Effect of Magnesium Chelators on the Regulation of Pyruvate Oxidation by Rabbit Heart Mitochondria†

Sheldon M. Schuster and Merle S. Olson*

ABSTRACT: The regulation of pyruvate oxidation in isolated rabbit heart mitochondria was investigated. A comparison of the effects of adding ATP, ADP, and AMP to uncoupled mitochondria oxidizing pyruvate plus L-malate indicated that ADP addition nearly completely inhibited pyruvate oxidation while ATP or AMP addition had little or no inhibitory effect. It was demonstrated that the availability of both ATP formed from the added ADP and intramitochondrial magnesium were critical components for the inhibition of pyruvate oxidation in this system. The inclusion of magnesium chelators in the mitochondrial incubations containing uncoupler and ADP resulted in prevention of the inhibition of pyruvate oxidation. It is proposed that the effect of magnesium chelators was due to a competition for free magnesium with the pyruvate dehydrogenase linked protein kinase which, in the presence of sufficient ATP and magnesium, results in phosphorylation and inactivation of the pyruvate oxidase complex of the mitochondrion. Nonchelating structural analogs of these chelator compounds or compounds which chelate divalent metals other than magnesium were found to be without effect for preventing the inhibition of mitochondrial pyruvate oxidation. The addition of magnesium to the mitochondrial incubations reversed the effect of the chelators. A linear relationship was obtained when the ability of a compound to prevent the inhibition of pyruvate oxidation was plotted against the logarithm of the stability constant of that compound for magnesium. It is proposed that the intramitochondrial free/bound magnesium ratio may be a potent regulator of the pyruvate dehydrogenase linked protein kinase in isolated mitochondrial systems.

Studies of the regulation of the pyruvate dehydrogenase multienzyme complex have indicated two types of mechanisms for controlling the conversion of pyruvate to acetyl-CoA. Garland (1964) and Garland and Randle (1964) have shown that two of the products of the pyruvate dehydrogenase reaction, NADH and acetyl-CoA, may act as feedback inhibitors of this enzyme. The more recent studies of Linn *et al.* (1969a,b, 1972), Reed (1969), Wieland and Jagow-Westerman (1969), and Wieland and Siess (1970) have suggested that the regulation of the pyruvate dehydrogenase complex may occur by covalent modification, *e.g.*, phosphorylation, resulting in an inactivation of the enzyme. By this mechanism the flow of carbon from pyruvate to acetyl-CoA would be controlled by an equilibrium established between a specific protein kinase and a specific phosphatase which control the degree of phosphorylation of the enzyme and hence, the catalytic activity of the multienzyme complex.

In a recent communication (Schuster and Olson, 1972) it was demonstrated that the rate of pyruvate oxidation in isolated rabbit heart mitochondria depended upon the availability of intramitochondrial ATP and free magnesium. It was proposed that this relationship between the rate of pyruvate oxidation and the availability of the ATP–magnesium complex was a result of the action of the protein kinase on the pyruvate dehydrogenase. This inhibitory effect was specific for the substrate, pyruvate, was accentuated by exogenous magnesium, and was neither due to an accumulation of NADH or acetyl-CoA, both inhibitors of pyruvate dehydrogenase.

The studies reported in this communication are an attempt to further elucidate the protein kinase mediated control of pyruvate dehydrogenase in intact mitochondria. The emphasis of the present studies will be to more clearly define the magnesium requirement of the proposed kinase-mediated regulation of pyruvate oxidation in isolated cardiac mitochondria.

Materials and Methods

Rabbit heart mitochondria were prepared according to the method of Von Korff (1965). The excised heart was homogenized in a media containing KCl, 0.18 M; EDTA, 5 mM; and bovine serum albumin, fraction V, 0.5% w/v, which had been defatted according to the method of Chen (1967). The isolated mitochondria were used in these experiments within 2 hr after their isolation, and were stored at 0–4° at a protein concentration of approximately 20 mg/ml prior to use. Mitochondrial protein concentrations were estimated using a biuret procedure (Layne 1957). Oxygen consumption was measured using a Clark-type oxygen electrode (Yellow Springs 5331) in a glass and Teflon reaction chamber. All experiments were performed at a constant temperature at 28°. Absorption measurements of the mitochondrial suspensions were made using a Hitachi-Perkin-Elmer Model 356 dual-beam spectrophotometer using the wavelength pair 340–374 nm to monitor intramitochondrial pyridine nucleotides. The measurement of ATP was accomplished on neutralized perchloric acid extracts of samples taken during various incubations using the procedures of Williamson and Corkey (1968). Labeled citric acid cycle intermediates and related amino acids were separated and identified using anion-exchange column chromatography as described by Von Korff (1968). The uncoupler *p*-trifluoromethoxyphenylhydrazone of carbonyl cyanide (FCCP) was the generous gift of Dr. P. G. Heytler of Du Pont. The following compounds were obtained from the indicated sources: 1,10-phenanthroline (*o*-phenanthroline), Sigma Chemical Co.; *m*-phenanthroline, Dr. Cyrle Parkanyi, Department of Chemistry, University of Texas at El Paso; 2,2-dipyridyl (α,α-dipyridyl), Fisher Scientific Co.; 4,4-di-

† From the Department of Biochemistry, College of Medicine, University of Arizona, Tucson, Arizona 85724. *Received May 8, 1972.* This work was supported by grants from the U. S. Public Health Service (AM 13967 and GM 01982) and the Arizona Heart Association.

pyridyl (γ,γ-dipyridyl), Aldrich Chemical Co.; ammonium purpurate (murexide), K and K Laboratories, Inc. L-Asparagine-U-^{14}C was obtained from New England Nuclear Corp. All other substrates and reagents were of reagent grade and were purchased from common suppliers.

