pyruvate dehydrogenase in intact mitochondria from inactivation. A concentration of 1 mM was completely effective in blocking decreases in dehydrogenase activity. Fig. 9 (lower traces) also shows that inhibition of the kinase was noncompetitive with respect to ATP. In contrast, Wieland et al. (24) described the inhibition of purified kinase by inorganic pyrophosphate but reported that the inhibitory effect of the pyrophosphate was abolished after hydrolysis of the pyrophosphate with pyrophosphatase. It should be noted, however, that the concentration of inorganic phosphate used in the present experiments was considerably greater than the concentration of pyrophosphate used in the studies with the purified soluble preparations.

ADP inhibited the inactivation of pyruvate dehydrogenase in intact coupled mitochondria. As illustrated in Fig. 10, approximately 50% of the dehydrogenase activity was retained with 0.1 mM ADP, in the presence of 0.5 mM ATP. The loss in dehydrogenase activity was prevented completely with 0.8 mM ADP. This protection of the dehydrogenase by ADP could be explained, in part, by the ADP inhibition, competitive with respect to ATP, of the kinase, as described previously with purified preparations of the enzyme (2, 5). In the intact mitochondrial system, as used in the present experiments, however, ADP could also prevent the inactivation of the dehydrogenase by being translocated, preferentially relative to ATP, into the mitochondria (25).

Although ADP in the presence of added ATP prevented the decrease in pyruvate dehydrogenase activity in coupled mitochondria, ADP had a different effect in mitochondria uncoupled with FCCP and in the absence of exogenous ATP. With these conditions inactivation increased with increases in added ADP (Fig. 11). This effect was dependent on the presence of FCCP because pyruvate dehydrogenase was not inactivated by ADP in the absence of uncoupler. Parallel experiments measuring ATP in the reaction when mitochondria were incubated at 30°C in MSHE media with 2 mM ADP, 4 #G FCCP, and oligomycin (4 nM) revealed that ATP had increased from 2.3 #mol/g of mitochondrial protein at zero time to 10.2 and 16.1 #mol/g of mitochondrial protein 30 s and 2 min later, respectively. Thus, pyruvate dehydrogenase inactivation could be correlated with increased ATP production. An inhibition of pyruvate oxidation in heart mitochondria incubated with ADP and FCCP was reported by Schuster and Olson (26). They related the inhibition to increases in ATP, presumably generated by adenylate kinase which had become activated by endogenous Mg²⁺ made available by the action of the uncoupler. In the present study, perhaps a similar mechanism accounted for the increase in ATP and, consequently, a decrease in pyruvate dehydrogenase activity. Some ATP could also be synthesized at the substrate level, concomitant with the oxidation of α-ketoglutarate found endogenously or formed from endogenous precursors during incubations.

The rate of inactivation of pyruvate dehydrogenase measured...
Kinase utilizing endogenous ATP would be insensitive to atractyloside. However, a concentration of A23187 as low as 0.2 μM brought about maximal inactivation. In the absence of ATP, pyruvate dehydrogenase activity was decreased approximately 50% by 50°C (MgATP) and 5 mM NaF, oligomycin (2 μg/mg of protein) were used in all experiments. Incubation media and conditions were as described in Fig. 1. ATP (0.5 mM) and oligomycin (2 μg/mg of protein) were present. Pyruvate dehydrogenase kinase activity was estimated by the decrease in pyruvate dehydrogenase activity between the zero time incubation sample and the incubated sample. Activity is expressed as units of pyruvate dehydrogenase activity per mg of mitochondrial protein after an incubation period of 5 min.

Fig. 11 (right). The increase in pyruvate dehydrogenase kinase activity in intact mitochondria by ADP in the presence of FCCP. Incubation media and conditions were as described in Fig. 1. ATP (0.5 mM) and NaF were omitted. Two micromolar FCCP and oligomycin (2 μg/mg of protein) were present. Mitochondrial protein was 1.2 mg. Pyruvate dehydrogenase kinase activity was estimated by the decrease in pyruvate dehydrogenase activity between the zero time incubation sample and the incubated sample. Activity is expressed as units of pyruvate dehydrogenase activity per mg of mitochondrial protein after an incubation period of 5 min.

