NEUROSPORA CRASSA PYRUVATE DEHYDROGENASE: INTERCONVERSION BY PHOSPHORYLATION AND DEPHOSPHORYLATION *

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1. Introduction

Interconvertible forms of glycogen phosphorylase and glycogen synthetase have been reported to occur in *Neurospora crassa* wild-type strain L 74 [1, 2]. It was our aim to establish whether in this organism pyruvate dehydrogenase is also regulated by covalent modification as known from the mammalian enzyme. As will be shown in this paper the pyruvate dehydrogenase complex from *N. crassa* strain L 74 contains the regulatory kinase and phosphatase subunits and is inactivated and reactivated by phosphorylation and dephosphorylation reactions.

2. Experimental

Conditions of culturing *N. crassa* wild-type strain L 74 and of harvesting and disruption of the hyphae by a grind mill were essentially as described [3]. (We are greately indebted to Dr. H. Weiss for carrying out this work at the Institut für Physiologische Chemie, University of Munich). After separation of the mitochondrial pellet at 8000 *g* the supernatant contained 75% of pyruvate dehydrogenase activity; the remainder was found in the mitochondria. Obviously, the major part of the mitochondria is broken up already in the grind mill which is also indicated by the small yield of mitochondria (26%) by this procedure [3]. For the following studies the 8000 *g* supernatant was used. Pyruvate dehydrogenase activity was determined spectrophotometrically by NADH-formation, as described [4].

* This work is part of the Diplomarbeit of Miss Ute Hartman, University of Tübingen, Germany.

Protein was determined by the Biuret method. All chemical reagents were analytical grade and purchased from Merck, Darmstadt. Enzymes and coenzymes were products of Boehringer, Mannheim.

3. Results and discussion

Upon incubation of the 8000 *g* supernatant fraction from *N. crassa* with ATP, pyruvate dehydrogenase is inactivated in a time dependent manner (fig. 1). Increasing the Mg^{2+}-concentration to 10 mM resulted in a gradual reappearance of enzyme activity which was completed after 120 min. At this time the activity was about 40% higher than at the beginning of the experiment indicating that the enzyme was present only partially in the active form. In fact, about the same gain in activity was observed when the enzyme was incubated with 10 mM Mg^{2+} without previous ATP-treatment (not shown). As may be noted in fig. 1 the effect of ATP is not complete and levels off at about 50% inactivation. This is explained by rapid cleavage of ATP due to ATP-ase activity of the crude *Neurospora* extract. For further studies pyruvate dehydrogenase was therefore purified starting from the 8000 *g* supernatant (table 1).

After gradient centrifugation the enzyme was still susceptible to ATP which led now to complete inactivation. However, reactivation was not achieved until – in addition to Mg^{2+} – protein from the top layer of the gradient was added. From these findings it would appear that the enzyme from *N. crassa* – like that from mammalian tissues [5] – exists in an active and an inactive form and is converted from one form to the other by an ATP-dependent kinase and a Mg^{2+}-dependent phosphatase the latter being separated during the gradient centrifugation step.

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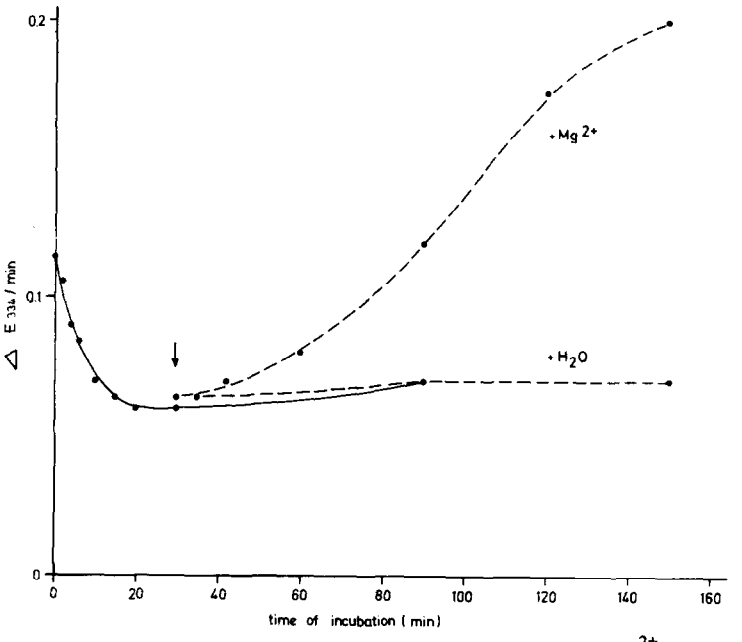