The stability constants of the chelator metal complexes were taken from O'Sullivan (1969). For the reaction between the metal (M) and the ligand (L), M + L = ML, the stability constant is defined as K = [ML]/[M][L]. The values of K, the metal–ligand stability constant, were determined at 25–30°, an ionic strength in the range of 0.01–0.5 and approximately neutral pH.

The reaction mixture used in these experiments contained KCl, 0.18 M; and potassium phosphate, 2 mM; pH 7.2. The exact reaction conditions of each experiment are detailed in the figure legends. Magnesium was added as the chloride unless otherwise noted. All chelators were added to the mitochondrial incubations as neutralized potassium salts.

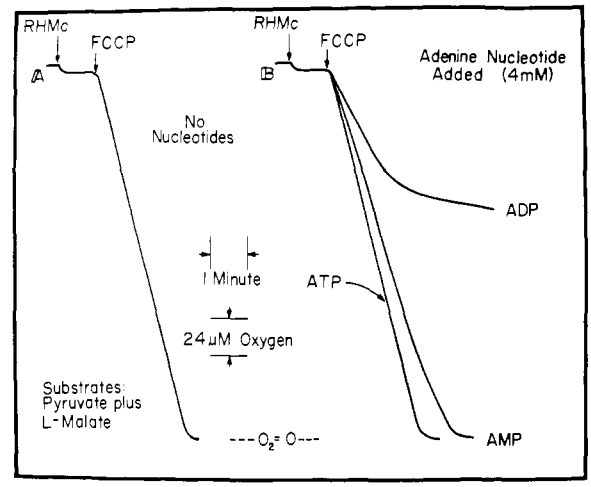

FIGURE 1: The effect of various adenine nucleotides on the rate of pyruvate oxidation in uncoupled isolated rabbit heart mitochondria. The incubation medium contained KCl, 0.18 M; K_2HPO_4, 2 mM; pH 7.2. The mitochondrial protein concentration was 0.52 mg/ml. Uncoupler (FCCP) was added to a final concentration of 1 μM and the concentrations of the substrates, pyruvate plus L-malate, were 1 mM. The adenine nucleotides were added as indicated to a final concentration of 4 mM. The rates of oxygen consumption with each of the indicated conditions was (in nmoles of oxygen/min per mg of protein): FCCP alone, 235; FCCP plus AMP, 205; FCCP plus ADP, 130; FCCP plus ATP, 245.

Results

Previous studies have established that regulation of pyruvate oxidation in isolated cardiac mitochondria may be accomplished through alterations of the intramitochondrial concentrations of the ATP–magnesium complex (Schuster and Olson, 1972). It was proposed that the ATP–magnesium complex served as a substrate for the pyruvate dehydrogenase kinase which catalyzed the phosphorylation and subsequent inactivation of the pyruvate dehydrogenase multienzyme complex (Linn *et al.*, 1969a,b).

The data presented in Figure 1 illustrate the effect of various adenine nucleotides on the rate of oxidation of pyruvate in isolated rabbit heart mitochondria. Rapid, linear rates of pyruvate oxidation were observed in uncoupled mitochondria (Figure 1, curve A) or in coupled mitochondria (not shown, see Figure 3, Schuster and Olson, 1972). As can be seen in Figure 1, curve B-ADP, the inclusion of ADP (4 mM) in the mitochondrial incubation in the presence of uncoupler caused a strong inhibition of pyruvate oxidation. It was previously shown (Schuster and Olson, 1972) that this inhibition was due both to the accumulation of ATP synthesized in the adenylate kinase reaction and to the mobilization of intramitochondrial magnesium. Furthermore, it was demonstrated that this inhibitory effect was prevented by atractyloside, was accentuated by exogenous magnesium, was specific for pyruvate oxidation, and was not due to the accumulation of either inhibitory product of the pyruvate dehydrogenase reaction, *i.e.*, NADH or acetyl-CoA. The oxygen electrode trace designated B-ATP in Figure 1 indicated that the addition of ATP (4 mM) to the uncoupled mitochondria did not affect the rate of pyruvate oxidation. Apparently, added ATP was not accessible to the site of inhibition of the pyruvate dehydrogenase complex in this mitochondrial system. Addition of AMP (4 mM) (curve B-AMP) caused only a slight inhibition of the rate of pyruvate oxidation. It was anticipated that an inhibition of pyruvate oxidation would not occur as the presence of AMP in the uncoupled mitochondria should have prevented the generation of sufficient ATP to cause the inhibitory effect. Even if a small amount of ATP was produced *via* the succinate thiokinase step of the citric acid cycle and the nucleoside diphosphokinase reaction, the presence of AMP and the adenylate kinase reaction should have converted the ATP plus AMP to two molecules of ADP. Under these conditions the pyruvate dehydrogenase linked protein kinase would have been deprived of its substrate, the ATP–magnesium complex. Hence, the experiments shown in Figure 1 indicate that ADP was the only adenine nucleotide effective in causing an inhibition of pyruvate oxidation in uncoupled rabbit heart mitochondria. It should be noted that ADP in the absence of the uncoupler did not cause an inhibition of pyruvate oxidation (Schuster and Olson, 1972).