Table I

| Atractyloside (μM) | Pyruvate dehydrogenase kinase activity (units/mg/5 min) |
|-------------------|--------------------------------------------------------|
| 0                 | 0.079                                                  |
| 10                | 0.051                                                  |
| 50                | 0.027                                                  |
| 100               | 0.033                                                  |

Incubation reactions contained MSHE medium, 0.5 mM ATP, 5 mM NaF, oligomycin (5 μg/mg of protein), 1 mg of mitochondrial protein, and atractyloside as indicated in a final volume of 0.5 mL. Pyruvate dehydrogenase kinase activity was estimated by the decrease in pyruvate dehydrogenase activity between the zero time incubation sample and the incubated sample. Activity is expressed as units of pyruvate dehydrogenase activity per mg of mitochondrial protein after an incubation period of 5 min.

In the presence of added 0.5 mM ATP, oligomycin, and NaF was sensitive to atractyloside, an inhibitor of the adenine nucleotide translocase (27). As shown in Table I, pyruvate dehydrogenase kinase activity was decreased approximately 50% by 50 μM atractyloside. This effect could be attributed to the inhibition of the transport of ATP into the mitochondria, perhaps suggesting that the kinase acted internal to the atractyloside-sensitive carrier. Higher concentrations of atractyloside had little additional effect on the loss of pyruvate dehydrogenase activity. However, kinase utilizing endogenous ATP would be insensitive to atractyloside.

The activity of pyruvate dehydrogenase in intact mitochondria was also decreased when the divalent cation ionophore, A23187, an uncoupler of oxidative phosphorylation and inhibitor of ATPase (28, 29) was added to the incubation mixture together with 0.5 mM ATP (Fig. 12). A concentration of A23187 as low as 0.2 μM brought about maximal inactivation. In the absence of ATP, the ionophore, in contrast to oligomycin, did not effect a significant reduction in dehydrogenase activity. Oligomycin added in conjunction with A23187 and ATP enhanced the rate of inactivation. Inasmuch as mitochondria incubated in the presence of A23187 and EGTA (in the MSHE medium) would be depleted of endogenous Mg2+ and Ca2+ (28), the action of the ionophore in increasing the loss of pyruvate dehydrogenase activity could be explained in part by the prevention of the hydrolysis of ATP by the Mg2+-requiring ATPase. However, also contributing to the mechanism by which A23187 decreased the dehydrogenase activity could be the action of the ionophore on pyruvate dehydrogenase phosphatase. As reported below, the phosphatase, which converts the inactive dehydrogenase to its active form in these mitochondrial preparations, was extremely sensitive to Mg2+ and Ca2+. Thus, a leakage of these divalent cations from the mitochondria would inhibit the phosphatase and tend to facilitate the inactivation of pyruvate dehydrogenase by the kinase.

After conversion of the pyruvate dehydrogenase in intact cardiac mitochondria to the inactive form, the enzyme remained inactive for several hours, provided the mitochondria were kept in an ice bath and EGTA (in the MSHE medium) was present. Although kept cold, the inactivated dehydrogenase slowly converted to the active form in the absence of EGTA. When the mitochondria were incubated in MSHE medium at 30°C, the inactivated dehydrogenase was completely reactivated. As shown in Fig. 13, the activity of the dehydrogenase returned to its original level prior to inactivation in approximately 40 min. Some variation in the time required for complete reactivation was noted in different mitochondrial preparations. Fig. 13 also shows that the addition of Mg2+ to the incubation medium accelerated the rate of reactivation whereas the addition of EDTA decreased the rate. Reactivation, and presumably dephosphorylation, was effectively blocked by 10 mM NaF, a known inhibitor of the purified phosphatase (5).

