

The pyruvate dehydrogenase complex of *Saccharomyces cerevisiae* is regulated by phosphorylation

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Abstract Mitochondria were isolated from *Saccharomyces cerevisiae* grown on different carbon sources prior to incubation with [γ - 32 P]ATP. A major 46,000-M_r phosphoprotein, corresponding in M_r value to the E1 α subunit of the yeast pyruvate dehydrogenase complex (PDC), was detected only in mitochondria isolated from cells grown on a fermentable carbon source such as galactose. Immunoprecipitation with subunit-specific antiserum to the E1 component of mammalian or yeast PDC confirmed the identity of this polypeptide. PDC activity in isolated yeast mitochondria could be inactivated in an ATP-dependent fashion and reactivated in the presence of Ca²⁺ ions.

Key words: Yeast; Pyruvate dehydrogenase complex; Phosphorylation; Inactivation and reactivation

1. Introduction

The mitochondrial pyruvate dehydrogenase multienzyme complex (PDC) occupies a key position in carbohydrate metabolism in the eukaryotic cell, catalysing the irreversible conversion of pyruvate to acetyl CoA. Activity of PDC from eukaryotic sources, including mammalian, avian, and plant tissues and *Neurospora crassa* is regulated by a phosphorylation-dephosphorylation cycle [1–3]. Phosphorylation by PDC kinase inactivates the complex, while dephosphorylation by a specific PDC phosphatase results in its reactivation. The phosphorylation sites in mammalian PDC are located on three particular serine residues in the α subunit of the pyruvate dehydrogenase (E1) component of the complex [4–6].

Attempts thus far to demonstrate PDC kinase activity in yeast have been unsuccessful [7]. However, the first indirect evidence that yeast PDC may be regulated in vivo by a phosphorylation-dephosphorylation mechanism was presented by Uhlinger et al. [8]. Highly purified PDC from *S. cerevisiae* can be phosphorylated and inactivated by a purified heterologous (bovine heart) PDC kinase, in the presence of [γ - 32 P]ATP in which protein-bound radioactivity was localised in the E1 α subunit of the complex. Furthermore, dephosphorylation and reactivation of the phosphorylated, inactive complex could be effected by PDC phosphatase isolated from bovine heart. Tryptic digestion of the 32 P-labelled complex yielded a single phosphopeptide, which was purified to homogeneity and shown to possess significant sequence homology to the major phosphorylation site peptide derived from the E1 α subunit of bovine kidney and heart PDC. Cloning of the subunits of mam-

malian PDC kinase has also revealed its similarity to an *E. coli* histidine kinase [9,10].

In this paper, the first direct evidence for phosphorylation of yeast PDC is derived from in situ analysis of intact yeast mitochondria purified from yeast cultures growing logarithmically on a fermentable carbon source.

2. Materials and methods

Saccharomyces cerevisiae, strains D273-10B and S288C, were kindly supplied by Professor J.R. Coggins, University of Glasgow and were grown routinely in culture medium (1% (w/v) yeast extract, 2% (w/v) peptone, 0.1M KP_i, pH 6.0) supplemented with 2% (w/v) galactose (YPG). Starved rats (Wistar strain, 200–220 g) were provided by the University animal house and killed by cervical dislocation. Rat liver and yeast mitochondria were prepared essentially as described by Chance and Hagihara [11] and Rickwood et al. [12], respectively. Radiolabelling experiments were carried out on freshly isolated, intact mitochondria suspended in the requisite isolation buffer in a final assay volume of 0.25 ml. Mitochondria (0.5 mg or 0.1 mg protein/assay) were labelled in the presence of 10 μ Ci [γ - 32 P]ATP (approx. 3 Ci/mmol). Each assay also included the following reagents to the denoted final concentrations: ATP (0.2 mM), NaF (25 mM), phenylmethylsulphonyl fluoride (0.5 mM), benzamidinium-HCl (0.5 mM), leupeptin (0.3 μ M). Following incubation at 37°C for 15 min, the mitochondria were pelleted (10,000 \times g for 5 min) and processed for analysis by SDS/polyacrylamide gel electrophoresis and autoradiography as described previously [13,14].

Purified PDC and its individual constituent enzymes isolated from bakers' yeast were kindly supplied by Dr Hans Bisswanger, University of Tübingen [7, 15]. Subunit-specific antibodies to the individual highly-purified E1, E2 and E3 enzymes were raised in rabbits and their specificity confirmed by immunoblotting against purified yeast and mammalian PDC.