It was deemed essential to design experiments which would alter the intramitochondrial concentration of the other component of the dehydrogenase kinase reaction, namely free magnesium. It was proposed (Schuster and Olson, 1972) that the magnesium serving with the ATP to inactivate the pyruvate dehydrogenase in this system was released or "mobilized" from bound magnesium in the mitochondrion by the combined action of the uncoupler and the added ADP by an, as yet, unknown mechanism (Kun *et al.*, 1969, 1970; Bogucka and Wojtczak, 1971). The following experiments were designed to demonstrate that the inactivation of the pyruvate dehydrogenase complex can be prevented by including compounds in the incubation medium which chelate intramitochondrial magnesium, released by uncoupler plus ADP addition in this system. It is proposed that the dehydrogenase kinase is inactive in the absence of available magnesium even though sufficient ATP may be present (see Table I). Chelator compounds were chosen for use in these studies both with regard to their stability constants for magnesium as well as for their possible physiological significance for regulating the ratio of free/bound magnesium in the mitochondrion.

Figure 2, trace A, illustrates the inhibitory effect of ADP addition to uncoupled mitochondria on pyruvate oxidation. Trace B demonstrates that the inclusion of the amino acid, L-asparagine (1 mM), in the mitochondrial incubation medium prior to the initiation of respiration with ADP and uncoupler caused a complete prevention of the inhibition of pyruvate oxidation. That this effect of L-asparagine was indeed due to its chelation of intramitochondrial magnesium was surmised from the fact that the inhibitory effect on pyruvate oxidation

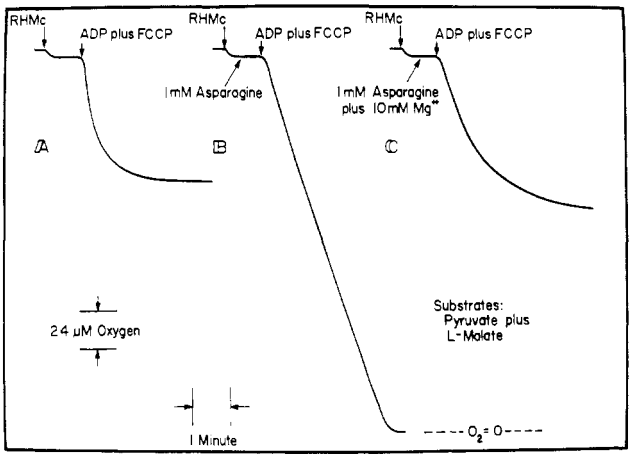

FIGURE 2: The effect of L-asparagine and L-asparagine plus magnesium on the oxidation of pyruvate by isolated rabbit heart mitochondria. Reaction conditions were identical with those described in the legend of Figure 1.

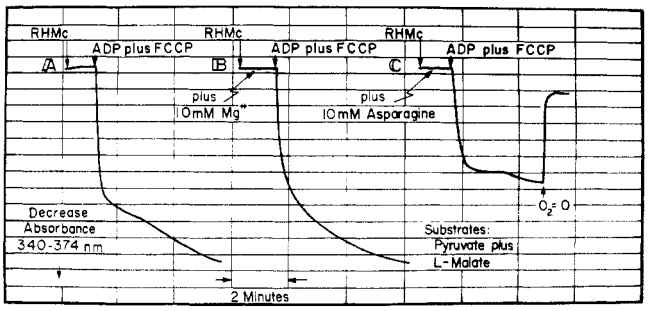

FIGURE 3: A comparison of the effect of magnesium and L-asparagine on the absorbance at 340 nm of mitochondrial suspension oxidizing pyruvate plus L-malate in the presence of uncoupler plus ADP. Reaction conditions are identical with those described in the legend of Figure 1. Pyridine nucleotide absorbance measurements were made as described in the Materials and Methods.

could be reestablished by the addition of magnesium (10 mM) to the incubation (Figure 2, curve C). L-Asparagine has a stability constant for magnesium of 1×10^4 and seems to be freely permeable to the mitochondrial membrane. L-Asparagine addition did not affect the rate of pyruvate oxidation in either coupled or uncoupled mitochondria (not shown). In addition, L-asparagine was not oxidized as a substrate when incubated with L-malate in this mitochondrial experiment. In this regard, when L-asparagine-*U*-^{14}C was incubated with ADP-supplemented, uncoupled mitochondria oxidizing pyruvate plus L-malate, label from L-asparagine-*U*-^{14}C was not incorporated into the intermediates in the citric acid cycle. The only metabolite which was apparently formed in this incubation was a very small amount of aspartate. The addition of L-asparagine to ADP-supplemented, uncoupled mitochondria oxidizing pyruvate plus L-malate did not cause an alteration of the acetyl-CoA level. The acetyl-CoA level of the L-asparagine-treated mitochondria was about 0.25 nmoles of acetyl-CoA/mg of protein, was maintained at a constant level during the experiment, and was identical with that in both the experiment in which pyruvate oxidation was inhibited (ADP plus FCCP[1]) and the uninhibited, FCCP control experiment (see Schuster and Olson, 1972). This experiment indicated that the prevention of the inhibition of pyruvate oxidation probably did not involve alterations of the level of acetyl-CoA which is a known inhibitor of the pyruvate dehydrogenase reaction.

TABLE I: Relationship between the Rate of Pyruvate Oxidation and the ATP Level of Isolated Rabbit Heart Mitochondria.