The relationship between the concentration of added Mg2+ and the reactivation of pyruvate dehydrogenase in cardiac mitochondria is illustrated in Fig. 14. Complete reactivation was found with 0.5 mM added Mg2+, in a 20 min incubation. The need for
FIG. 13. The time-course of reactivation of pyruvate dehydrogenase in intact heart mitochondria. The dehydrogenase in mitochondria, approximately 10 mg of protein, was inactivated as described in the text and the mitochondria were resuspended in 4 ml of MSHE medium. The suspension (0.25 ml) was mixed with 0.25 ml of medium containing the final concentrations of Mg²⁺, EDTA, or NaF, as indicated. At the times indicated, an aliquot (40 µl) was assayed for pyruvate dehydrogenase, as reported in the text. The original activity of the dehydrogenase, prior to inactivation, is indicated by the dashed line.

FIG. 14. The effect of the concentration of added Mg²⁺ on the reactivation of pyruvate dehydrogenase in intact heart mitochondria. The experiments were carried out as described in Fig. 13. Only 0.5 mM added Mg²⁺ for maximal activity with the intact mitochondrial system contrasted with the requirement of approximately 10 mM Mg²⁺ for the solubilized purified pyruvate dehydrogenase phosphatase (5, 7, 30). As shown in Fig. 14, however, the inactive pyruvate dehydrogenase in these mitochondria was partially reactivated in the 20-min incubation period in the absence of added Mg²⁺, as was evident also from the findings described in Fig. 13. This may suggest the presence of endogenous Mg²⁺ in the mitochondria, as isolated, in the environment of the phosphatase.

In addition to Mg²⁺, Ca²⁺ markedly enhanced the rate of the reactivation of the inactivated dehydrogenase in intact mitochondria. As shown in Fig. 15, a free Ca²⁺ concentration of 10⁻⁶ M increased pyruvate dehydrogenase activity, approximately 4- to 5-fold in a 10-min incubation, compared to an incubation carried out in the presence of EGTA (Ca²⁺ < 10⁻⁸ M). Using Ca²⁺-EGTA buffers prepared according to Portzehl et al. (31), a physiological concentration of free Ca²⁺ in the range of 10⁻⁶ to 10⁻⁷ was found to be effective in reactivating the inactive dehydrogenase in cardiac mitochondria.

The reactivation of dehydrogenase was sensitive to the ionophore A23187. Table II shows that 0.1 µM A23187 strongly inhibited the rate of reactivation. At an ionophore concentration of 0.2 µM, reactivation was minimal, less than 20% of the control value after an incubation period of 40 min. This inhibition was probably due to the depletion from the mitochondria of endogenous Mg²⁺ and Ca²⁺. Table II also shows that A23187 completely prevented the increased rate of reactivation induced by added Ca²⁺ and Mg²⁺. Conversely, neither added Ca²⁺ nor Mg²⁺ reversed the inhibition effected by the ionophore.

Inasmuch as it was shown above that FCCP increased the rate of inactivation of pyruvate dehydrogenase, whereas pyruvate and inorganic phosphate decreased the rate and, presumably, both kinase and phosphatase are present in these mitochondria and the rates of interconversion of phosphorylated and dephosphorylated forms of pyruvate dehydrogenase depend on the relative activities of the kinase and phosphatase reactions, each

![Graph](https://via.placeholder.com/150)

**Effect of ionophore A23187 on rate of reactivation of pyruvate dehydrogenase in intact heart mitochondria**

The inactivation and reactivation of pyruvate dehydrogenase were carried out as described in the text. After inactivation of the dehydrogenase, the mitochondrial suspension was mixed with an equal volume of MSHE medium containing the additions as indicated. At the times indicated, an aliquot (40 µl) was assayed for pyruvate dehydrogenase, as reported in the text. The concentration of free Ca²⁺ was obtained with Ca²⁺-EGTA buffer. The activity of the dehydrogenase prior to inactivation is considered 100%.

