Protein phosphorylation/dephosphorylation in the inner membrane of potato tuber mitochondria

André Struglics[a,1], Kenneth M. Fredlund[b,2], Yuri M. Konstantinov[b,3], John F. Allen[a,4], Ian M. Møller[b,*]

[a] *Plant Cell Biology, Lund University, P.O. Box 7007, S-220 07 Lund, Sweden*
[b] *Plant Physiology, Lund University, P.O. Box 117, S-221 00 Lund, Sweden*

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Abstract Inside-out inner mitochondrial membranes free of matrix proteins were isolated from purified potato tuber (*Solanum tuberosum* L.) mitochondria and incubated with [γ-^{32}P]ATP. Proteins were separated by SDS–PAGE and visualized by autoradiography. Phosphorylation of inner membrane proteins, including ATPase subunits, was strongly inhibited by the phosphoprotein phosphatase inhibitor NaF. We propose that an inner membrane phosphoprotein phosphatase is required for activation of the inner membrane protein kinase. When prelabelled inner membranes were incubated in the absence of [γ-^{32}P]ATP, there was no phosphoprotein dephosphorylation unless a soluble matrix fraction was added. This dephosphorylation was inhibited by NaF, but not by okadaic acid. We conclude that the mitochondrial matrix contains a phosphoprotein phosphatase that is responsible for dephosphorylation of inner membrane phosphoproteins.

Key words: Protein phosphorylation; (Plant) mitochondrion; Inner membrane; Matrix; Protein kinase; Protein phosphatase; Protein kinase phosphatase

1. Introduction

Protein phosphorylation/dephosphorylation by protein kinases and protein phosphatases is a ubiquitous mechanism in eukaryotes and prokaryotes for modulating the activity of intracellular proteins in response to extracellular signals [1].

In mitochondria, two well-characterized enzymes, the α-subunit of pyruvate dehydrogenase and the α-subunit of branched-chain 2-oxoacid dehydrogenase complex, both located in the matrix are under the control of phosphorylation/dephosphorylation [2]. Four inner membrane phosphoproteins have been identified by N-terminal sequencing: the 18 kDa AQDQ subunit of complex I [3]; the 17 kDa subunit IV of cytochrome *c* oxidase [4]; and the 22 kDa δ′-subunit and the 28 kDa β-subunit of the F_0F_1-ATPase [5]. The phosphorylation of a number of unidentified proteins has also been demonstrated in bovine heart mitochondria [6,7] and in potato tuber mitochondria [5,8–10].

When inside-out submitochondrial particles (IO-SMP) from plant mitochondria are incubated with [γ-^{32}P]ATP, more than 20 proteins are phosphorylated mostly on serine/threonine residues [11]. The basic properties of the phosphorylation, specificity, time course, pH dependence, K_m(ATP), have been characterized and two putative kinases/kinase subunits of 16.5 and 30 kDa identified [11]. In the present study, we show that two distinct phosphoprotein phosphatases are involved in the protein phosphorylation/dephosphorylation in IO-SMP, one bound to the mitochondrial inner membrane and another, soluble, in the matrix.

2. Materials and methods

Highly purified intact mitochondria free from plastids (amyloplasts) and peroxisomes were purified from potato tubers (*Solanum tuberosum* L. cv. Bintje or Ukama) [12]. The mitochondria were subfractionated and inside-out inner mitochondrial membrane (IO-SMP) vesicles lacking any substrates or matrix proteins were prepared as in [10]. The matrix fraction was concentrated in an Amicon 8010 Stirred Ultrafiltration Cell with a 10 kDa cut off filter (Amicon YM 10).

Protein phosphorylation assays were carried out in a volume of 50 μl containing 250 μg IO-SMP proteins [11]. Final concentrations in the reaction mixture were as follows: 0.3 M sucrose, 50 mM HEPES–KOH (pH 7.5), 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 0.2 mM (0.4–1.0 Ci mmol^{-1}) [γ-^{32}P]ATP. After 2–15 min incubation at room temperature (22–23°C), the reaction was stopped in either of two ways: (A) by addition of sample buffer [13] and thereafter boiling at 100°C for 2 min, or (B) by addition of trichloroacetic acid (TCA) (6.5% w/v final concentration), and after 30 min at room temperature the samples were pelleted by centrifugation at 13 000 × *g* for 6 min, resuspended in 100 μl 50 mM HEPES–KOH (pH 7.5) and pelleted again. The washed pellets were then resuspended in the sample buffer.