Experiment	Addition	Concentration (mM)	Rate of Oxygen Consumption (nmoles of O_2/min per mg of Protein)	ATP Level (nmoles of ATP/mg of Protein)
1	FCCP	1[a]	218[b]	2.6[b]
2	ADP	5	196	2500
3	FCCP	1[a]	0	200
	ADP	5		
4	FCCP	1[a]		
	ADP	5	191	230
	L-Asparagine	1		
5	FCCP	1[a]	20	1090
	ADP	5		
	L-Asparagine	1		
	$MgCl_2$	10		

[a] Concentration given as μM. [b] Rate of oxygen consumption and the ATP level were determined after 3 min of incubation under the conditions noted in the table and the legend for Figure 1.

As shown in Table I, nearly equivalent, rapid rates of pyruvate oxidation (218 compared to 196 nmoles of oxygen/min per mg of protein) obtained in mitochondria incubated in the presence of a low ATP level, *i.e.*, under uncoupled conditions (2.65 nmoles of ATP/mg of protein), and in the presence of a high ATP level, *i.e.*, under coupled conditions (2500 nmoles of ATP/mg of protein). Addition of the uncoupler (FCCP) and ADP resulted in a complete inhibition of pyruvate oxidation at an ATP level 100 times greater than that of the uncoupled mitochondria in the absence of ADP. Addition of L-asparagine prevented the inhibition of pyruvate oxidation under these conditions and resulted in an ATP level of 230 nmoles of ATP/mg of protein. The addition of magnesium to the L-asparagine-treated mitochondria reestablished the inhibition of pyruvate oxidation and also resulted in an ATP level of 1090 nmoles of ATP/mg of protein. The point of this experiment is that the rate of pyruvate oxidation cannot be correlated solely with the ATP level of the mitochondrial incubation.

Figure 3 demonstrates the effect of ADP and uncoupler (FCCP) addition to rabbit heart mitochondria oxidizing pyruvate plus L-malate on the oxidation–reduction state of the intramitochondrial pyridine nucleotides as measured by the absorbance of the mitochondrial suspension using the wavelength pair 340–374 nm. ADP plus uncoupler caused an initial rapid oxidation followed by a slower oxidation of pyridine nucleotides (Figure 3, trace A). Addition of mag-

[1] Abbreviation used is: FCCP, *p*-trifluoromethoxyphenylhydrazone of carbonyl cyanide.

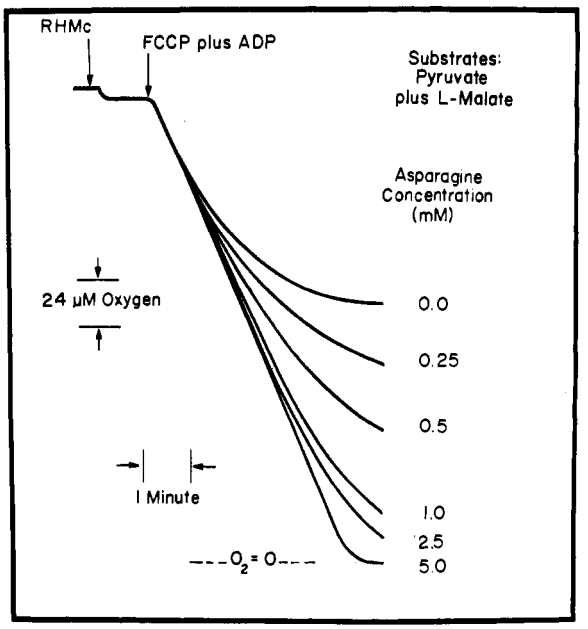

FIGURE 4: A comparison of the rates of pyruvate oxidation of rabbit heart mitochondria in the presence of both ADP and uncoupler (FCCP) with varying concentrations of L-asparagine. The reaction conditions were as described in the legend to Figure 1. The maximum rate of oxidation (in the presence of 5 mM L-asparagine) was 220 nmoles of oxygen/min per mg of protein.

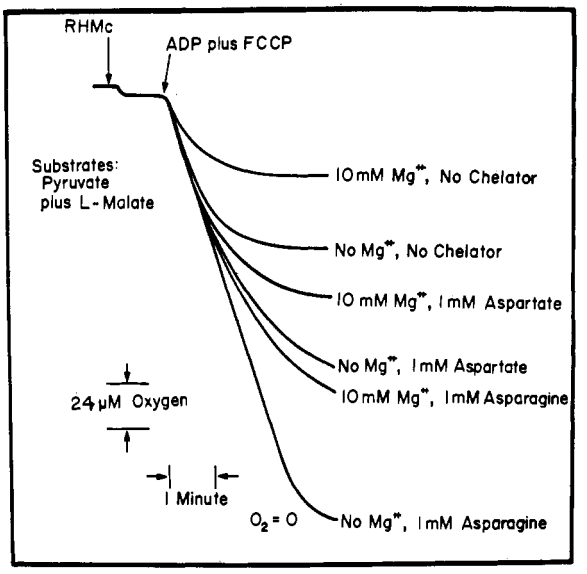

FIGURE 5: A comparison of the effects of L-asparagine, L-aspartate, and magnesium on the rate of pyruvate oxidation by rabbit heart mitochondria in the presence of both ADP and FCCP. The reaction conditions were identical with those described in the legend to Figure 1 except that the mitochondrial protein concentration was 0.4 mg/ml. The maximum rate of oxygen consumption (in the presence of no Mg^{2+}, and 1 mM asparagine) was 241 nmoles of oxygen/min per mg of potein.

nesium chloride (10 mM) to the mitochondrial incubation which accentuates the inhibition of pyruvate oxidation under these conditions caused a similar two-phase oxidation of intramitochondrial pyridine nucleotides (Figure 3, trace B). Pyruvate oxidation in both traces A and B was nearly completely inhibited and, hence, the mitochondrial suspension did not become anaerobic with even prolonged incubation. Trace C indicates that addition of ADP and FCCP to mitochondria containing L-asparagine (10 mM) caused a less extensive oxidation of pyridine nucleotides and anaerobiosis occurred within 3 min following the initiation of respiration with ADP plus FCCP. Under the conditions of this experiment pyruvate oxidation was not inhibited, yet the oxidation–reduction state of the intramitochondrial pyridine nucleotides was considerably more reduced than the experiments in which pyruvate oxidation was completely inhibited (see traces A and B). This experiment implies that NADH accumulation was not involved in the inhibition of the pyruvate oxidase system in these mitochondrial experiments.

