

## Reversible ATP-induced inactivation of branched-chain 2-oxo acid dehydrogenase

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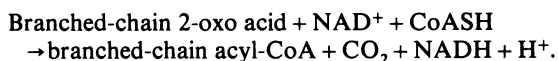
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The branched chain 2-oxo acid dehydrogenase from rat skeletal muscle, heart, kidney and liver mitochondria can undergo a reversible activation–inactivation cycle *in vitro*. Similar results were obtained with the enzyme from kidney mitochondria of pig and cow. The dehydrogenase is markedly inhibited by ATP and the inhibition is not reversed by removing the nucleotide. The non-metabolizable ATP analogue adenosine 5'-[ $\beta$ -imido]triphosphate can block the effect of ATP when added with the nucleotide, but has no effect by itself, nor can it reverse the inhibition in mitochondria preincubated with ATP. These findings suggest that the branched chain 2-oxo acid dehydrogenase undergoes a stable modification that requires the splitting of the ATP  $\gamma$ -phosphate group. In skeletal muscle mitochondria the rate of inhibition by ATP is decreased by oxo acid substrates and enhanced by NADH. The dehydrogenase can be reactivated 10–20-fold by incubation at pH 7.8 in a buffer containing  $Mg^{2+}$  and cofactors. Reactivation is blocked by NaF (25 mM). The initial activity of dehydrogenase extracted from various tissues of fed rats varies considerably. Activity is near maximal in kidney and liver whereas the dehydrogenase in heart and skeletal muscle is almost completely inactivated. These studies emphasize that comparisons of branched chain 2-oxo acid dehydrogenase activity under various physiological conditions or in different tissues must take into account its state of activation. Thus the possibility exists that the branched chain 2-oxo acid dehydrogenase may be physiologically regulated via a covalent mechanism.

The oxidation of the branched-chain amino acids appears to influence the production of alanine and the turnover of protein in skeletal muscle (Goldberg & Odessey, 1972; Buse *et al.*, 1973, 1975; Odessey *et al.*, 1974; Fulks *et al.*, 1975; Chang & Goldberg, 1978*a,b*; Buse & Reid, 1976). The activity of this pathway changes in response to hormonal and dietary signals (Wohlhueter & Harper, 1970; Goldberg & Odessey, 1972; Buse *et al.*, 1973, 1975; Goodman, 1977, 1978; Sullivan *et al.*, 1978). An increased oxidation of these amino acids also appears to be a metabolic consequence of trauma (Ryan *et al.*, 1974).

Recent studies (Odessey & Goldberg, 1979; Goodman, 1977, 1978) have demonstrated that the rate of oxidation of leucine, isoleucine and valine is regulated by the activity of branched chain 2-oxo acid dehydrogenase (EC 1.2.4.4), the enzyme that

converts the 2-oxo acid analogue to their respective acyl-CoA esters:



Investigations of crude and purified preparations indicate that a single mitochondrial enzyme degrades all these branched chain 2-oxo acids with  $K_m$  values of 20–50  $\mu\text{M}$  (Parker & Randle, 1978*a*; Pettit *et al.*, 1978; Danner *et al.*, 1978; Odessey & Goldberg, 1979).

A number of hypotheses have been advanced to explain the regulation of this enzyme. End-product inhibition has been suggested on the basis of inhibitory effects of NADH and branched chain acyl-CoA (Pettit *et al.*, 1978; Bremer & Davis, 1978; Parker & Randle, 1978*a*). An alternative (although not mutually exclusive) hypothesis is that the enzyme activity may be controlled by phosphorylation and dephosphorylation. In support of

Abbreviation used: p[NH]ppA, adenosine 5'-[ $\beta$ -imido]triphosphate.

this hypothesis, it has been shown that ATP markedly inhibits activity (Johnson & Connelly, 1968; Parker & Randle, 1978*b*; Odessey & Goldberg, 1979) and that nonmetabolizable analogues fail to do so (Odessey & Goldberg, 1979). However, two groups (Pettit *et al.*, 1978; Danner *et al.*, 1979), working with highly purified preparations, have failed to observe ATP inhibition as significant incorporation of radioactivity from [ $\gamma$ - $^{32}\text{P}$ ]ATP into enzyme protein. To resolve these apparent contradictions, I have studied the effects of ATP on dehydrogenase activity.

### Materials and methods

Male rats (Sprague-Dawley) weighing 250–500 g were obtained from Hilltop Laboratories, Scottsdale, PA, U.S.A. and provided with Purina laboratory chow and water *ad libitum*.

All biochemicals and enzymes were of the highest grade available and were obtained from Sigma. p[NH]ppA was obtained from Boehringer. 4-Methyl-2-oxo[1- $^{14}\text{C}$ ]pentanoate was prepared as described previously (Odessey & Goldberg, 1979).