**Table II**

| Pyruvate dehydrogenase activity | Time | Control | A23187 (0.1 µM) | A23187 (0.2 µM) | Ca²⁺ (10⁻⁶ M) | 8 X 10⁻⁷ M Ca²⁺ + | 0.2 mM Mg²⁺ + | 0.2 mM Ca²⁺ + Mg²⁺ |
|--------------------------------|------|---------|----------------|----------------|---------------|-----------------|----------------|------------------|
| min                            |      |         | A23187         | A23187         | Ca²⁺          | Ca²⁺ + 8 X 10⁻⁷ M | Ca²⁺ + 0.2 mM Mg²⁺ | Ca²⁺ + 0.2 mM Ca²⁺ + Mg²⁺ |
| 0                              | 2    | 2       | 2              | 3              | 31            | 16              | 16              | 0                |
| 10                             | 6    | 9       | 3              | 31             | 94            | 16              | 16              | 2                |
| 20                             | 93   | 13      | 9              | 99             | 26            | 10              | 10              | 3                |
| 30                             | 93   | 20      | 9              | 99             | 26            | 10              | 10              | 3                |
| 40                             | 96   | 41      | 18             | 100            | 30            | 17              | 17              | 17               |

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Fig. 16 (left). The increase in the rate of reactivation of pyruvate dehydrogenase in intact heart mitochondria by FCCP. Experimental conditions were as described in Fig. 13. The effect of pyruvate on the reactivation of pyruvate dehydrogenase activity in intact cardiac mitochondria. The experiments were carried out as described in Fig. 13. In the incubations (C), the mitochondrial suspension was mixed with an equal volume of MSHE medium containing the final concentrations of 2 mM pyruvate and 10 μM rotenone. The control incubations (●) contained MSHE medium plus the rotenone.

Fig. 18. The effect of inorganic phosphate on the reactivation of pyruvate dehydrogenase in intact heart mitochondria. The experiments were carried out as described in Fig. 13. The activity of the dehydrogenase prior to inactivation is considered 100%. In the inset, activities were measured after an incubation period of 10 min.

effector was tested for its potential effect on the reactivation of the inactivated dehydrogenase. As shown in Fig. 16, FCCP accelerated the rate of reactivation of pyruvate dehydrogenase in the intact mitochondria. At 5 μM FCCP, the dehydrogenase activity was enhanced approximately 5 fold in a 20 min incubation, compared to an incubation without uncoupler. This increase in reactivation may reflect the FCCP-induced mobilization of membrane-bound Mg²⁺ and Ca²⁺ (22, 23, 26, 32), perhaps making the divalent cations more accessible to the phosphatase. Pyruvate, a noncompetitive inhibitor of the kinase (5), had no effect on the rate of reactivation of pyruvate dehydrogenase (Fig. 17). In contrast, inorganic phosphate, also a noncompetitive inhibitor of the inactivation of pyruvate dehydrogenase, increased reactivation. Fig. 18 shows that a concentration of 0.2 mM inorganic phosphate accelerated the rate severalfold and that the dehydrogenase in the intact mitochondria was completely reactivated by 1 mM inorganic phosphate within 10 min.

**DISCUSSION**

The present results demonstrate that pyruvate dehydrogenase in isolated intact cardiac mitochondria is inactivated by ATP, and this suggests the action of pyruvate dehydrogenase kinase in phosphorylating the dehydrogenase (5). Concomitant with this decrease in dehydrogenase activity is a decrease in the mitochondrial oxidation of pyruvate via the tricarboxylate cycle.