The experiment shown in Figure 4 illustrates the concentration dependence of the effect of L-asparagine on the rate of pyruvate oxidation in cardiac mitochondria. Without the addition of L-asparagine the rate of pyruvate oxidation in ADP-supplemented, uncoupled mitochondria was strongly inhibited. Progressively increasing the L-asparagine concentration from 0 to 5 or 10 mM caused a nearly complete prevention of the inhibition of pyruvate oxidation. It is proposed that as the concentration of chelator was increased, the level of magnesium available for use in this system decreased, and the ATP–magnesium complex, the substrate for the pyruvate dehydrogenase kinase, was presumed to be limiting.

Figure 5 shows a series of experiments designed to compare the effects of L-asparagine, which is a reasonably good chelator of magnesium (stability constant, 1×10^4), to a naturally occurring structural analog of L-asparagine, L-aspartate, which is not as good a chelator of magnesium (stability constant, 6×10^2). In the absence of the chelator the rate of pyruvate oxidation in ADP-supplemented, uncoupled mitochondrial was strongly inhibited. The addition of magnesium chloride (10 mM) accentuated the inhibition of pyruvate oxidation (Schuster and Olson, 1972). The addition of L-aspartate (1 mM) effected a partial release of the inhibition of pyruvate oxidation. Addition of magnesium chloride (10 mM) in the presence of the added L-asparatate reestablished a rather strong inhibition of pyruvate oxidation.

Addition of L-asparagine (1 mM) to the inhibited system caused a nearly complete prevention of the inhibition of pyruvate oxidation while the inclusion of magnesium chloride (10 mM) in the presence of L-asparagine resulted in an oxygen trace nearly equivalent to the reaction to which L-aspartate alone was added. Neither L-aspartate nor L-asparagine was metabolized under the conditions of these experiments.

To further establish that the release or prevention of the inhibition of pyruvate oxidation in this system was mediated by the chelation of intramitochondrial magnesium, other magnesium chelators and their structural analogs were employed. The oxygen electrode tracings illustrated in Figure 6 demonstrate that addition of well-established magnesium chelators such as *o*-phenanthroline (trace A) and α,α-dipyridyl (trace C) to the inhibitory system caused a complete prevention of the inhibition of pyruvate oxidation. The inclusion of nonchelating isomers of these two compounds, *m*-phenanthroline (trace B) and γ,γ-dipyridyl (trace D), caused little change in the inhibition of pyruvate oxidation in the ADP-supplemented, uncoupled mitochondria. Again, the addition of compounds with a high binding affinity for magnesium caused a competition to occur for intramitochondrial magnesium with ATP and the pyruvate dehydrogenase kinase. That the chelation of magnesium and not calcium was of primary importance in the reversal or prevention of the inhibition of pyruvate oxidation may be seen in the exper-

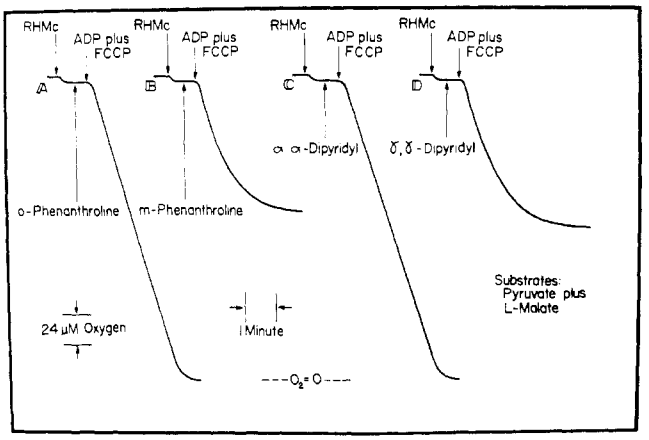

FIGURE 6: A comparison of the effects of known magnesium chelators and their structural analogs on the rate of pyruvate oxidation by rabbit heart mitochondria in the presence of both ADP and FCCP. The compounds were added to a final concentration of 1 mM to a reaction mixture that was the same as that described in the legend to Figure 1. The maximum rates of oxygen consumption were 236 and 224 nmoles of oxygen/min per mg of protein for curves A and C, respectively.

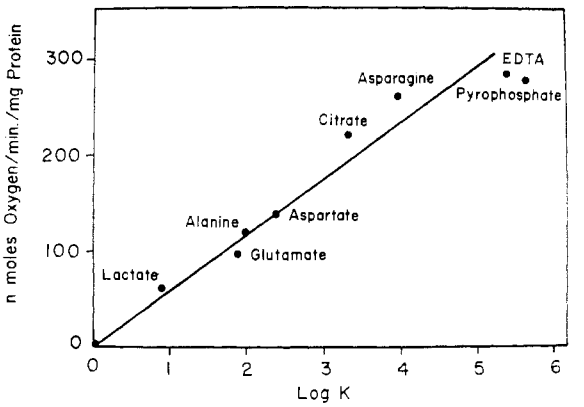

FIGURE 8: The relationship between the rate of pyruvate oxidation of rabbit heart mitochondria in the presence of both ADP and FCCP with the stability constants of various compounds for magnesium. Reaction conditions were the same as those described in the legend to Figure 1. The compounds noted were added to a final concentration of 4 mM. The oxygen consumption rate reported was that observed after respiration had proceeded for 2.5 min.

iment described in Figure 7. In this experiment, the inhibition of pyruvate oxidation by ADP and FCCP (trace B) was prevented by the magnesium chelator α,α-dipyridyl (trace A). On the other hand, the inhibitory situation was unaffected by the inclusion of murexide, a specific calcium chelator, at the same concentration as α,α-dipyridyl in the incubation mixture.