Skeletal muscle mitochondria were prepared as described previously (Odessey & Goldberg, 1979). Mitochondria from rat hearts were prepared by the same method. The mitochondria were suspended in the following buffer: 50 mM-Tris/HCl (pH 7.8 at 27°C)/5 mM-EGTA/1.97 mM-MgCl<sub>2</sub>/50 mM-KCl/0.25 M-sucrose. Mitochondria from other tissues were prepared as described by Schnaitman & Greenawalt (1968). The mitochondria (0.5 ml) (approx. 1 mg of protein/ml) were mixed with 0.5 ml of assay buffer [50 mM-Tris-HCl (pH 7.8)/11.6 mM-MgCl<sub>2</sub>/50 mM-KCl/20 mM-NaHCO<sub>3</sub>/2 mM-dithiothreitol (final Mg<sup>2+</sup> concentration 5.0 mM)]. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (0.5  $\mu\text{M}$ ) and oligomycin (5  $\mu\text{g/ml}$ ) were also included to enable external regulation of intramitochondrial ATP and ADP levels and eliminate respiratory control (Klingenberg, 1970; Hansford, 1976). This mixture was used for both preincubations and assays. To assay the activity, NaF (25 mM) and Triton X100 (1 mg/ml) were added to the reaction mixture (except where indicated otherwise). Where indicated lipoic dehydrogenase (Sigma) (from pig heart) was added to the final assay mixture at a concentration of 2.5 U/ml. Dehydrogenase activity was assayed by measuring the decarboxylation of 4-methyl-2-oxo[1- $^{14}\text{C}$ ]pentanoate (100  $\mu\text{M}$ ) in a 5–10 min incubation as described previously (Odessey & Goldberg, 1979). Protein was measured by the biuret method (Gornall *et al.*, 1949). ATP was assayed by using purified firefly luciferase and luciferin (Lundin *et al.*, 1976). Estimates of  $K_m$  and  $V_{max}$  were made with a non-linear curve-fitting program (Cleland, 1967) with data from five

concentrations of substrate ranging from 10 to 100  $\mu\text{M}$ .

### Results

Dehydrogenase activity in mitochondria isolated from skeletal muscle of fed rats is very low (0.5–2 nmol/h per mg of protein) compared with other tissues (Shinnick & Harper, 1976). Preincubation for 20–40 min in assay buffer results in a 5–20-fold increase in activity (Fig. 1). Addition of ATP to the activated enzyme decreases activity below initial levels within 5 min (Fig. 1). Extensive washing of the mitochondria with cold buffer containing NaF (25 mM) does not reactivate the dehydrogenase. The residual ATP content of washed mitochondria containing activated enzyme (0.45 nmol/mg) is not significantly ( $P > 0.2$ ) different from that of mitochondria containing ATP-inhibited enzyme (0.52 nmol/mg). The inhibition by ATP is reversible, since further incubation with the

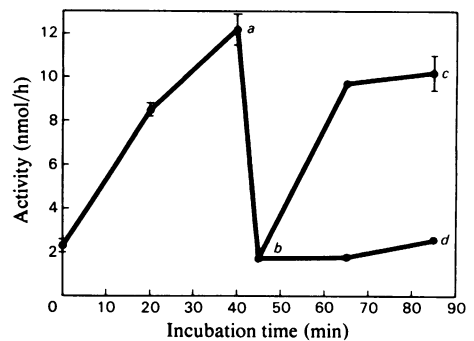


Fig. 1. Branched-chain 2-oxo acid dehydrogenase activation and its inhibition by ATP

Mitochondria were isolated from skeletal muscle and were suspended in the incubation mixture. After incubation for the time shown, NaF (25 mM) was added. At point *a*, ATP (0.2 mM), phosphoenolpyruvate (2 mM) and pyruvate kinase (5 U/ml) (the ATP regenerating system) were added. The mixture was further incubated for 5 min. At point *b*, samples were removed and the mitochondria were centrifuged and washed to remove ATP. The pellets were resuspended in the incubation medium and further incubated in the presence (*d*) or absence (*c*) of NaF. To assay, Triton X-100 was added to all samples at a final concentration of 1 mg/ml. The reaction was started by addition of 100  $\mu\text{M}$  4-methyl-2-oxo [1- $^{14}\text{C}$ ]pentanoate and incubated for 10 min. The reaction was terminated by injection of 0.5 ml of 1 M-HClO<sub>4</sub> and  $^{14}\text{CO}_2$  was collected as described previously (Odessey & Goldberg, 1979). Each point represents the mean  $\pm$  S.E.M. of three assays.

Mg<sup>2+</sup>-containing buffer in the absence of NaF reactivates the enzyme with a 6-fold increase in activity (Fig. 1). This activation can be blocked by the presence of NaF (Fig. 1). Although incubation with NaF prevents reactivation, mitochondrial ATP content does not vary significantly.