For the dephosphorylation assay, inside-out inner mitochondrial membrane proteins were first phosphorylated using [γ-^{32}P]ATP as described above. The membranes were then pelleted (1 min at 100 000 × *g*) with an Airfuge (Beckman, rotor A-100) and the supernatant containing excess ATP was discarded. The membranes were resuspended in 50 mM HEPES–KOH (pH 7.5), 5 mM MOPS (pH 7.2), 0.3 M sucrose, 125 mM mannitol, 7 mM MgCl, 0.1 mM $CaCl_2$ and 0.4 mM EDTA in the presence or absence of 400 μg matrix proteins in a total volume of 50 μl. After incubation for 1–60 min, the inner membranes were pelleted as above and the supernatant, which contained matrix proteins, was removed. TCA was added to the inner membranes to a final concentration of 6.5% (w/v) and after 30 min at room temperature proteins were pelleted at 13 000 × *g* for

*Corresponding author. Fax: (46)-46-222 4113.
E-mail: ian_max.moller@fysbot.lu.se

[1] Present address: Active Biotech Research AB, P.O. Box 724, S-220 07 Lund, Sweden.
[2] Present address: Novartis Seeds AB, P.O. Box 302, S-261 23 Landskrona, Sweden.
[3] Present address: Laboratory of Plant Genetic Engineering, Siberian Institute of Plant Physiology and Biochemistry, P.O. Box 1243, Irkutsk 664033, Russia.
[4] Present address: Department of Plant Biochemistry, Lund University, P.O. Box 117, S-221 00 Lund, Sweden.

Abbreviations: IO-SMP, inside-out submitochondrial particles (inner membranes); TCA, trichloroacetic acid

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6 min. The pellet was resuspended in 100 μl 50 mM HEPES–KOH (pH 7.5) buffer and treated as above before gel electrophoresis.

SDS–PAGE was performed according to [13] on a 10–15% T (C = 2.7%) gradient gel with a Protean II xi Slab Cell (Bio-Rad) apparatus. Gels were stained with Coomassie brilliant blue R250, destained and dried. The phosphoproteins were visualized in two ways: (1) the gels were placed on a phosphorimager plate (Molecular Dynamics) for 2–5 days. The plate was then screened in a phosphorim-