In conclusion, Figure 8 shows the effects of a series of compounds of differing stability constants for magnesium on the prevention of the inhibition of pyruvate oxidation in ADP-supplemented, uncoupled mitochondria. It is apparent that at equimolar concentrations of the magnesium chelator, the greater the binding constant for magnesium, the faster the rate of pyruvate oxidation or, if you will, the less the inhibitory effect of the ATP–magnesium complex on the pyruvate oxidase system. The deviations from a straight-line relationship may be related to permeability restrictions of the various compounds employed in this intact mitochondrial system. Also, the fact that EDTA and pyrophosphate fall below the straight line indicates that the pyruvate oxidase system may be operating at maximal velocity when the rate reached 250–280 nmoles of oxygen/min per mg of protein. It may be appropriate to note the possible physiological significance of some of the common substrates used in this experiment which may affect the bound/free magnesium ratio in various cellular compartments by virtue of their ability to chelate and, thus, compete for free magnesium which may regulate enzymatic functions such as the pyruvate oxidase complex.

It should be noted at this point that the magnesium chelators which prevent the inhibition of pyruvate oxidation were without effect when they were added following the establishment of the inhibitory condition. This observation implies, but certainly does not prove, that the combined effects of intramitochondrial ATP and magnesium cannot be reversed simply by adding chelator compounds which may compete with ATP for free magnesium. This observation is compatible with a phosphorylation mechanism for the regulation of the pyruvate oxidase in this mitochondrial system.

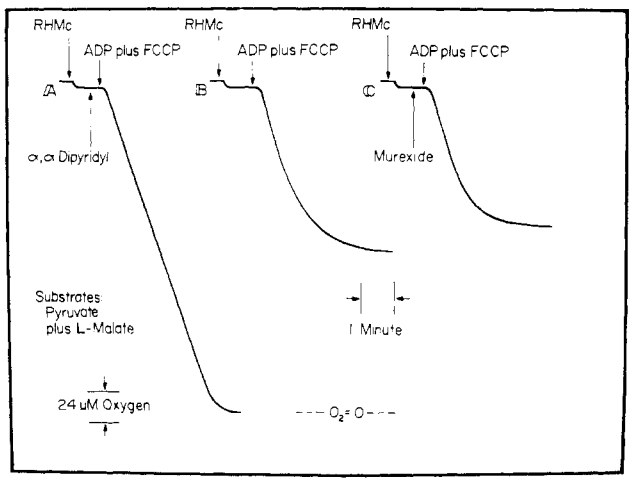

FIGURE 7: A comparison of the effect of a magnesium chelator and a calcium chelator on the rate of pyruvate oxidation of rabbit heart mitochondria in the presence of both ADP and FCCP. The reaction conditions were the same as those described in the legend of Figure 1. The concentration of α,α-dipyridyl in the experiment described by curve A was 1.0 mM while the concentration of murexide in the experiment shown in curve C was 1.0 mM. The rate of oxygen consumption of curve A was 224 nmoles of oxygen/min per mg of protein.

Discussion

The regulation of energy production in the mitochondrial fraction of the cell is an often studied, complex consideration. Prominent among the various mechanisms which have been proposed for the regulation of mitochondrial energy production are: (a) a respiratory control, and (b) small molecule effector regulation of individual enzymes in the citric acid cycle. Respiratory control depends upon the coupling relationship which exists between all the primary dehydrogenase reactions of the mitochondrion and the processes of electron transport and oxidative phosphorylation, while the effector control of individual mitochondrial enzymes merely relates to specific activations and/or inhibitions of enzymes pertinent to the process of energy production by various small molecules.

Small molecule effector mediated regulation of the entry of acetyl units from pyruvate into the citric acid cycle as a prelude to oxidative energy generation has been proposed to occur by a regulation of pyruvate dehydrogenase by acetyl-CoA or NADH (Garland, 1964; and Garland and Randle, 1964) or citrate synthase by adenine nucleotides (Jangaard *et al.*, 1968; and Shepherd and Garland, 1969) and by acyl-CoA derivatives (Wieland and Weiss, 1963; Smith and Williamson, 1971). These suggested regulators of citrate synthase in most cases must be substantiated in intact metabolic systems (*e.g.*, see Olson and Williamson, 1971, and Olson and Allgyer, 1972).

The importance of metal ions in enzymatic catalysis has been recently reviewed (Mildvan, 1970). That divalent ions may be important in the regulation of the activity of pyruvate dehydrogenase has been pointed out by Linn *et al.* (1972). In addition, the work of Reed and his colleagues (Linn *et al.*, 1969a,b, 1972; Reed *et al.*, 1969) and of Wieland (Wieland and Jagow-Westerman, 1969; Wieland and Siess, 1970) has demonstrated that the regulation of pyruvate dehydrogenase from a variety of tissues may occur by a covalent modification of the enzyme complex, *i.e.*, phosphorylation and inactivation by a specific protein kinase and dephosphorylation and reactivation by a specific phosphatase.