This behaviour is not unique to the dehydrogenase from skeletal muscle. Heart mitochondria isolated in the presence of NaF have a low initial activity (Fig. 2a). Incubation under the conditions described in Fig. 1 stimulates activity 45-fold. Subsequent addition of ATP (0.2 mM) results in an 87% inhibition within 5 min. After removal of ATP by centrifugation, and resuspension of the pellet in fresh medium, further incubation for 30 min causes a 12-fold reactivation of the enzyme. However, if the incubation medium contains NaF (25 mM) little reactivation is observed. Thus, the behaviour of branched-chain 2-oxo acid dehydrogenase in heart

mitochondria resembles in all respects that of the enzyme from skeletal muscle (Fig. 2b).

When a similar experiment is carried out on the enzyme from kidney mitochondria, qualitatively similar results are obtained, except that initially the activity is high and is not further stimulated by incubation. However, the enzyme from the kidney can be inhibited by ATP and its reactivation is blocked by NaF (Fig. 2c). Exactly analogous results have been obtained with mitochondria isolated from bovine and pig kidney.

The dehydrogenase present in liver mitochondria is less responsive to the experimental manipulations than that of the other tissues so far examined (Fig. 2d). Initial activity is high and is not further increased by incubation. Addition of ATP results in a 30–50% decrease in activity. After removal of ATP by centrifugation, further incubation in fresh medium increases activity but does not restore it to initial levels.

Pettit *et al.* (1978) reported that lipolic dehydrogenase, a component of the branched chain 2-oxo acid dehydrogenase complex, is only loosely associated with the enzyme and is easily lost during purification. To test whether any of the effects of ATP and activation could be due to alterations in the association of this component with the dehydrogenase complex, the experiments described in Fig. 2 were repeated in the presence of added lipolic dehydrogenase. The addition of this enzyme does not affect the dehydrogenase activity of heart (Fig. 2a) or skeletal muscle (Fig. 2b). Under some conditions, the activity of the dehydrogenase from kidney and liver is stimulated (Fig. 2c and 2d). However, no qualitative differences in the results are apparent. In fact, when assayed in the presence of lipolic dehydrogenase, the liver enzyme that was incubated in the absence of NaF has activity equal to that of controls. The results of Fig. 2 taken together indicate that the regulation of the branched-chain 2-oxo acid dehydrogenase by ATP is qualitatively similar in all tissues studied.

The above experiments suggest that once the dehydrogenase is inhibited by exposure to ATP, the nucleotide does not need to be present to maintain the inhibition. However, since intact mitochondria were used in the experiments described above, other tests were performed to show that internal compartmentation of residual nucleotide could not account for differences in activity.

It has been found that high concentrations of non-ionic detergents disrupt mitochondria so that enzyme activity is found primarily (98%) in the 35000g supernatant. When skeletal muscle mitochondria are incubated with ATP, washed, dissolved with Triton X-100 (30 mg/ml) and centrifuged at 35000g for 10 min, inhibition is still observed in the supernatant fraction although ATP

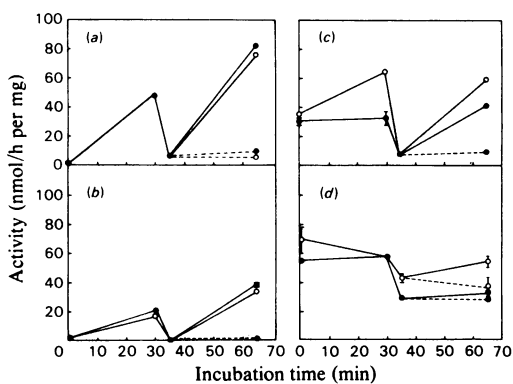


Fig. 2. Regulation of branched-chain 2-oxo acid dehydrogenase in various tissues: effect of lipoid dehydrogenase

Mitochondria from various tissues were isolated as described in the Materials and methods section and suspended in the incubation medium. One sample was taken for assay of initial activity and the remainder was incubated for 30 min at 27°C. Further manipulations were the same as described in the legend to Fig. 1 with one exception. One sample of the washed ATP-inhibited pellet was resuspended in buffer containing both NaF (25 mM) and Triton X-100 (1 mg/ml) rather than NaF alone (as in Fig. 1). For assay all samples were made up to 25 mM-NaF and 1 mg Triton X-100/ml. ●—●, Samples assayed with no further additions; ●—●, incubated with NaF (25 mM) and Triton X-100 (1 mg/ml) and assayed with no further additions; ○—○, samples assayed in the presence of lipoid dehydrogenase (2.5 U/ml); ○—○, incubated with NaF (25 mM) and Triton X-100 (1 mg/ml) and assayed in the presence of lipoid dehydrogenase (2.5 U/ml). Each point represents the mean ± S.E.M. of three determinations. (a) Heart; (b) skeletal muscle; (c) kidney; (d) liver.

