Cellular Regulation by Protein Phosphorylation

Edited by

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DYNAMIC PHOSPHORYLATION OF A SMALL CHLOROPLAST PROTEIN EXHIBITING SO FAR UNDESCRIBED LABELLING PROPERTIES

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A 19 kDa phosphoprotein from mixed envelope membranes of spinach chloroplasts with extreme labelling kinetics has been characterized. Its localization between the inner and the outer envelope membrane can be deduced by the differential labelling between intact and broken chloroplasts (Table 1), (Soll and Bennett 1988, Soll et al. 1989).

Table 1
Differential labelling of proteins from intact and lysed chloroplasts. Phosphorylation was done at 10 nM ATP at 4 °C for 30 sec. Values are expressed in dpm μg chlorophyll^{-1} x min^{-1}

	intact chloroplasts	lysed chloroplasts	ratio
thylakoid/LHCP	53	125	0.424
stroma a	12	110	0.109
stroma b	11	100	0.11
outer envelope (86 kDa)	63	16	3.9
intermembrane space (64 kDa)	98	54	1.8
19 kDa protein	2340	375	6.24

If the 19 kDa protein is indeed localized in the envelope lumen, intact and broken chloroplasts should differ in their labelling kinetics. Intact chloroplasts still contain residual, endogenous ATP, this means that during labelling of intact chloroplasts two ATP pools exist, with different specific activity which is encountered by envelope membrane proteins and another with low specific activity which is encountered by proteins inside the chloroplast. In intact chloroplasts the outer envelope 86 kDa protein, the 64 kDa and the 19 kDa pro-

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tein were labelled much earlier and stronger than the stromal and thylakoid phosphoproteins.

The function of the interenvelope space is unknown; the dynamically phosphorylated 19 kDa protein has been purified from mixed envelope membranes (Table 2) and characterized. It seems reasonable that it participates in a signal transduction process. The first purification step was a mild sonication followed by anion exchange chromatography on DEAE cellulose of the supernatant (Table 2). Most of the protein eluted at 125 mM NaCl. Active protein fractions were pooled and purified further on a hydroxylapatite column from which it could be eluted at 60 mM phosphate buffer pH 7.6.

Table 2
Purification of spinach envelope 19 kDa protein

Step	Volume ml	Protein µg	Total units	Specific activity units/mg	Recovery %	Purification fold
Envelope membranes	1	6950	63.2	9.1	100	1
Sonication supernatant	0.89	1510	55	36.4	87	4.6
DEAE chromatography	5.6	8.9	28.5	3200	45	352
Hydroxylapatite	4.5	0.54	4.4	8161	7	897

1 unit equals 1 fmol ^{32}p incorporated into the 19 kDa protein from [γ-^{32}P]-ATP x min^{-1}

The 19 kDa protein shows an extreme affinity for ATP and GTP as demonstrated by the low K_m values of 8 nM and 5 nM for ATP and GTP respectively (Fig. 1 A). The phosphorylation, that is trichloracetic acid or acetone precipitable, is dependent on the presence of divalent cations (Mg^{2+} and Mn^{2+}) (Fig. 1 B).

The cation Ca^{2+} has no effect. ADP and GDP inhibit phosphorylation (Fig. 1 D). The optimal pH for phosphorylation is in the range between pH 7 and pH 9 (Fig. 1 C). The pI of the phosphorylated enzyme has the value 6.2, whereas the pI of the non phosphorylated enzyme is 6.3.

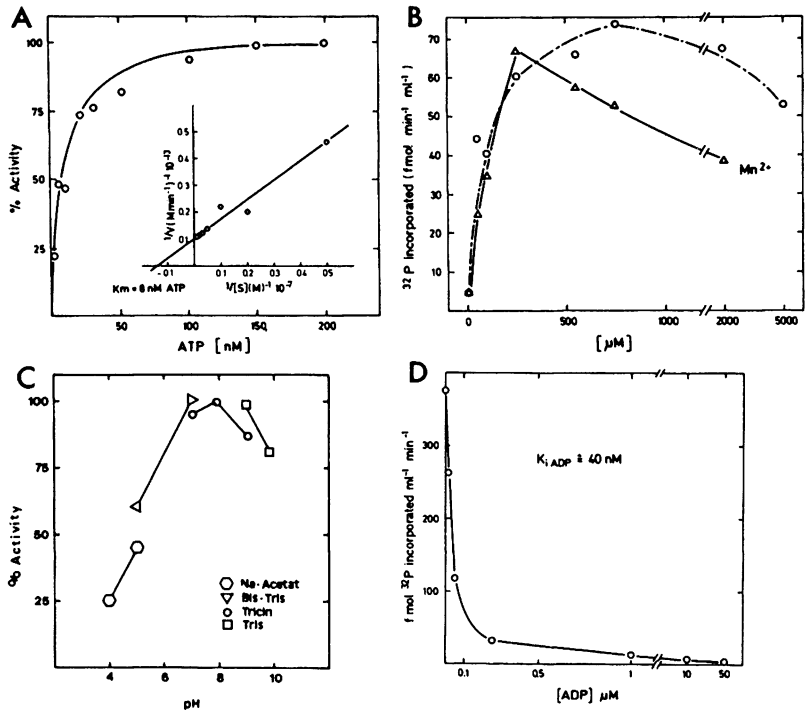

Fig. 1) Characterization of the phosphorylation reaction of the partially purified 19 kDa protein. A) Determination of the Km value for ATP. B) Influence of divalent cations. C) pH dependence. D) The phosphorylation is inhibited by ADP.