The present studies were designed to investigate factors affecting the regulation of pyruvate oxidation in isolated mitochondrial systems. Specifically we have attempted to demonstrate the potentiality that pyruvate oxidation in mitochondrial systems may be regulated by kinase-mediated phosphorylation of the pyruvate dehydrogenase. We have presented evidence previously that the level of intramitochondrial ATP and the availability of magnesium were of critical importance for the inactivation of pyruvate oxidation in cardiac mitochondria (Schuster and Olson, 1972). The experiments described in the present communication were performed to amplify the importance of the divalent cation magnesium in the regulation of pyruvate oxidation.

That the availability of ATP was necessary, but not sufficient alone for the regulation of the mitochondrial pyruvate oxidizing system, is shown in Figure 1 and Table I. High ATP levels and rapid rates of pyruvate oxidation persisted in coupled mitochondria (ADP), in ADP-supplemented, uncoupled mitochondria in the presence of the magnesium chelator L-asparagine and in ATP-supplemented, uncoupled mitochondria. Low ATP levels with rapid pyruvate oxidation were observed with uncoupled mitochondria and with AMP-supplemented, uncoupled mitochondria. Pyruvate oxidation was inhibited only under conditions in which an elevated ATP level occurred and in which intramitochondrial magnesium has been released or "mobilized" by the combined action of the uncoupler and ADP. These conditions obtained in the case of ADP-supplemented, uncoupled mitochondria are seen in Figure 1 and Table I, experiment 3. In addition, the addition of exogenous magnesium to the ADP-supplemented, uncoupled mitochondria accentuated the inhibition of pyruvate oxidation (*e.g.*, see Figure 5) and the addition of exogenous magnesium to ADP-supplemented, uncoupled mitochondria in the presence of L-asparagine also caused severe inhibition of pyruvate oxidation. Soluble magnesium salts other than the chloride (*e.g.*, acetate) gave a result identical with that described for magnesium chloride. Hence, it seems clear that the availability of both ATP and magnesium was necessary for the inactivation of pyruvate oxidation in this isolated mitochondrial system. It may be appropriate to note that the addition of exogenous magnesium to coupled mitochondria (ADP), uncoupled mitochondria (FCCP), and ATP-supplemented, uncoupled mitochondria did not result in an inhibition of pyruvate oxidation (experiments not shown).

That L-asparagine was acting to prevent the inhibition of pyruvate oxidation by chelating intramitochondrial magnesium was implicated by the following observations: (a) inhibition of pyruvate oxidation was reestablished in L-asparagine-treated mitochondria by adding excess magnesium; (b) L-aspartate, a structural analog of L-asparagine with a much lower stability constant for magnesium, was not effective in preventing the inhibition of pyruvate oxidation; (c) D-asparagine and L-asparagine both had similar effects on the prevention of the inhibitory effect; (d) L-asparagine was not metabolized in this system and, thus, apparently exerted its effect through some "nonmetabolic" effect; and (e) other amino acids such as glycine and glutamine which were not metabolized and which have reasonably high (10^3–10^4) stability constants for their magnesium complex gave similar results to that obtained with asparagine.

The proposal that intramitochondrial magnesium was an important component in the process of inactivation of the pyruvate oxidase complex was further supported by the experiment illustrated in Figure 6. Magnesium chelators such as *o*-phenanthroline and α,α-dipyridyl completely prevented the inhibition of pyruvate oxidation while their nonchelating structural analogs *m*-phenanthroline and γ,γ-dipyridyl had no effect on the pyruvate oxidase inhibition. When using any family of metal ion chelators, care must be taken that the compounds may be forming stable complexes with other metals than magnesium. In mitochondrial systems the other divalent metal ion which is of crucial importance in a variety of processes is calcium. The experiment shown in Figure 7 indicates that the addition of murexide, a compound which forms a stable complex with calcium and not magnesium, did not prevent the inhibition of pyruvate oxidation. Again, this experiment implies that one of the components involved in this inhibitory system must be magnesium.

If the ability of a compound to tie up magnesium or compete with the pyruvate dehydrogenase linked protein kinase for magnesium depends upon the stability constant of its magnesium complex, a linear relationship should obtain when the logarithm of the stability constant is plotted against the ability of the compound to prevent the inhibition of pyruvate oxidation. That this was the case for the series of compounds tested in this study may be seen in Figure 8. If the pyruvate oxidation rates were plotted against the stability constant of these compounds for either calcium or manganese, a linear relationship was not evident (data not shown).

It seems clear from the data presented in this and the previous communication from this laboratory (Schuster and Olson, 1972) that the regulation of pyruvate oxidation in intact rabbit heart mitochondria may depend upon both the levels of intramitochondrial ATP and free, unsequestered magnesium. That various naturally occurring substrates or compounds by virtue of their ability to form stable complexes with metal ions may serve as regulators of various metabolic reactions which depend upon these metal ions is an intriguing possibility.

References

Bogucka, K., and Wojtczak, L. (1971), *Biochem. Biophys. Res. Commun. 44*, 1330.

Chen, R. F. (1967), *J. Biol. Chem. 242*, 173.