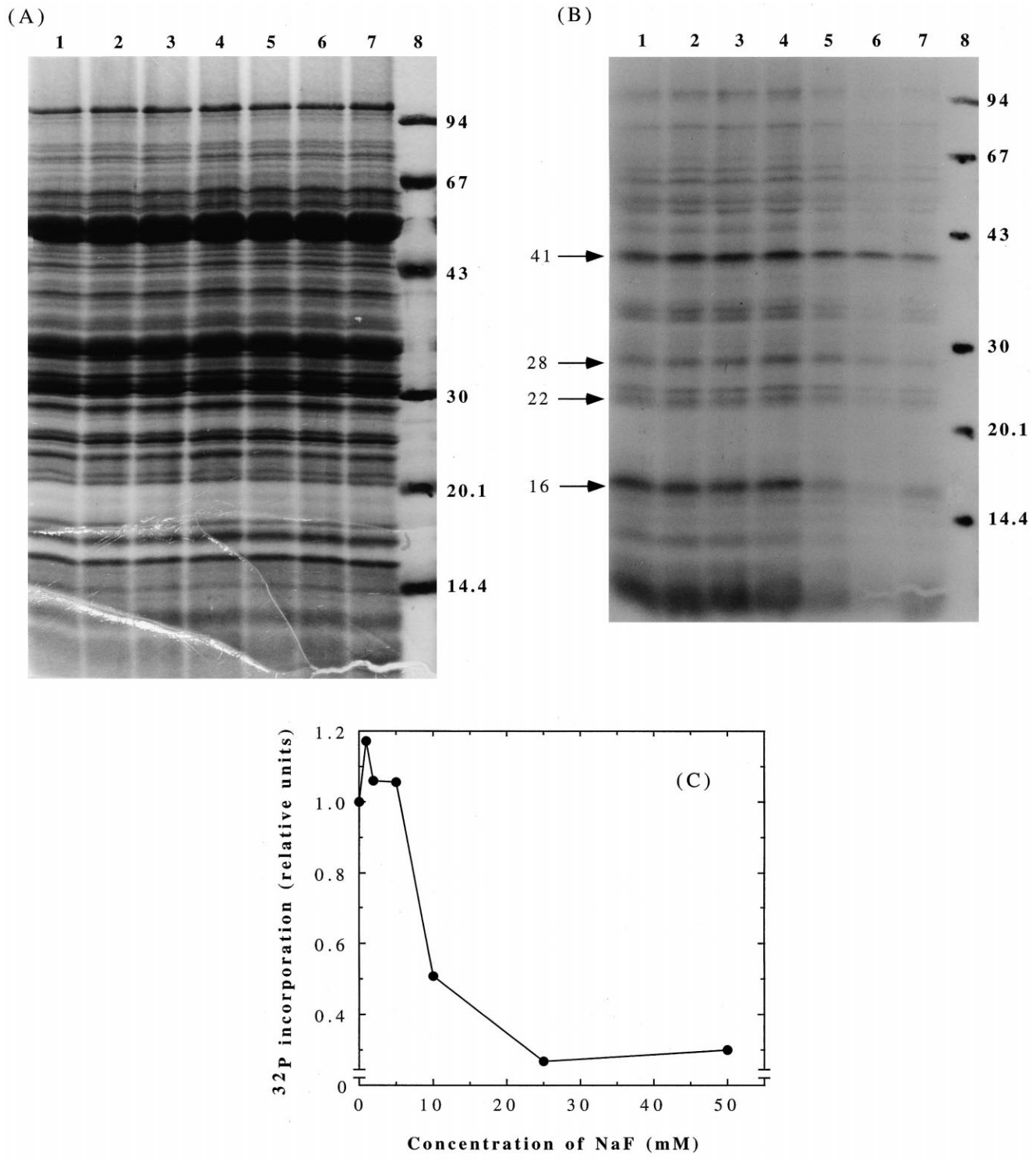

Fig. 1. The effect of NaF on the phosphorylation of mitochondrial inner membrane proteins. (A) Coomassie-stained gel identical to that used in B. (B) Autoradiograph of IO-SMP proteins which had been phosphorylated for 2 min in the absence or presence of NaF at different concentrations following separation using SDS–PAGE. Lane 1, no NaF; lane 2, 1 mM NaF; lane 3, 2 mM NaF; lane 4, 5 mM NaF; lane 5, 10 mM NaF; lane 6, 25 mM NaF; lane 7, 50 mM NaF. Lane 8 shows molecular mass standards (in kDa) visualized using a ^{35}S marker pen on the Coomassie-stained gel before autoradiography. Phosphoproteins discussed in the text are indicated by arrows and size in kDa. (C) Relative inhibition of ^{32}P incorporation at different NaF concentrations given as the average value for all detected phosphoproteins in IO-SMP. Results from B.

ager (Molecular Dynamics SI) and the data analyzed with a Microsoft Windows NT version 3.1 program. (2) The gels were placed on a screen with autoradiographic film (Hyper film MP, Amersham) for 5–7 days at −80°C. Protein concentration was determined by use of Coomassie brilliant blue G250 according to the instructions from Bio-Rad using IgG as the standard.

All experiments were carried out at least twice, and only consistently reproducible results are presented.

3. Results and discussion

Incubating IO-SMP vesicles with [γ-^{32}P]ATP in the presence of divalent cations results in phosphorylation of more than 20 proteins as judged by SDS–PAGE and autoradiography [5,11]. In the experiment shown in Fig. 1, the involvement of phosphatases was investigated by incubating the IO-SMP in the presence or absence of the phosphatase inhibitor NaF. The Coomassie-stained gel showed equal loading of all lanes (Fig. 1A). A comparison with the autoradiograph (Fig. 1B) showed that the polypeptide and phosphoprotein patterns were quite different. The labelling time was only 2 min, the time giving half-maximal phosphorylation [11] to maximize the sensitivity to inhibition, resulting in somewhat less ^{32}P incorporation than in subsequent experiments (cf. Fig. 2A).