Phosphoproteins were immunoprecipitated from [γ - 32 P]ATP-labelled yeast mitochondria by employing the method of Jones and Yeaman [14]. Experiments were performed on 32 P-labelled mitochondrial pellets containing 0.1 or 0.5 mg protein, prepared as described above and levels of antisera determined (50 μ l) which promoted complete precipitation of the 46,000-M_r phosphoprotein from detergent-solubilised mitochondrial extracts. Pre-immune rabbit serum and subunit-specific antiserum to the yeast and mammalian PDC E1 subunits were prepared as described previously [13]. The dissociated immunoprecipitates were processed for analysis by SDS/polyacrylamide gel electrophoresis and autoradiography.

For analysis of PDC activation and reactivation in yeast grown on galactose, freshly-isolated mitochondria were suspended in the final isolation buffer at 2 mg \cdot ml⁻¹ mitochondrial protein and incubated in the presence of 2 mM Mg-ATP with vigorous stirring. Separate batches of mitochondria were also treated with ATP until complete inactivation was achieved (30–40 min), then pelleted and resuspended in isolation medium containing 2 mM CaCl₂ or 30 mM MgCl₂. Samples (400 μ l) were taken at various time intervals and the mitochondria pelleted by centrifugation in Eppendorf tubes at 14,000 \times g for 10 min. Mitochondria were lysed in isolation buffer containing 0.25% (v/v) Triton X-100 and PDC activity assayed in a reaction mixture containing 2.6 mM cysteine, 2.5 mM NAD⁺, 2 mM thiamine pyrophosphate, 1 mM MgCl₂, 0.13 mM CoASH, 2 mM sodium pyruvate and 50 mM KH₂PO₄, pH 7.4.

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Abbreviations: PDC, pyruvate dehydrogenase complex; BCOADC, branched-chain 2-oxoacid dehydrogenase complex.

The concentration of protein in mitochondrial extracts was determined routinely by the method of Lowry et al. [16] as modified by Markwell et al. [17].

3. Results and discussion

3.1. Profile of phosphoproteins in rat liver and yeast mitochondria and differing sensitivities to dichloroacetate

Initially optimal conditions for obtaining maximal incorporation of radioactivity from [γ - 32 P]ATP into isolated mitochondria were developed employing these organelles purified from rat liver. In addition to serving as a positive control in subsequent [γ - 32 P]ATP-labelling of isolated yeast mitochondria, these pilot studies facilitated the establishment of suitable conditions for obtaining similar radiolabelling of yeast mitochondria. Fig. 1 illustrates the [γ - 32 P]ATP-dependent incorporation of radioactivity into rat liver mitochondrial proteins under the conditions described in section 2. The two prominent polypeptides observed in lanes 1 and 2 are presumed to represent the E1 α subunits of the pyruvate and branched-chain 2-oxo acid dehydrogenase multienzyme complexes (PDC and BCOADC). These radiolabelled components with M_r values 41,000 and 46,000, respectively, have been previously identified as the only two reversibly phosphorylated proteins known to be present in mammalian mitochondria [3–5,14]. Both PDC and BCOADC contain distinct intrinsic kinases which co-purify with the complexes, inactivating them in an ATP-dependent reaction. The identity of the phosphorylated subunits in Fig. 1 is confirmed by the observation that 32 P incorporation into these polypeptides is sensitive to the mammalian kinase inhibitor, dichloroacetate (lanes 3–6), an analogue of pyruvate which is a substrate for both complexes. It is also evident that the BCOADC kinase is inhibited more rapidly at lower concentrations of dichloroacetate than its counterpart. The presence of two additional phosphorylated species with lower M_r values than the E1 α subunits, is apparent in lanes 1 and 2. The origin of these polypeptides is unknown but they may represent minor proteolytic fragments of the two main phosphoproteins since their phosphorylation is also inhibited by dichloroacetate.