Garland, P. B. (1964), *Biochem. J. 92*, 10c.
Garland, P. B., and Randle, P. J. (1964), *Biochem. J. 91*, 6c.
Jangaard, N. O., Unkeless, J., and Atkinson, D. E. (1968), *Biochim. Biophys. Acta 151*, 225.
Kun, E., Kearney, E. B., Lee, N. M., and Wiedemann, I. (1970), *Biochem. Biophys. Res. Commun. 38*, 1002.
Kun, E., Kearney, E. B., Wiedemann, I., and Lee, N. M. (1969), *Biochemistry 8*, 4443.
Layne, E. (1957), *Methods Enzymol. 3*, 447.
Linn, T. C., Pettit, F. H., Hucho, F., and Reed, L. J. (1969a), *Proc. Nat. Acad. Sci. U. S. 64*, 227.
Linn, T. C., Pettit, F. H., and Reed, L. J. (1969b), *Proc. Nat. Acad. Sci. U. S. 62*, 234.
Linn, T. C., Pelley, J. W., Pettit, F. H., Hucho, F., Randall, D. D., and Reed, L. J. (1972), *Arch. Biochem. Biophys. 148*, 327.
Mildvan, A. S. (1970), *in* The Enzymes, Vol. 2, 3rd ed, Boyer, P. D., Ed., New York, N. Y., Academic Press, p 445.
Olson, M. S., and Allgyer, T. (1972), *Biochim. Biophys. Acta 267*, 238.
Olson, M. S., and Williamson, J. R. (1971), *J. Biol. Chem. 246*, 7794.
O'Sullivan, W. J. (1969) *in* Data for Biochemical Research, Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M., Ed., New York, N. Y., Oxford University Press, p 423.
Reed, L. J. (1969), *Curr. Top. Cell. Regul. 1*, 233.
Schuster, S. M., and Olson, M. S. (1972), *J. Biol. Chem. 247*, 5088.
Shepherd, D., and Garland, P. B. (1969), *Biochem. J. 114*, 597.
Smith, C. M., and Williamson, J. R. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 18*, 35.
Von Korff, R. W. (1965), *J. Biol. Chem. 240*, 1351.
Wieland, O., and Jagow-Westerman, B. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 4*, 271.
Wieland, O., and Siess, E. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 947.
Wieland, O., and Weiss, L. (1963), *Biochem. Biophys. Res. Commun. 13*, 26.
Williamson, J. R., and Corkey, B. E. (1968), *Methods Enzymol. 13*, 434.

Regulation and Kinetics of Glucose-6-phosphate Dehydrogenase from *Candida utilis*†

Adeyinka Afolayan

ABSTRACT: Torula yeast (*Candida utilis*) glucose-6-phosphate dehydrogenase catalyzes only the NADP-dependent oxidation of glucose-6-P. When $NADP^+$ is the variable substrate, a hyperbolic rate–concentration response curve was obtained below 1 mM of the coenzyme. At higher $NADP^+$ concentration, a marked substrate inhibition and/or inactivation was observed. NADPH is an allosteric effector as well as a competitive inhibitor. The response curves at various ($NADP^+$):(NADPH) ratios at two different total pyridine nucleotide concentrations of 50 and 200 μM, respectively, were found to be sigmoidal. It is not unlikely that both the absolute concentration of $NADP^+$ and NADPH and the ratio of their levels might be important for regulation. While ATP severely inhibited the enzyme, neither AMP nor 6-phosphogluconate had any effect on the enzyme. Both spermidine and 3′,5′-cyclic AMP are activators. The extent of activation by cyclic AMP depends on the concentration of the cyclic nucleotide itself as well as on $NADP^+$ concentration. Thus, cyclic AMP relieves torula yeast glucose-6-P dehydrogenase of substrate inhibition observed at high $NADP^+$ concentration. The physiological implications of these results are discussed. The nature of reaction mechanism inferred from the kinetic data is also presented and discussed. A simple ordered sequential mechanism with $NADP^+$ binding first to the enzyme has been proposed.

Glucose-6-phosphate dehydrogenase (glucose 6-phosphate:NADP oxidoreductase, EC 1.1.1.49)[1] is an enzyme which has been highly purified and extensively studied from many sources including microorganisms and mammalian tissues (Glaser and Brown, 1955; Noltmann *et al.*, 1961; Marks *et al.*, 1961; Kirkman and Hendrickson, 1962; Chung and Langdon 1963; Yoshida, 1966; Luzzatto and Afolayan, 1968, 1971; Afolayan, 1969; Afolayan and Luzzatto, 1971; Sanwal, 1970; Olive *et al.*, 1971). In addition, one noteworthy development is a growing interest in the regulation and control of the activity of this enzyme by some metabolites, cations and nucleotides. However, the mechanism of regulation and control is not clearly understood.

Since glucose-6-P dehydrogenase is the initial enzyme of the pentose phosphate pathway, a branching sequence from the glycolytic pathway and which produces NADPH and pentose, the existence of sophisticated mechanism for the control and regulation of its activity is not surprising. Moreover, the reduced coenzyme is the major source of metabolic hydrogen in aerobic organisms and is necessary for the formation of glutamate, lipids, deoxyribonucleotides, cell walls, and other cellular components. Recently, Luzzatto (1967), Afolayan and Luzzatto (1971), and Luzzatto and Afolayan (1971) showed that both the absolute amount of NADP and NADPH concentrations as well as their ratio are crucial in

† From the Division of Biochemistry, Department of Biological Sciences, University of Ife, Ile-Ife, Nigeria. *Received January 19, 1972.*

[1] Abbreviations used are: $NADP^+$, oxidized nicotinamide–adenine dinucleotide phosphate; NADPH, reduced nicotinamide–adenine dinucleotide phosphate; NAD^+ and NADH, oxidized and reduced nicotinamide–adenine dinucleotide.