Fig. 1. ATP-dependent inactivation of *N. crassa* pyruvate dehydrogenase and reactivation by Mg^{2+}. 240 μl of 8000 *g* supernatant were incubated at 25° in a medium containing 20 mM potassium phosphate buffer, pH 7.0, 0.1 mM $MgCl_2$, and 0.25 mM ATP, final volume 300 μl. After 30 min. (at arrow) $MgCl_2$ was added to give a final concentration of 10 mM. Another portion received H_2O instead of Mg^{2+}. 10 μl samples were withdrawn at the times indicated and assayed spectrophotometrically for pyruvate dehydrogenase activity [4].

Table 1
Purification of pyruvate dehydrogenase from *N. crassa* strain L 74.

Step*	Vol (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification factor	Recovery (%)
(1) 8000 *g* supernatant	900	5400	117	0.022	1	100
(2) First $(NH_4)_2SO_4$ precipitate	55	1870	110	0.059	2.7	94
(3) Phosphate gel	170	1020	69.7	0.068	3.1	60
(4) Second $(NH_4)_2SO_4$ precipitate	6	252	48	0.19	8.7	41
(5) Glycerol gradient centrifugation	6	3	9.6	3.2	145	8.2

* (1) The procedure leading to the 8000 *g* supernatant was as described [3].
(2) Add 250 g $(NH_4)_2SO_4$, centrifuge, dissolve precipitate in 20 mM potassium phosphate buffer, pH 7, dialyse 3 hr against same buffer.
(3) To 50 ml (2) add 43 g $Ca_3(PO_4)$-Gel (dry weight 28 mg/ml), centrifuge. Fractional elution (1 × with 80 ml, 3 × with 30 ml each) of a mixture of 0.5 M $(NH_4)_2SO_4$, 1 mM $MgCl_2$, 2 mM mercaptoethanol adjusted to pH 7.6 with NH_3.
(4) To combined eluates (3) add solid $(NH_4)_2SO_4$ to reach a concentration of 2 M (check by $BaCl_2$-Titration [11]), centrifuge, dissolve in 5 ml 20 mM phosphate buffer, pH 7 containing 2 mM dithiothreitol, dialyse 4 hr against same buffer.
(5) Spin 7 hr at 27000 rpm (SW 27) on linear gradient from 10–50% glycerol essentially as desribed [12]. Collect 2 ml fractions, combine fractions 4–8 and concentrate by ultrafiltration. Phosphatase activity is present in the last three (top) fractions.

The steps were essentially adopted from the procedure described for pig heart pyruvate dehydrogenase purification [8].

This view was confirmed by labelling studies with [γ-^{32}P]ATP.

As may be seen from fig. 2 pyruvate dehydrogenase inactivation is accompanied by concurrent ^{32}P-incorporation into the protein. Upon addition of the (phosphatase containing) gradient fraction this situation is reversed. Due to the difficulty to obtain a more active phosphatase preparation ^{32}P-release and reactivation proceed rather sluggish and could not be followed to completeness.

ITP, GTP, CTP, and UTP were only little effective when compared to ATP in the inactivation reaction. There was no influence on the rate of inactivation by ADP (0.01–1 mM) and only little effect of pyruvate both of them effectively protecting mammalian pyruvate dehydrogenases from ATP inactivation [6, 7]. Inorganic phosphate, 5 mM, definitely stimulated inactivation of the *Neurospora* enzyme by ATP. Reactivation was maximal at 10 mM Mg^{2+} (which could be replaced by Mn^{2+}) and was not influenced by 5′-AMP or 3′, 5′-cyclo APM.