NaF at concentrations of 10 mM and above inhibited ^{32}P incorporation into all bands strongly (Fig. 1B). The phosphorylation of all the proteins was reduced by 75% on average at 25 mM NaF (Fig. 1C). Specifically, NaF reduced the labelling of the two F_0F_1-ATPase subunits of 22 and 28 kDa [5], the prominent, but as yet unidentified, 16 kDa polypeptide, as well as the 41 kDa α-subunit of the pyruvate dehydrogenase (sometimes associating with the IO-SMP, [8]) (Fig. 1B).

To test whether the effect of 25 mM NaF on phosphorylation was due to increased ionic strength, we included 25 mM NaCl instead in a control experiment but it had no effect on ^{32}P incorporation into the inner membrane proteins (results not shown). Okadaic acid, which is a phosphatase 1 and 2A inhibitor, used in mammalian systems [14], had no effect on protein phosphorylation of the inner membrane of potato tuber mitochondria even at a concentration of 1 μM (results not shown).

NaF has been demonstrated to be an effective phosphoseryl and phosphothreonyl protein phosphatase inhibitor [15] in plant chloroplasts [16], and in mammalian [17] and plant [18] mitochondria. In these systems, NaF usually enhances ^{32}P incorporation into proteins by inhibiting protein phosphatases whose activity compete with that of the protein kinases. Since NaF inhibits protein phosphatases and not protein kinases, the decrease in ^{32}P incorporation in the presence of NaF (Fig. 1) was unexpected. We suggest that phosphorylation of the inner membrane proteins is dependent on a protein kinase that is activated by a protein phosphatase. The possible mechanism will be discussed later.

Protein phosphatase activity was investigated as follows: the inner membrane proteins were phosphorylated using labelled ATP. After 15 min of labelling, to achieve maximum labelling of the phosphoproteins, the ATP was removed and