concentrations in the assay mixture are less than  $0.1\mu\text{M}$  (Table 1). Similarly, dehydrogenase activity in 35000 g Lubrol supernatants from kidney mitochondria is inhibited by ATP. Inhibition remains after removal of the ATP by precipitating the enzyme with 50%-satd.  $(\text{NH}_4)_2\text{SO}_4$ . This inhibition of activity could not have been due to residual nucleotide since a high concentration of ATP (0.2mM) when added to the  $(\text{NH}_4)_2\text{SO}_4$  fraction (derived from preparations not previously exposed to ATP) does not inhibit activity (Table 1). In another experiment, mitochondria after incubation with ATP were washed and then frozen and thawed as described by Pettit *et al.* (1978). This procedure releases the enzyme from the organelle. When assayed in the mitochondria-free supernatant, the inhibition of the enzyme was retained (Table 1).

Further evidence for the covalent nature of the ATP inhibition is obtained from experiments with the non-metabolized ATP analogue p[NH]ppA (Table 2). This analogue by itself has no effect on dehydrogenase activity from skeletal muscle. When added before ATP, it markedly decreases the inhibitory effect of ATP, which is consistent with its role as an effective competitive inhibitor of many ATP-utilizing enzymes (Yount *et al.*, 1971). However, if mitochondria are first preincubated with ATP, washed and resuspended in fresh buffer

containing the analogue, the inhibition is not affected. These experiments strongly suggest that the inhibition by ATP is not due to a reversible ligand-binding reaction.

Table 2. Effect of the non-metabolized analogue p[NH]ppA on inhibition of dehydrogenase activity by ATP

Skeletal muscle mitochondria were preincubated for 30min as described in the legend to Fig. 1. One sample was assayed (control). Other samples were further incubated for 5min with p[NH]ppA (1.0mM) alone, ATP (0.2mM) with a regenerating system (cf. legend to Fig. 1) (ATP alone) or with an ATP regenerating system + p[NH]ppA (p[NH]ppA before ATP). The mitochondria were centrifuged, washed and resuspended in fresh buffer containing NaF (25mM) and Triton X-100 (1mg/ml). To one sample of mitochondria that had been preincubated with ATP alone p[NH]ppA (1mM) was also added to the assay mixture (ATP before p[NH]ppA). Each value is the mean ( $\pm$ S.D. of three determinations).

Sample	Activity (% of control)
Control	100 $\pm$ 3
ATP alone	14 $\pm$ 1
p[NH]ppA alone	106 $\pm$ 9
p[NH]ppA before ATP	68 $\pm$ 7
ATP before p[NH]ppA	14 $\pm$ 2

Table 1. Effect of ATP on branched-chain 2-oxo acid dehydrogenase activity in detergent-treated or freeze-thawed preparations of skeletal muscle and kidney mitochondria

Skeletal muscle mitochondria were preincubated for 30min. Then ATP (0.2mM) or water was added and the incubation was continued for 5min. The mitochondria were then centrifuged at 10000 g for 10min, the pellet was washed in buffer and resuspended in fresh buffer containing 30mg of Triton X100/ml and assayed (Expt. 1). One sample of kidney mitochondria was incubated for 5min with or without an ATP (0.2mM)-regenerating system as described in the legend to Fig. 1. The mitochondria were washed as described above and assayed in the usual manner (Expt. 2). Another sample of kidney mitochondria was treated with Lubrol (0.15 mg/mg of protein) and centrifuged at 35000 g for 10min. One part of the supernatant was incubated for 5min with or without ATP as described above and assayed immediately (Expt. 3). Another part was precipitated with  $(\text{NH}_4)_2\text{SO}_4$  (50% satd.). The precipitate was washed and resuspended in fresh buffer and assayed (Expt. 4). Finally, some of the Lubrol-treated 35000 g supernatant was precipitated with  $(\text{NH}_4)_2\text{SO}_4$  before incubation with ATP. The precipitate was resuspended in fresh buffer and incubated with ATP and assayed as described for Expt. 3 of the Table (Expt. 5). In Expt. 6, kidney mitochondria were incubated with or without ATP, washed and then frozen and thawed three times as described by Pettit *et al.*, (1978). After centrifugation at 19000 g for 30min the supernatants were assayed. Activity is based on protein concentration of the freeze-thaw supernatant. All values are the means of three determinations.