S. cerevisiae, strains D273-10B and S288C, were initially grown in YPG and in culture media supplemented with a vari-

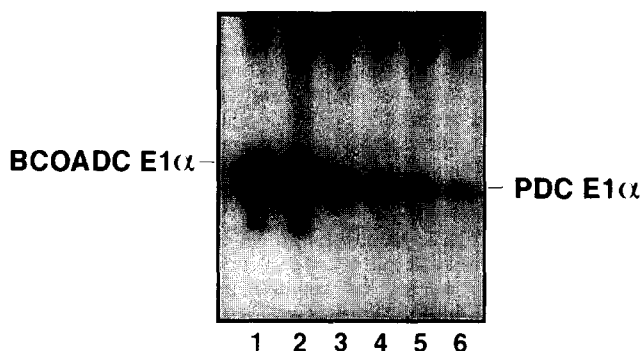


Fig. 1. PDC kinase and BCOADC kinase activities in rat liver mitochondria. Mitochondria (0.5 mg/assay) were incubated with [γ - 32 P]ATP (10 μ Ci/assay) and in some cases, the mammalian kinase inhibitor, dichloroacetate as described in section 2. Mitochondria were pelleted and solubilised in Laemmli sample buffer prior to resolution on a 12.5% (w/v) SDS/polyacrylamide gel which was processed for autoradiography. Lanes: 1 and 2, ATP-radiolabelled rat liver mitochondria; 3–6, in the presence of dichloroacetate (1, 2, 5 and 10 mM, respectively).

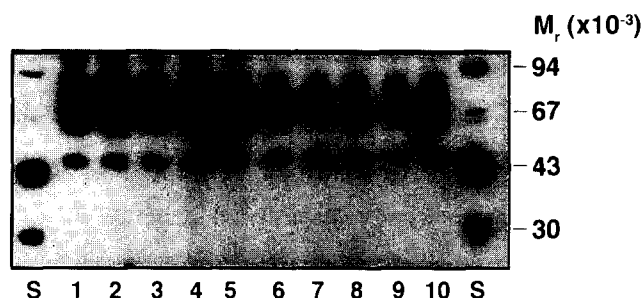


Fig. 2. Incubation of yeast mitochondria with [γ - 32 P]ATP. Yeast (*S. cerevisiae* strains D273-10B and S288C) mitochondria (0.5 mg/assay) were incubated with [γ - 32 P]ATP (10 μ Ci/assay) and in some cases, the mammalian kinase inhibitor, dichloroacetate as described in section 2. Mitochondria were pelleted and solubilised in Laemmli sample buffer prior to resolution on a 12.5% (w/v) SDS/polyacrylamide gel which was processed for autoradiography. Lanes: 1, ATP-radiolabelled D273-10B mitochondria; 2–5, in the presence of dichloroacetate (1, 2, 5 and 10 mM, respectively); 6–10, identical to lanes 1–5 except using S288C mitochondria; S, 125 I-labelled M_r markers.

ety of carbon sources: 2% (w/v) glucose (YPD); 2% (v/v) glycerol, 3% (v/v) ethanol (YPEG); 0.1% (w/v) glucose, 3% (v/v) glycerol (YPDG); 2% (w/v) sodium succinate (YPS); 2% (w/v) sodium lactate (YPL). Mitochondria were isolated and labelled with [γ - 32 P]ATP as described for the equivalent mammalian organelles. Specific incorporation of radioactivity into a 46,000- M_r polypeptide was detected only in mitochondria isolated from yeast grown on galactose as a carbon source. The 46,000- M_r yeast mitochondrial phosphoprotein was not observed after growth of either strain on YPD, YPEG, YPDG, YPS or YPL media.

After growth on YPG, the presence of a 46,000- M_r phosphoprotein, which may represent PDC E1 α , was apparent in mitochondrial extracts originating from both strains of yeast (Fig. 2). However, in each case, no reduction was observed in phosphorylation of the putative PDC E1 α subunit in the presence of increasing concentrations of dichloroacetate (lanes 2–5 and 7–10). This indicates that the kinase responsible for phosphorylating this polypeptide is unaffected by or less susceptible to dichloroacetate inhibition than its mammalian counterpart. A similar lack of inhibition was observed in detergent-solubilised mitochondrial extracts indicating that this observation did not merely reflect lack of permeability of yeast mitochondria to dichloroacetate. Recently, it has been found that phosphorylation of plant mitochondrial PDC is also not subject to inhibition by dichloroacetate over the same concentration range (R.M. Cook, J.G. Lindsay and H.G. Nimmo, unpublished observations). Mitochondria from the two yeast strains exhibited identical phosphorylation patterns with both displaying additional phosphorylated bands, one with an approx. M_r value of 94,000 and a broad band in the M_r range 70–80,000. The identities of these phosphoproteins are unknown at present.