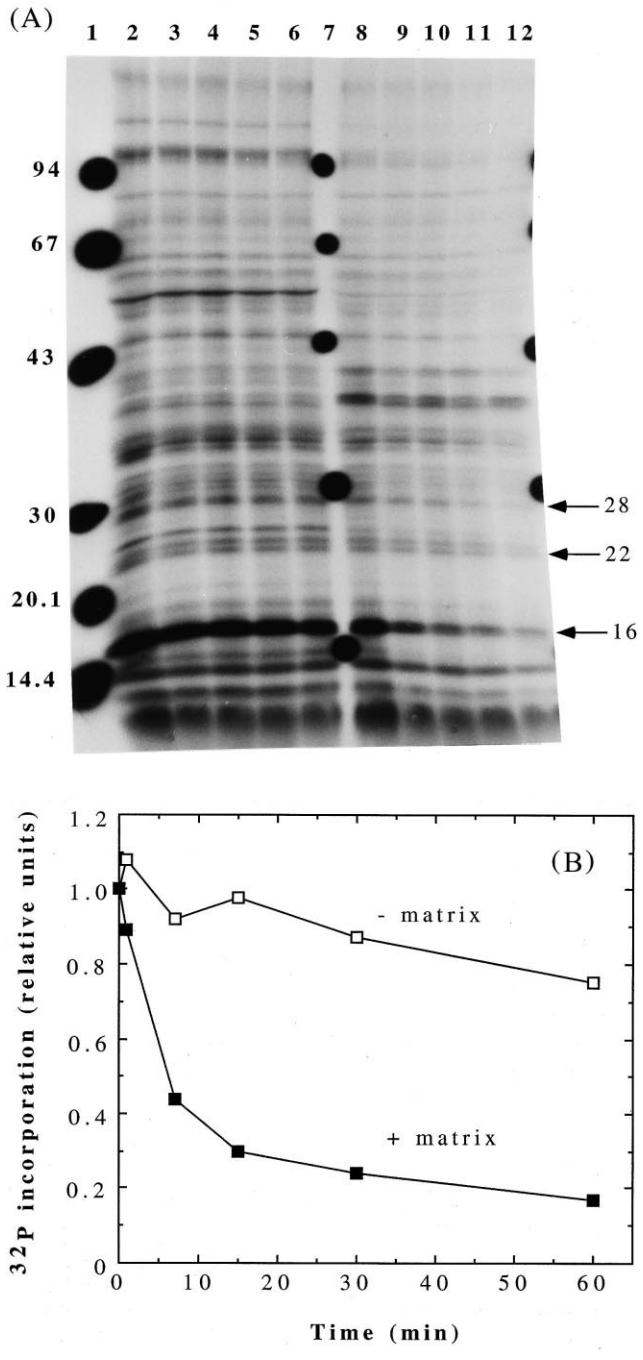

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Fig. 2. The effect of the matrix fraction on the time-dependent dephosphorylation of inner membrane phosphoproteins. A: Autoradiograph of inner membrane phosphoproteins subjected to dephosphorylation for different times in the absence (lanes 2–6) or presence (lanes 8–12) of the matrix fraction. An inner membrane fraction was first phosphorylated for 15 min, then the ATP/[γ-^{32}P]ATP was removed by centrifugation and the labelled inner membranes were incubated at room temperature for different times to become dephosphorylated. Lane 1, molecular weight standards (in kDa) visualized as in Fig. 1; lane 2, 1 min without matrix fraction; lane 3, 2 min without matrix fraction; lane 4, 7 min without matrix fraction; lane 5, 15 min without matrix fraction; lane 6, 60 min without matrix fraction; lane 7, molecular weight standards (in kDa); lane 8, 1 min with matrix fraction; lane 9, 2 min with matrix fraction; lane 10, 7 min with matrix fraction; lane 11, 15 min with matrix fraction; lane 12, 60 min with matrix fraction. The arrow indicates the position of phosphoproteins discussed in the text. The Coomassie-stained gel showed equal loading of proteins and identical polypeptide patterns in all lanes. B: Dephosphorylation profile of the 16 kDa phosphoprotein. Results from A.

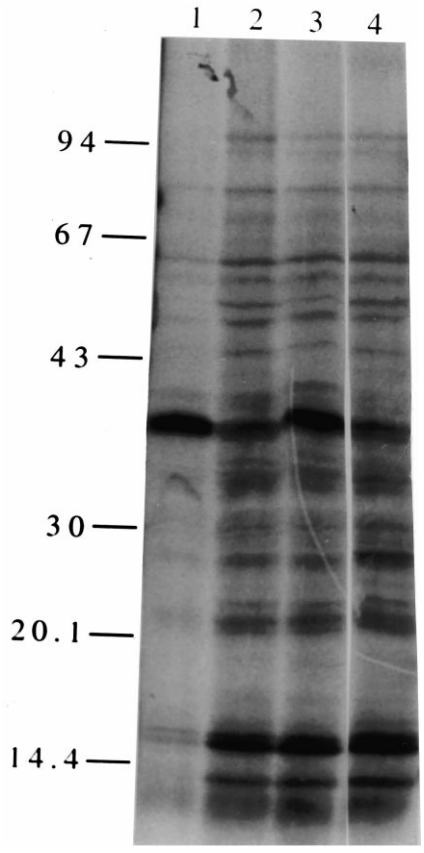

Fig. 3. Autoradiograph of the mitochondrial inner membrane phosphoproteins subjected to dephosphorylation under different conditions. Lanes 1–3, the ATP/[γ-^{32}P]ATP was removed after 10 min phosphorylation and the samples were further incubated for 60 min with the following additions: lane 1, matrix fraction; lane 2, buffer only; lane 3, matrix fraction+25 mM NaF. Lane 4, control sample labelled for 10 min with no subsequent incubation. Molecular mass standards are indicated at the left side of the figure (in kDa).