Expt.	Tissue	Fraction incubated $\pm$ ATP	Fraction assayed	ATP removed prior to assay	Enzyme activity (nmol/h per mg of protein)		Inhibition (%)
					-ATP	+ATP	
1.	Skeletal muscle	Mitochondria	Triton X-100-treated 35000 g supernatant	Yes	9.9	1.2	88
2.	Kidney	Mitochondria	Mitochondria	Yes	105	27.2	74
3.	Kidney	Lubrol WX-treated 35000 g supernatant	Lubrol WX-treated 35000 g supernatant	No	107	7.3	93
4.	Kidney	Lubrol WX-treated 35000 g supernatant	$(\text{NH}_4)_2\text{SO}_4$ ppt.	Yes	188	15.9	93
5.	Kidney	$(\text{NH}_4)_2\text{SO}_4$ ppt.	$(\text{NH}_4)_2\text{SO}_4$ ppt.	No	64.5	61.8	5
6.	Kidney	Mitochondria	Freeze-thaw supernatant	Yes	1284	0	100

Since p[NH]ppA by itself has no effect on enzyme activity, inhibition of dehydrogenase activity by ATP appears to require the transfer of the  $\gamma$ -phosphate. However, the ATP metabolites ADP and AMP have little or no effect on enzyme activity (Table 3). In addition, preincubation of skeletal muscle mitochondria with ATP (in the absence of a regenerating system) results in complete hydrolysis of the nucleotide after 20–30 min without inhibition of the enzyme unless NaF is also present (Table 3).

At 27°C the dehydrogenase is inhibited extremely rapidly by ATP. Maximal inhibition is obtained in less than 1 min in mitochondria from skeletal muscle. To examine the kinetics, the reaction rate was decreased by cooling to 0°C. At this temperature, 50% inhibition occurs in approximately 6 min (Fig. 3) (although some variability in the time course is observed). The inhibition by ATP is blocked completely by p[NH]ppA at this temperature (Table 4). Therefore, to study the effect of test compounds on the rate of ATP inhibition, the reaction is initiated by the addition of the test agent in the presence or absence of ATP. After a 5 min incubation period at 0°C, the addition of 1 mM-p[NH]ppA blocks any further inhibition by ATP, although it does not affect the activity of the enzyme inactivated during the incubation. After centrifugation to wash out the additions, the mitochondria are suspended in fresh buffer and assayed.

4-Methyl-2-oxopentanoate, a substrate for the dehydrogenase, markedly decreases the rate of

inhibition by ATP (Table 4). The high concentration of pyruvate (1 mM) also protects against ATP inhibition. However, at lower concentrations (0.2 mM) pyruvate has little effect on the rate of inhibition by ATP. By contrast, NADH, an end product of the reaction, potentiates the inhibition by ATP. Dichloroacetate (1 mM), ADP (1 mM) and sodium pyrophosphate (2 mM), inhibitors of pyruvate dehydrogenase kinase (Cooper *et al.*, 1974), do not block ATP-stimulated inhibition of branched chain 2-oxo acid dehydrogenase. In fact, ADP and pyrophosphate are themselves inhibitory, probably due to a stimulation of ATP synthesis (Table 4). [This possibility is supported by the observation that addition of an ADP-regenerating system and AMP, to shift the adenylate kinase equilibrium, blocks the inhibitory effect of ADP (Table 3)]. Similar results have been reported by Parker & Randle (1978b).

The properties of the reactivation reaction of skeletal muscle mitochondria have also been studied. NaF, an inhibitor of phosphoprotein phosphatases (Linn *et al.*, 1972) blocks activation (Table 5 and Fig. 1). However, this compound has no effect on the activated enzyme or on mitochondrial ATP concentrations. Activation requires  $Mg^{2+}$  and is stimulated several-fold by enzyme cofactors, NAD and CoA. However,  $Ca^{2+}$  (0.1 mM) and phosphate (20 mM) have no effect on activation. Moreover,

Table 3. Effect of preincubation with nucleotides and their metabolites on activity of dehydrogenase

Skeletal muscle mitochondria were preincubated for 5 or 30 min. For the 5 min preincubation, nucleotides were added with NaF (25 mM) as follows: ATP, 0.2 mM with a regenerating system (see the legend to Fig. 1); ADP, 1 mM with a regenerating system (10 U of hexokinase/ml, 20 mM-glucose and 5 mM-AMP); AMP, 1 mM. For the 30 min preincubation ATP (0.2 mM) was added without a regenerating system or in the presence or absence of NaF (25 mM). After the preincubations the mitochondria were centrifuged, washed and assayed as described in the legend to Fig. 1. [The ADP used was freed of contaminating ATP by the procedure described by Lowry & Passonneau (1972)].