3.2. Immunological identification of the 46,000 M_r -phosphorylated polypeptide of yeast mitochondria

Positive identification of the 46,000- M_r yeast mitochondrial phosphoprotein as the E1 α component of PDC was achieved by specific immunoprecipitation of this protein from 32 P-labelled mitochondrial extracts. As illustrated in Fig. 3, the



Fig. 3. Immunoprecipitation of the E1 α subunit from [γ - 32 P]ATP-labelled yeast mitochondria. Yeast (*S. cerevisiae* strain D273-10B) mitochondria were incubated with [γ - 32 P]ATP (10 μ Ci/assay) and subjected to immunoprecipitation with anti-yeast E1 serum, anti-bovine heart E1 serum or pre-immune rabbit serum as described in section 2. Immunoprecipitates were dissociated by boiling in Laemmli sample buffer prior to resolution on a 12.5% (w/v) SDS/polyacrylamide gel which was processed for autoradiography. Equal proportions of detergent-solubilised mitochondrial extracts (20%, 0.5 mg/assay) were subjected directly to SDS-PAGE as a control (lane 1) or employed for immunoprecipitation so that it was possible to visualise the extent of immunoprecipitation of the 46,000-M $_r$ species. Lanes: 1, ATP-radiolabelled D-273-10B mitochondria (0.5 mg/assay); 2–4, ATP-radiolabelled D273-10B mitochondria (0.5 mg/assay) immunoprecipitated with anti-yeast E1 serum (10 and 50 μ l) and normal rabbit serum (50 μ l), respectively; 5–7, identical to lanes 2–4 except with 0.1 mg/assay D273-10B mitochondria; 8–13, identical to lanes 2–7 except with anti-bovine heart E1 serum; S, 125 I-labelled M $_r$ markers.

46,000-M $_r$ 32 P-phosphoprotein was selectively immunoprecipitated from D273-10B mitochondrial extracts (0.5 or 0.1 mg/assay) with anti-yeast PDC E1 serum (lanes 2–7) while similar results were obtained with anti-bovine heart E1-specific serum (lanes 8–13). The specificity and quantitative nature of this immunoprecipitation is apparent from comparison of the intensities of the 46,000 M $_r$ species in the control track (lane 1) with that obtained under conditions of optimal immunoprecipitation in lanes 3 and 9 (0.5 mg mitochondrial protein/assay and 50 μ l antisera). It is also clear (lanes 2 and 8) that lower amounts of antisera (10 μ l) give rise to incomplete immune complex formation. In contrast, in lanes 5,6 and 11,12 which are equivalent immunoprecipitates performed on detergent-solubilised extracts of mitochondria treated with [32 P- γ]ATP at 0.1 mg/assay, 10 μ l of antiserum is sufficient to promote complete precipitation and the total amount of radiolabelled 46,000-M $_r$ species is also correspondingly reduced. In addition, this band was found to be removed quantitatively from the supernatant fluid in conditions where immunoprecipitation was maximal (data not shown). Finally no non-specific immune complex formation was observed with pre-immune serum (lanes 4, 7, 10 and 13). These data provide the strongest evidence to date for the presence of an intrinsic PDC kinase activity in yeast mitochondria and were not confined to strain D-273-10B as similar data were also obtained for strain 288C.

Final confirmation of the presence of the regulation of PDC by phosphorylation/dephosphorylation kinase in isolated yeast mitochondria is demonstrated in Fig. 4. Incubation of mitochondria (isolated from galactose-grown yeast) in the presence of ATP and Mg $^{2+}$ resulted in the rapid disappearance of PDC activity. Subsequent reactivation of PDC could be achieved in a Ca $^{2+}$ dependent manner, suggesting Ca $^{2+}$ -mediated activation of PDC phosphatase in this organism. In contrast, treatment

of intact mitochondria with 20 mM MgCl $_2$ failed to restore PDC activity in organellar extracts. These data suggest that phosphatase activity in yeast is similar to that in mammalian cells and unlike that in plants, where PDC has been shown to be re-stimulated by high levels of Mg $^{2+}$ [18]. In addition, preliminary results indicate that little or no inactivation of PDC (<10%) could be achieved under similar conditions employing mitochondria isolated from yeast grown on glycerol (not shown).