Pyruvate dehydrogenase in cardiac mitochondria is converted to an inactive form when sufficient ATP is available. Previously, an inverse relationship between pyruvate dehydrogenase activity and ATP content in rat heart mitochondria was observed (10). It is evident from the present study that ATP may be supplied exogenously or generated endogenously during oxidative phosphorylation. Moreover, the content of the adenine nucleotide in mitochondria, as isolated, is adequate to effect a decrease in pyruvate dehydrogenase activity, provided the level is maintained, e.g., by low ATPase activity. In coupled mitochondria the concentration of ATP required to half-inactivate the dehydrogenase is 0.24 mM, a value considerably less than the concentration of ATP in heart mitochondria. In FCCP-uncoupled mitochondria the apparent Kₐ is decreased to 60 μM, approaching the value obtained for the solubilized highly purified kinase (5). Possible explanations for the lowered apparent Kₐ in uncoupled mitochondria include the facilitation of ATP entrance into the mitochondria when the membrane potential is dissipated and the release of membrane-bound Mg²⁺ making the divalent cation accessible to the kinase. On the other hand, the lowering of the apparent Kₐ for ATP by FCCP is not due to inhibition of pyruvate dehydrogenase phosphatase, because the reactivation of the inactive dehydrogenase in these mitochondria is accelerated by uncoupler. Nor is the value of 60 μM a reflection of the apparent Kₐ of the adenine nucleotide translocase, which is approximately 1 μM in the presence of FCCP (25). Seemingly in contrast to the results described here, Schuster and Olson (33) reported that the combination of added ATP and FCCP did not inhibit pyruvate oxidation in heart mitochondria. It should be noted, however, that neither oligomycin nor NaF was included in their reaction mixtures, contrary to the present experiments. Our finding that the inactivation of pyruvate dehydrogenase in intact mitochondria by exogenous ATP was sensitive to atractyloside, an inhibitor of the translocase, suggests that pyruvate dehydrogenase kinase acts internal to the atractyloside-sensitive barrier. Recently, Berger and Hommes (14) observed that added ATP decreased pyruvate oxidation by liver mitochondria and that this decrease was not as marked in the presence of atractyloside. This finding is in accord with the suggested localization of the kinase.

The present studies on the regulation of the expression of pyruvate dehydrogenase activity in intact mitochondria indicate control by ATP, ADP, pyruvate, inorganic phosphate, and divalent cations. ADP, added to the incubation mixture together with ATP, inhibited the inactivation of pyruvate dehydrogenase. This finding is consistent with the previously described inhibition by ADP of the purified enzyme complex system (2, 5) and with the report that ADP, in the absence of exogenous respiratory substrate and ATP, stimulate formation of the active form of pyruvate dehydrogenase in isolated liver mitochondria (13). This protection of the dehydrogenase in intact cardiac mitochondria by ADP may result from both a direct effect on the kinase, in that ADP competes with ATP (2, 5), and an indirect effect, in that ADP competes with ATP at the site of translocation (25). The observations that the decrease in pyruvate dehydrogenase activity is highly sensitive to the ATP generated from endogenous ADP in coupled mitochondria, somewhat less sensitive to the ATP produced from exogenous ADP, presumably by adenylate kinase, in uncoupled mitochondria, plus the finding that inactivation of the dehydrogenase by added ATP is but partially inhibited by atractyloside may suggest that it is the intramito-
chondrial [ATP]:[ADP] ratio which effects directly pyruvate dehydrogenase kinase activity, whereas the cytosolic (ATP):[ADP] ratio acts indirectly. Martin et al. (16) pointed out, however, that a decreased mitochondrial (ATP):[ADP] ratio also may enhance pyruvate dehydrogenase activity by activating pyruvate dehydrogenase phosphatase with Mg\(^{2+}\) and Ca\(^{2+}\). The concentrations of free divalent cations would tend to increase because the affinities of ADP for these ions are less than those of ATP.

Pyruvate, in the presence of rotenone to block its oxidation, strongly inhibited the inactivation of pyruvate dehydrogenase in intact heart mitochondria. The half-maximal effect was found with 0.4 mM. This finding is in accord with the action of pyruvate on the purined soluble kinase (5) and with a reported increase in pyruvate dehydrogenase activity during State 4 oxidation of pyruvate by liver mitochondria (13). This suggests that pyruvate facilitates its own oxidation. Thus, increases in pyruvate dehydrogenase activity by substrate may provide a modulating influence on the utilization of pyruvate via the tricarboxylic cycle.

Inorganic phosphate also prevented the decrease in pyruvate dehydrogenase activity during incubation with ATP. Although it was also found that phosphate accelerated the reactivation of the inactivated pyruvate dehydrogenase, this cannot explain the action of phosphate in preventing the inactivation of the dehydrogenase because NaF was added to the incubations to block the presumed dephosphorylation of the dehydrogenase. Although the mechanisms by which inorganic phosphate exerts its effects remain to be clarified, its action in inhibiting the inactivation and simultaneously enhancing the reactivation may suggest a possible role in increasing pyruvate dehydrogenase activity.