Preincubation (min)	Additions	Activity (% of control)
5	None	100
5	ATP (0.2 mM)	14
5	ADP (1 mM)	79
5	AMP (1 mM)	100
5	p[NH]ppA (1 mM)	123
30	ATP (no NaF)	89
30	ATP (25 mM-NaF)	11

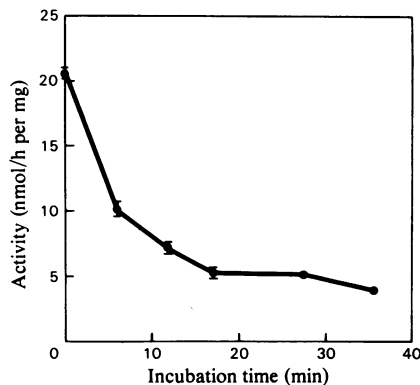


Fig. 3. Inhibition of dehydrogenase activity by ATP at 0°C

Skeletal muscle mitochondria were incubated for 30 min at 27°C as described in the legend to Fig. 1. NaF (25 mM) was added and the mixture was cooled to 0°C. After taking a sample for assay of initial activity (to which 1 mM-p[NH]ppA was added) the incubation was started by the addition of ATP (0.2 mM). The incubation was terminated by addition of p[NH]ppA (1 mM). All samples were centrifuged, washed and resuspended in fresh buffer containing NaF (25 mM) and Triton X-100 (1 mg/ml) and assayed. Each point represents the mean  $\pm$  S.E.M. of three determinations.

Table 4. *Effect of substances on inhibition of dehydrogenase activity by ATP at 0°C*

Skeletal muscle mitochondria were preincubated for 30min at 27°C. The mixture was divided into portions and cooled to 0°C. Then the test substance in the presence or absence of ATP (0.2mM) was added and incubated for 5 min. The incubation was terminated by addition of p[NH]ppA (1mM). Controls contained p[NH]ppA prior to addition of ATP or the test substances. The mixtures were centrifuged at 0°C at 35000g for 2min. The supernatant was discarded and the pellet was rinsed with buffer. The pellets were then resuspended in assay mixture and assayed. (To test the effect of p[NH]ppA the reaction mixture was centrifuged immediately and the pellet was washed; total time 6min.) Values represent the means of three determinations.

Expt.	Addition	Activity (% of control)		Change (%)
		-ATP	+ATP	
1	None	100	51	-49 ± 1.2†
	p[NH]ppA (1mM)	94	95	0 ± 0.4*
	4-Methyl-2-oxopentanoate (1mM)	162	139	-16 ± 1.2*†
	Pyruvate (1mM)	111	92	-17 ± 3.9*†
2	None	100	79	-21 ± 1.2†
	4-Methyl-2-oxopentanoate (0.2mM)	92	96	+4 ± 2.1*
	Pyruvate (0.2mM)	95	82	-14 ± 3.0†
	2-Oxoglutarate (0.1mM)	92	76	-19 ± 3.0†
	NADH (0.64mM)	83	53	-36 ± 2.0*†
3	None	100	46	-54 ± 3.4†
	Dichloroacetate (1mM)	100	49	-51 ± 1.2†
	Sodium pyrophosphate (2mM)	50	37	
	ADP	50	39	

\* Significant effect of addition on ATP inhibition,  $P < 0.01$ .

† Significant effect of ATP,  $P < 0.01$ .

Table 5. *Effect of various treatments on activation of branched-chain 2-oxo acid dehydrogenase in skeletal muscle mitochondria*

From freshly isolated skeletal muscle mitochondria, one sample was taken for assay of initial activity and one sample was preincubated for 30min as described in the legend to Fig. 1 with the additions or omissions or conditions shown in the Table, and then assayed. The pH of all mixtures was adjusted to 7.8 before incubation. Sonication was performed before preincubation with a Branson Sonifier (setting 4) for 3 × 20s intervals at 0°C. Unlike previous and subsequent experiments NaF and Triton X-100 were present in the assay mixtures only where indicated. Since the amount of mitochondria in all experiments was approximately equal (approx. 1mg/ml), this may account for the differences in initial values due to a small degree of activation during the 5min assay. Note also that incubation mixtures contain EGTA (2.5mM) and MgCl<sub>2</sub> (5mM). Therefore, addition of EDTA represents the removal of Mg<sup>2+</sup> and the CaCl<sub>2</sub> added represents a free [Ca<sup>2+</sup>] of 0.1mM. Each point represents the mean of three determinations. All values in a particular experiment represent equal amounts of enzyme.

Treatment	Activity (nmol/h)		Activation (Preincubated/ initial)
	Initial	Preincubated	
1. Control	3.2	15.5	4.8
+Triton X-100 (1mg/ml)	1.4	1.3	0.9
2. Control*	0.3	6.9	23
+CaCl <sub>2</sub> (2.6mM)	0.3	6.3	21
+CaCl <sub>2</sub> + Triton X-100	0.3	0.3	1.0
3. Control	2.6	16.0	6.2
+NaF (25mM)	1.4	1.4	1.0
4. Control	3.4	13.5	4.0
+EDTA (7mM)	3.2	1.8	0.6
5. Control	3.4	13.5	4.0
+P <sub>i</sub> (30mM)	2.5	11.9	4.8
6. Control	1.4	13.6	9.7
+Sonication	1.4	1.1	0.8
7. Control	3.4	13.5	3.9
-NAD, CoA, TPP†	3.7	7.0	2.0

\* NaF added to assay mixture after preincubation.