The molecular weight of the phosphorylated protein was estimated by SDS gel electrophoresis and found to be 18.8 kDa (Fig. 2 A). The phosphoryl turnover is extremely rapid, as deduced from a pulse-chase experiment. If the protein was labelled in the presence of 8 nM [γ-^{32}P]-ATP for 60 sec and 10 μM cold ATP was added at this time point, 90 % of the labelled phosphoryl-groups in the protein are turned over within 15 sec.

The determination of the phosphorylated amino acid residue demonstrated that no hydroxylated amino acid was phosphorylated, firstly the phosphate bond was labile to acidic conditions;

secondly after acid hydrolysis of the protein and high voltage electrophoresis no radioactivity was detectable in P-Ser, P-Thr or P-Tyr (Soll et al. 1989). Extraction of the phosphorylated protein by chloroform methanol at pH 1 resulted in no detectable label in the organic solvent phase, but the total radioactivity was still bound to the protein. Exposure of the phosphoprotein to hydroxylamine or pyridine buffered in acetate showed a concentration dependent base catalyzed enhancement of the hydrolysis rate (Fig. 2 B) and excluded most likely aspartate and glutamate as phosphorylgroup acceptor, as those are not susceptible to pyridine treatment (Sabato and Jencks 1961,Hokin et al. 1965). The label was also labile at alkaline pH (Stelte and Witzel 1986). At the moment it seems most likely that we deal with a lysine or histidine phosphate. The phosphorylation of the 19 kDa protein is inhibited by TNP-ATP and by erythrosin (Fig. 2 C,D).

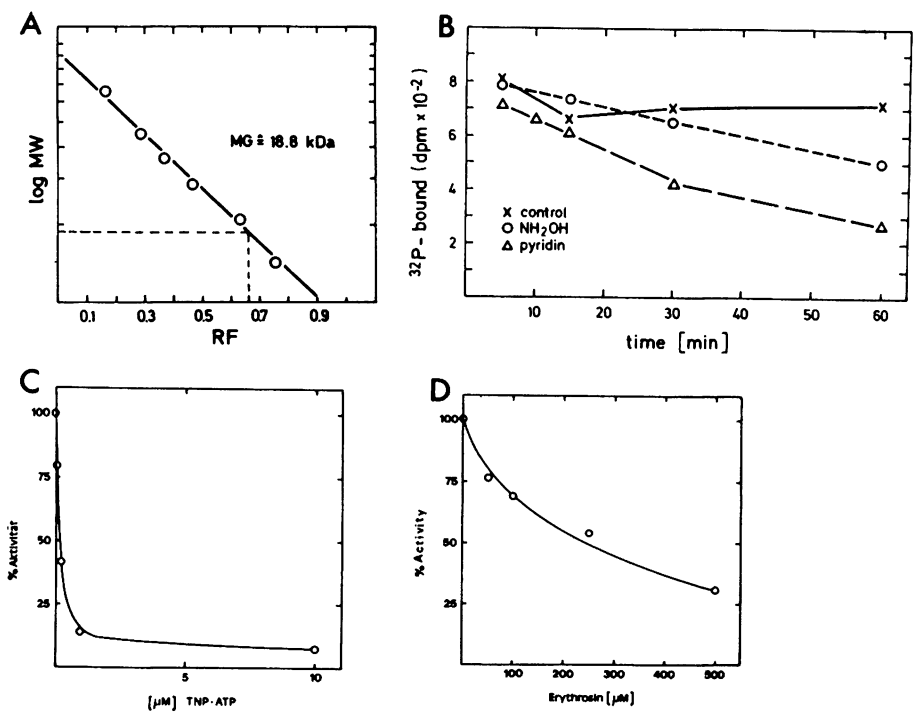

Fig. 2) A. Determination of the molecular weight of the phosphorylated form of the purified 19 kDa protein by SDS-PAGE B. Time course of hydrolysis of the phosphate bond in 1 M acetate buffer pH 5.5 in the presence of 0.1 M hydroxylamin or pyridin. C. Inhibition of the ^{32}P-incorporation by TNP-ATP. D. Inhibition of 19 kDa protein phosphorylation by erythrosine

The purified 19 kDa protein did not show significant ATPase activity (not shown). These findings are corroborated by results (Table 3), which demonstrate the effect of various ATPase inhibitors on the phosphorylation of the 19 kDa protein (Sze et al. 1987, Serrano 1988, Sze and Randall 1987). From further experiments it seems likely that ^{32}p-incorporation into the 19 kDa protein is due to autophosphorylation.

Table 3
Inhibition of 19 kDa protein phosphorylation by various substrates. The purified protein was phosphorylated by [γ-^{32}P]-ATP in the presence of different effectors. A minimum of five different effector concentrations was used in every case.

effector	max.concentration	% inhibition
NaN_3	10 mM	0
NaF	20 mM	0
$NaF/AlCl_3$	10 mM/50 μM	0
ortho vanadate	0.5 mM	29
molybdate	2.0 mM	0
nitrate	10 mM	0
oligomycin	0.5 mM	0
DCCD[1)]	0.5 mM	0
ouabain	125 μM	0
dihydroxyacetonephohate	2 mM	60
NaCl	150 mM	50

1) The purified protein was preincubated with DCCD for to 2h.

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