Previous attempts to detect yeast PDC kinase [7, 8] have been unsuccessful, probably because PDC has been purified from bakers' yeast strains, as it now appears that yeast must be cultured under carefully defined conditions to detect phosphorylation of the complex. Moreover, in our hands, it has proved difficult to assay PDC kinase routinely in crude mitochondrial extracts using endogenous yeast PDC, purified exogenous yeast or bovine PDC as substrates with reproducible phosphorylation only occurring in intact mitochondria. Thus PDC kinase may prove (a) to be a labile enzyme; (b) be rapidly degraded in detergent-solubilised extracts or (c) subject to inactivation by negative allosteric effectors or inhibitory proteins.

The reason for detection of the phosphorylated 46,000-M $_r$ band, mainly in mitochondria from galactose-grown cells is

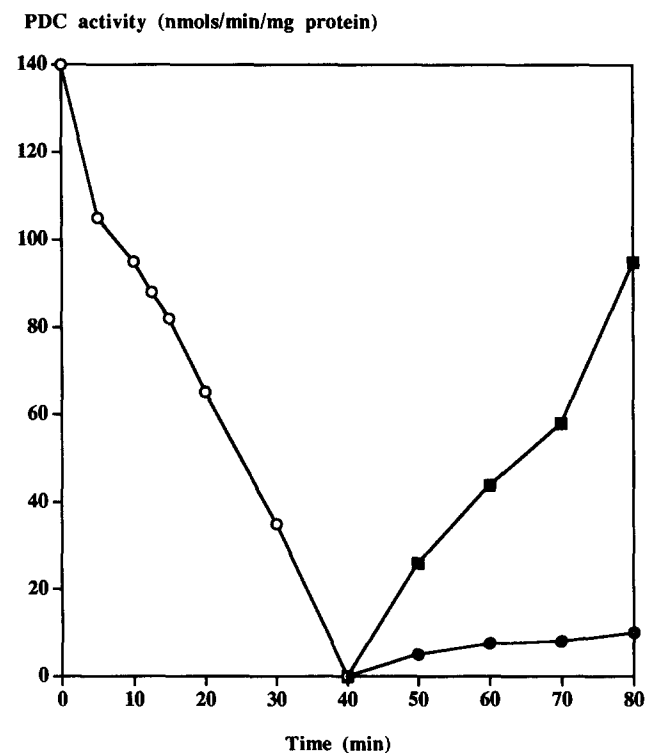


Fig. 4. ATP-inactivation and Ca $^{2+}$ -dependent reactivation of PDC in yeast mitochondria. Yeast mitochondria (isolated from *S. cerevisiae* 288C grown on galactose) were suspended at 2 mg·ml $^{-1}$ in final isolation buffer and incubated in the presence of 2 mM Mg-ATP with vigorous stirring. Samples were removed at the times indicated and treated as described in section 2. Mitochondria, pretreated with ATP to inactivate PDC, were also resuspended in the same buffer containing 2 mM CaCl $_2$ or 30 mM MgCl $_2$ and assayed for PDC activity as described in section 2. Open circles (○) show time course of decrease in PDC activity in mitochondria treated with 2 mM Mg-ATP. Closed circles (●) depict reactivation of PDC in mitochondria pretreated with Mg-ATP, pelleted by centrifugation and resuspended in buffer containing 30 mM MgCl $_2$; closed squares (■) indicate a similar profile of reactivation performed in the presence of 2 mM CaCl $_2$.

also unclear although occasional faint phosphorylation was also observed in yeast cultures using glucose as a carbon source. This may be related to glucose-induced catabolite repression of mitochondrial enzymes. The flux of intermediates into the TCA cycle may be occurring at maximal rates in the presence of non-fermentable carbon sources such as lactate and glycerol since energy production is strictly dependent on aerobic metabolism in these cases. Under these conditions, regulation by PDC kinase may be unnecessary so it may be maintained in an inactive state or be totally absent if it proves to be an inducible enzyme. In contrast, growth can proceed under anaerobic or oxygen-limited conditions in the presence of galactose or glucose when it will be necessary to limit or prevent the flux of two carbon units into the citric acid cycle by altering the activity (phosphorylation) state of the PDC. Clearly, however, now that it has been established that this key multienzyme complex is susceptible to covalent modification, further research is necessary to understand its regulation in yeast.

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