† Cofactors added back to mixture during assay for activity.

Table 6. *Effect of various substances on activated dehydrogenase from skeletal muscle*

Skeletal muscle mitochondria were preincubated for 30 min and then were assayed in the usual manner with the additions or omissions shown in the table. Each value is the mean of three determinations.

Addition (+) or omission (-)	Activity (% of control)
None	100
+ Triton X-100 (2 mg/ml)	101
+ NaF (25 mM)	101
+ EDTA (10 mM)	86
+ Ca <sup>2+</sup> (0.5 mM)	95
- NAD, CoA	2
Sonication	60
+ P <sub>i</sub> (30 mM)	104

Table 7. *Effect of activation and ATP on dehydrogenase kinetics*

From freshly isolated muscle mitochondria, samples were taken for assay of initial activity. The remaining mitochondria were preincubated for 30 min and then were assayed. In a separate experiment samples of preincubated mitochondria were further incubated with ATP as described in the legend to Fig. 1. The mitochondria were centrifuged, washed and assayed. Five concentrations of substrate (10–100  $\mu$ M) were used for evaluation of the  $K_m$  and  $V_{max}$ , as described in the Materials and methods section.

Sample	$K_m$ ( $\mu$ M)	$V_{max}$ (nmol/h)
Unincubated	17.4 $\pm$ 4.5	3.6 $\pm$ 0.3
Incubated for 30 min	24.2 $\pm$ 3.5	36.9 $\pm$ 1.8
Incubated for 30 min	22.0 $\pm$ 4.1	34.8 $\pm$ 1.4
Incubated + ATP (0.2 mM)	27.3 $\pm$ 5.2	0.78 $\pm$ 0.06

Ca<sup>2+</sup> cannot restore the ability to activate the detergent-treated enzyme. The activating activity appears to be quite labile, since treatment with Triton X-100 or sonication eliminates activation (Table 5). The effects of these compounds on activation are clearly different from their effects on enzyme activity. Neither detergent, F<sup>-</sup> nor divalent cations significantly affect activated enzyme activity under these conditions (Table 6). In addition, the pH optimum for activation (pH 8.3) is higher than the optimum for the activated dehydrogenase (pH 7.4).

If activation and inhibition represent processes acting on the same site, they should affect the same kinetic property. Preincubation for 30 min increases the  $V_{max}$ , 10-fold but has no significant effect on  $K_m$  for 4-methyl-2-oxopentanoate (Table 7). Addition of ATP for 5 min to the preincubated mitochondria followed by washing results in a 40-fold reduction in  $V_{max}$ , but has little effect on  $K_m$ . Therefore, both

activation and inhibition by ATP affect only the  $V_{max}$  of the dehydrogenase, not the apparent affinity for its substrate.

## Discussion

The experiments reported here show that inhibition of branched chain 2-oxo acid dehydrogenase is effected by a brief preincubation with ATP, which need not be present during the subsequent assay for enzyme activity (Fig. 1). This inhibition can be reversed in Mg<sup>2+</sup>-containing buffers and the regulatory system appears qualitatively similar in all tissues studied (i.e., heart, skeletal muscle, kidney and liver) (Fig. 2).

Since p[NH]ppA, a sterically similar analogue that cannot be cleaved in the  $\gamma$ -phosphate position (Yount *et al.*, 1971) has no inhibitory effect on the enzyme (Tables 1 and 2), the inhibition by ATP appears to require transfer of the terminal phosphate group. In addition, AMP and ADP do not inhibit to any great extent (Table 1). The inhibition probably occurs by covalent modification rather than by ligand association and dissociation. Thus, the non-metabolizable analogue p[NH]ppA competitively blocks the inhibition when added with ATP, but has no effect when added to the ATP-inhibited enzyme (Table 2). The inhibition is not reversed by dilution or removal of ATP even in mitochondria disrupted with high concentrations of detergent or by freeze-thawing (Table 1). Inhibition remains even after precipitation of the enzyme with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Direct products of ATP metabolism are not inhibitory (Table 3). Possible inhibitors (e.g. NADH) that may have been produced by the mitochondria in the presence of ATP should have been removed by the washing procedures and the detergent treatment. Furthermore, the minute amounts of ATP remaining in washed mitochondria are not significantly different in active and inactive preparations. These data support the contention that the inhibition is covalent in nature and occurs with the dehydrogenases from several different tissues.

Parker & Randle (1978b) working with rat heart mitochondria have obtained data on branched-chain 2-oxo acid dehydrogenase activity that are consistent with the present data. These investigators also noted a 5–15-fold increase in activity upon incubation of mitochondria in the absence of substrate. The activated enzyme was rapidly re-inhibited by incubation with ATP at 0°C. Addition of succinate prevented activation, presumably by stimulating the synthesis of intramitochondrial ATP. This effect could be blocked by an uncoupler of oxidative phosphorylation. 4-Methyl-2-oxopentanoate blocked inhibition by exogenous ATP or by succinate-stimulated intramitochondrial ATP. Interestingly, these investigators could not reactivate the ATP-

inhibited enzyme. This may be due to their use of Triton X-100 (Table 5) during the incubation or to unfavourable conditions in the mitochondria after their manipulations to remove the nucleotide (e.g. low  $[Mg^{2+}]$ , low pH).