the incubation continued. There was no detectable dephosphorylation of the phosphoproteins even after a 60 min chase (Fig. 2A, lanes 2–6). Upon addition of the matrix fraction to inner membrane vesicles containing ^{32}P-labelled inner membrane proteins, dephosphorylation of all phosphoproteins in the inner membrane, including the 22 and 28 kDa subunits of the F_0F_1-ATPase, was observed (Fig. 2A, lanes 8–12). As an example, the time course of dephosphorylation of the 16 kDa inner membrane phosphoprotein, when treated in the presence or absence of a concentrated matrix fraction, is shown in Fig. 2B. The half-time of dephosphorylation of the 16 kDa phosphoprotein in the presence of matrix proteins was about 6 min. When NaF was added together with matrix proteins to prelabelled inner membrane proteins, the dephosphorylation was prevented (Fig. 3), while okadaic acid had no such effect (result not shown). When a heat-denatured matrix fraction was added to phosphorylated inner membrane proteins lacking ATP, there was no detectable dephosphorylation (results not shown). There was no detectable proteolysis of ^{32}P-labelled IO-SMP proteins, and the protease inhibitors phenylmethylsulphonyl fluoride and E64 (L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)-butane) had no effect on the dephosphorylation pattern of IO-SMP proteins (results not shown). These findings suggest that a phosphoprotein phosphatase in the mitochondrial matrix is responsible for the dephosphorylation of the inner membrane phosphoproteins.

There are at least two possible explanations for the NaF inhibition of protein phosphorylation in mitochondrial inner membranes (Fig. 1): (i) The substrates for the protein kinase are already phosphorylated when the IO-SMP fraction is prepared, and an active phosphatase is needed to dephosphorylate the IO-SMP proteins before they can be labelled with [γ-^{32}P]ATP.

(ii) The protein kinase itself is inactive in the phosphorylated form and it is either phosphorylated in vivo or becomes phosphorylated upon addition of ATP. It will then require an active protein kinase phosphatase for activation of the protein kinase and phosphorylation of its substrates.

Since the substrate phosphoprotein phosphatase is located in the matrix and not in the inner membrane (Fig. 2), then there must be another phosphatase that is inhibited when NaF is added to inner membranes resulting in a decrease of ^{32}P incorporation into IO-SMP proteins (Fig. 1). Therefore the decrease in ^{32}P incorporation must be the result of an inhibition of a kinase phosphatase in the IO-SMP. This supports the second proposal above: the protein kinase is in a phosphorylated and inactive form at the start of the experiment or when ATP is added to the membranes. Activation of a kinase then requires an active phosphatase.

Sommarin et al. [18] and Petit et al. [9] showed that total ^{32}P incorporation into intact mitochondria reached a maximum incorporation after 1–2 min. A time course experiment on phosphorylation of inner membrane proteins showed that the maximum ^{32}P incorporation was reached only after 15–23 min [11]. The differences between these results could be explained by the fact that in intact mitochondria, an equilibrium between kinase activities and phosphatase activities is rapidly reached. In the inner membrane vesicles where the substrate phosphatase is missing, an equilibrium is not reached, and thus it takes much longer to reach maximum ^{32}P incorporation.

The half-time for the dephosphorylation of prelabelled inner membrane phosphoproteins by a phosphatase in the matrix was 6 min when 250 μg IO-SMP proteins was incubated with 400 μg matrix proteins in a total volume of 50 μl (Figs. 2 and 3). The relative amounts of inner membrane and matrix proteins are close to that in intact mitochondrion (I.M. Møller, A.G. Rasmusson and K.M. Fredlund, unpublished). However, the actual concentration of matrix proteins in the assay (8 mg ml^{-1}) is more than an order of magnitude below that in the intact mitochondrion where it may be as much as 500 mg ml^{-1} [19], a concentration it is not possible to reach in vitro. Thus, the potential rates of dephosphorylation in vivo may well be much higher than those estimated here.

4. Conclusions

- Plant mitochondria contain a protein kinase phosphatase in the inner membrane which dephosphorylates, and thereby activates, the intrinsic protein kinase.
- Plant mitochondria contain a protein phosphatase in the matrix which can dephosphorylate all the phosphoproteins on the inner matrix surface of the inner membrane.

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