The divalent cations, Mg\(^{2+}\) and Ca\(^{2+}\), have profound effects on the regulation of pyruvate dehydrogenase activity in intact cardiac mitochondria. The cations accelerate the rate of reactivation of the inactivated enzyme, presumably by enhancing the rate of dephosphorylation of the phosphorylated form of the dehydrogenase. Addition of 0.5 mM Mg\(^{2+}\) maximally activates the dehydrogenase, when measured after an incubation period of 20 min. This relatively low concentration of added Mg\(^{2+}\) with the intact mitochondrial system contrasts with the requirement for 10 mM Mg\(^{2+}\) for maximal activation of the isolated pyruvate dehydrogenase phosphatase (5, 30). However, some reactivation of the dehydrogenase in intact mitochondria is found in the absence of added Mg\(^{2+}\) and this suggests the presence of considerable endogenous Mg\(^{2+}\), even when the incubation is carried out with EDTA in the medium. An alternative explanation for the difference, that the isolated phosphatase may have been modified during solubilization and purification to increase its requirement for Mg\(^{2+}\), has not been ruled out, however. It should be noted also that added Mg\(^{2+}\) is needed for maximal reactivation of the dehydrogenase, whereas the addition of exogenous Mg\(^{2+}\) is not necessary for maximal inactivation of the dehydrogenase by the kinase in these intact heart mitochondria. These findings are in accord with observations with the purified enzymes that the phosphatase requires a higher concentration of Mg\(^{2+}\) for optimal activity than does the kinase (5, 30). Hence, changes in the concentration of free Mg\(^{2+}\) in the mitochondria may alter the relative activities of the phosphatase and the kinase and, hence, modify the activity of pyruvate dehydrogenase.

The finding that free Ca\(^{2+}\) at the physiological concentration of 10\(^{-4}\) to 10\(^{-7}\) increases the rate of reactivation of the phosphorylated dehydrogenase in intact heart mitochondria can be related to the observation that Ca\(^{2+}\) facilitates the binding of the phosphatase to the pyruvate dehydrogenase complex (34). The present finding may be of added significance in that it suggeststhat the activity of pyruvate dehydrogenase in intact mitochondria also can be controlled by changes in the intramitochondrial concentration of free Ca\(^{2+}\). Alterations in the concentration of free Ca\(^{2+}\) may occur with changes in the rates of uptake and release of the divalent cation as well as with changes in the ratio of [ATP]:[ADP], the concentration of free Ca\(^{2+}\) tending to increase by less chelation as the ratio decreases (35).

The present study also shows that FCCP accelerates the rate of reactivation of pyruvate dehydrogenase in isolated heart mitochondria. The mode of action of the uncoupler in this intact system is probably complex. Walajtys et al. (15) recently have reported that in rat liver mitochondria FCCP causes the loss of mitochondrial adenine nucleotides, decreases the intramitochondrial ATP:ADP ratio, and increases the percentage of pyruvate dehydrogenase in the active form. In contrast, the uncoupler also depletes the mitochondria of Ca\(^{2+}\) (15) and mobilizes mitochondrial membrane-bound Mg\(^{2+}\) (29, 23, 26). Indeed, in the presence of exogenous ATP or ADP plus oligomycin, the rate of inactivation of pyruvate dehydrogenase by the kinase is enhanced by FCCP. This effect has been attributed in part to the facilitation of ATP entry and perhaps to the making of Mg\(^{2+}\) more accessible to the kinase relative to the phosphatase. Therefore, observations on the action of FCCP are difficult to interpret unless experimental conditions are strictly defined and concentrations of the different effectors in the extramitochondrial as well as the various intramitochondrial compartments are known.

These studies, on the kinetics of the inactivation of pyruvate dehydrogenase in intact cardiac mitochondria and on the actions of several effectors on the reactivation of the enzyme, provide insight into how pyruvate dehydrogenase activity may be regulated in a more physiologically intact mitochondrial system as found in the mammalian heart.

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