Gubler & Malquist (1979) have reported that high concentrations of  $Mg^{2+}$  stimulate branched-chain 2-oxo acid dehydrogenase activity of rat liver mitochondria in the presence of ATP. While consistent with present findings, their results are difficult to interpret, since extra- and intramitochondrial conditions are unknown, especially after the long incubations used (90 min).

These properties are consistent with the possibility that the dehydrogenase is regulated by enzyme phosphorylation. A protein kinase is postulated, which would inhibit the active enzyme. Dehydrogenase activity would be restored by the action of a phosphoprotein phosphatase. However, other possible ATP-requiring modifications such as adenylation (Shapiro & Stadtman, 1968), covalent attachment of ADP-ribose groups (Adler *et al.*, 1975) and methylation (Aswad & Koshland, 1974) cannot be ruled out. A definitive test of the phosphorylation mechanism must await the demonstration of  $^{32}P$  incorporation into the purified enzyme, as has been shown in the case of pyruvate dehydrogenase (Linn *et al.*, 1969; Wieland & Siess, 1970) and several other enzymes (Rubin & Rosen, 1975; Nimmo & Cohen, 1977).

Although the activation-deactivation mechanism occurs in mitochondria of a number of tissues, including kidney of several species, two groups (Pettit *et al.*, 1978; Danner *et al.*, 1979) have failed to find any inhibition of the dehydrogenase by ATP in mitochondrial extracts or purified enzyme from bovine kidney. Possibly, the freeze-thawing procedure employed by this group to obtain a 'soluble' mitochondrial extract may have caused the removal or destruction of the inactivating activity. In this context it is interesting that the rat kidney dehydrogenase, after precipitation by  $(NH_4)_2SO_4$ , was also not inhibited by ATP (Table 1). However, Lubrol-solubilized extracts of rat kidney mitochondria are inhibited by ATP (Table 1).

The activation-deactivation cycle is quite sensitive to the substrates and products of the branched-chain 2-oxo acid dehydrogenase. Inhibition is potentiated by the product (NADH) and decreased by the substrates (4-methyl-2-oxo pentanoate and pyruvate) (Table 4). Conversely NAD, CoA and TPP together stimulated activation of the dehydrogenase. Activation is blocked by removal of divalent cations (with EDTA) and by NaF (Table 5). These findings are similar in some respects to what is known of the regulation of pyruvate dehydrogenase kinase and phosphatase (Cooper *et al.*, 1974; Pettit *et al.*, 1975; Randle, 1976). However, there also also

significant differences. Thus, dichloroacetate, a potent inhibitor of pyruvate dehydrogenase kinase (Cooper *et al.*, 1974) has no effect on the inhibition by ATP of the branched-chain 2-oxo acid dehydrogenase (Table 4). Also,  $Ca^{2+}$ , which is required for the activation of pyruvate dehydrogenase, has no effect on activation of branched-chain 2-oxo acid dehydrogenase (Table 5).

The present findings indicate that branched-chain 2-oxo acid dehydrogenase can be controlled by its substrates and products in two distinct ways: (a) by reversible ligand binding as has been shown for the pure or semipure enzyme (Parker & Randle, 1978a; Pettit *et al.*, 1978; Odessey & Goldberg, 1979) and as regulators of an activation-inactivation cycle (Tables 4 and 5). Thus, as in the case of pyruvate dehydrogenase (Randle & Denton, 1976) the mitochondrial redox potential (NAD/NADH ratio) may alter branched-chain 2-oxo acid dehydrogenase activity by end-product inhibition or by affecting the extent of covalent modification. Studies on incubated diaphragm raise the possibility that the redox potential may influence leucine oxidation in intact tissue (Buse *et al.*, 1976). Also, the ability of the branched-chain 2-oxo acids to block the inactivation of the enzyme (Table 4; Parker & Randle, 1978b) may help explain how alterations in the amount of dietary protein can influence branched-chain amino acid catabolism (Wohlhueter & Harper, 1970; Sketcher *et al.*, 1974).

The ability of the dehydrogenase to undergo an inactivation by ATP that remains stable during isolation may also explain in part the great variability of enzyme activity in the hands of various investigators (Dancis *et al.*, 1961; Goedde & Keller, 1967; Connelly *et al.*, 1968; Sketcher *et al.*, 1974). These present results imply that comparisons of dehydrogenase activity (Paul & Adibi, 1976, 1978a,b) in different tissues or under different conditions must take into account the state of activation of the enzyme.

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