As shown previously, purified heart muscle phosphatase is strongly inhibited by EGTA and this is overcome by Ca^{2+} indicating that the enzyme – in addition to Mg^{2+} – requires Ca^{2+} as a cofactor [8]. No such an effect of EGTA could be observed with the *N. crassa* preparation.

The phospho-forms of mammalian pyruvate dehydrogenase complexes are dephosphorylated not only by their tissue homologous phosphatases but also by phosphatases derived from other tissues [6, 7]. As illustrated in fig. 3 purified pig heart phosphatase was able to reactivate the phospho-form of *N. crassa* pyruvate dehydrogenase although the reaction sets in only after a certain lag period. Conversely, the phosphatase preparation from *N. crassa* did also exhibit activity towards the phosphorylated heart muscle enzyme yet at a much slower rate than obtained with the pig heart phosphatase.

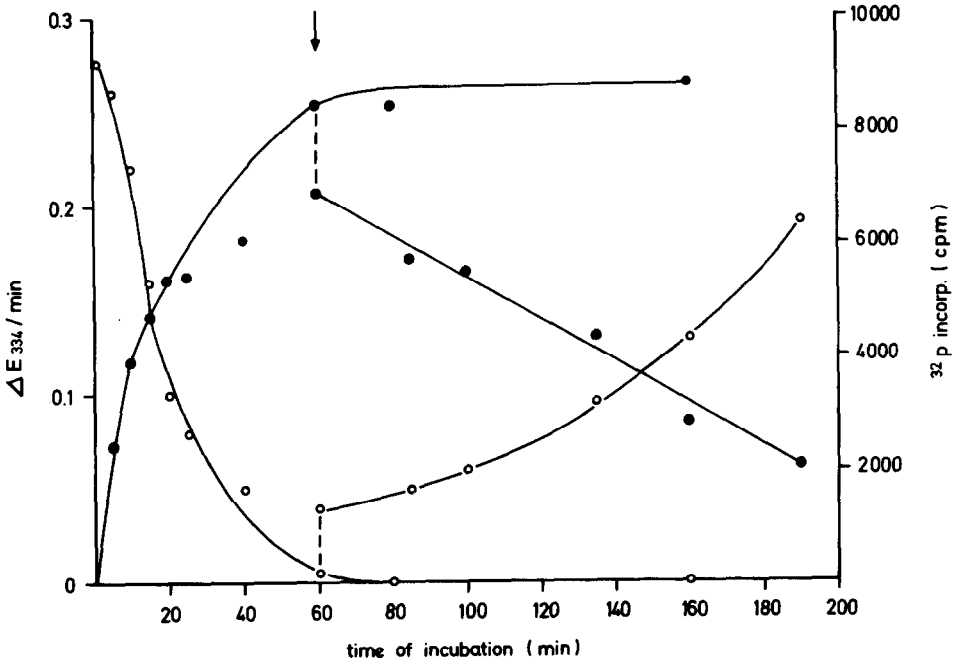

Fig. 2. Inactivation and phosphorylation of *N. crassa* pyruvate dehydrogenase with [γ-^{32}P]ATP. 280 μl pyruvate dehydrogenase (glycerol gradient fraction, see table 1) corresponding to 140 μg protein were incubated at 25° in a mixture consisting of 0.1 mM [γ^{32}P]ATP (corresponding to 3.6×10^6 cpm), 0.1 mM $MgCl_2$, and 20 mM potassium phosphate buffer, pH 7, final volume 400 μl. After 60 min a 85 μl sample of the reaction mixture was removed and after mixing with 15 μl of 0.1 M $MgCl_2$ and 50 μl (= 19.5 μg protein) of glycerol gradient top layer containing phosphatase activity, further incubated at 25°. At the times indicated 10 μl samples were taken for measuring pyruvate dehydrogenase activity (o—o—o) and ^{32}P-incorporation into the protein (•—•—•) according to [10].

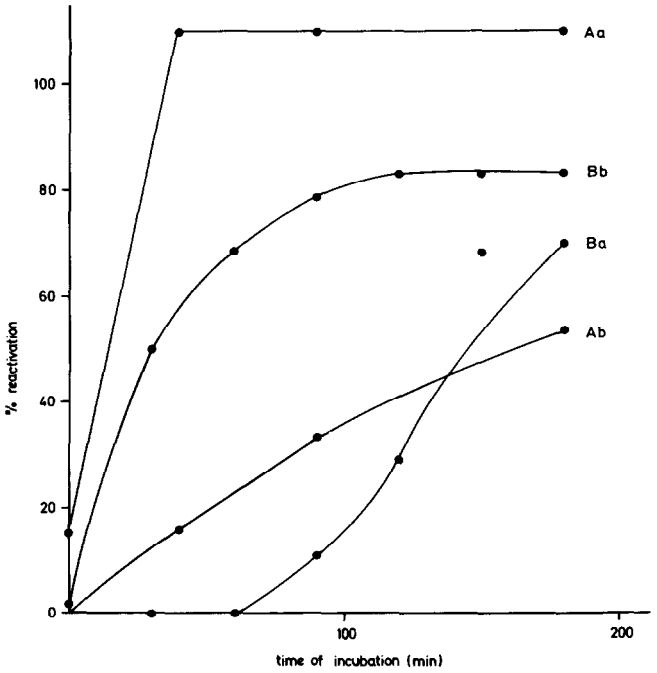

Fig. 3. Cross reactivity of *N. crassa* phosphatase with pig heart phospho pyruvate dehydrogenase and vice versa. Purified pyruvate dehydrogenase from pig heart and from *N. crassa*, respectively was first inactivated by incubation with 0.05 mM ATP in 20 mM potassium phosphate buffer, pH 7 containing 0.1 mM $MgCl_2$ for 30 min at 25°. Thereafter 50 µl samples were withdrawn and, after increasing Mg^{2+} concentrations to 10 mM, reactivation was started by addition of phosphatase. Aa) pig heart phospho-PDH + 75 µg pig heart phosphatase; Ab) pig heart phospho-PDH + 34 µg *N. crassa* phosphatase; Bb) *N. crassa* phospho-PDH + 34 µg *N. crassa* phosphatase; Ba) *N. crassa* phospho-PDH + 75 µg pig heart phosphatase. On replacement of phosphatases by buffer solution no reactivation was detectable.

Table 2
Kinetic parameters of *N. crassa* pyruvate dehydrogenase.

		N. crassa	Heart muscle *
Substrates:		(K_m)	(K_m)
	Present study	Ref. [9]	
Pyruvate	2.4×10^{-4}	2.6×10^{-4}	2.8×10^{-5}
NAD^+	2.3×10^{-4}	1.5×10^{-4}	4×10^{-5}
CoASH	1.1×10^{-5}	1.1×10^{-5}	5×10^{-6}
Inhibitors:		(K_i)	(K_i)
Acetyl-CoA	1.1×10^{-5}	1.8×10^{-5}	2.9×10^{-5}
NADH	4.9×10^{-5}	3.4×10^{-5}	

* Data from O. Wieland, B. v. Jagow-Westermann and B. Stukowski, Hoppe-Seyler's Z. Physiol. Chem. 350 (1969) 329.

Table 2 summarizes some kinetic parameters of *N. crassa* pyruvate dehydrogenase as compared to the enzyme from pig heart muscle. As may be seen the *N. crassa* enzyme is also inhibited by acetyl-CoA (competitively with CoA-SH) and by NADH (competitively with NAD^+). Our data are in close accordance with the findings of Harding et al. who have also observed an inhibition by ATP [9]. The mechanism of this inhibition, however, was not explained by these authors. Since they have studied a different strain of *N. crassa* the possibility exists that ATP acted in a different manner. Investigations to clarify this point are now in progress.

Acknowledgement

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