Mn^{2+}-DEPENDENT ATPase OF THE ENVELOPE OF SPINACH CHLOROPLASTS

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1. Introduction

We have previously described a Mg^{2+}-dependent ATPase insensitive to *N, N'*-dicyclohexylcarbodiimide in the envelope of spinach chloroplasts [1]. This ATPase differs strikingly from the latent ATPase activity of coupling factor I [1]. The exact role of the envelope ATPase remains to be determined, but it is tempting to think that the enzyme is a part of an active transport system for cations such as Mg^{2+} [2,3], and Mn^{2+} [4–9].

This paper describes the properties of an ATPase from the envelope fraction of chloroplasts which requires either Mg^{2+} or Mn^{2+}.

2. Materials and methods

Chloroplasts from whole green spinach (*Spinacia oleracea* L.) leaves were prepared according to the method of Walker [10]. Purification of intact chloroplasts (B type) [11] was carried out according to a modification of the method of Leech [12]. The envelope, thylakoids and stroma of the chloroplasts, were prepared and characterized as described previously [1]. Gentle swelling of intact chloroplasts caused rupture and total detachment of the envelope with the complete liberation of the stroma material [10].

The following enzyme assays were carried out on the various fractions: Trypsin-activated Ca^{2+}-dependent ATPase activity of the coupling factor I [13], fructose 1,6-diphosphatase [14] and NAD(P)H: Cytochrome *c* oxidoreductase [15]. Galactolipids synthesis was measured at 37°C as described previously [16].

ADP–ATP exchange was assayed in a 0.16 ml reaction mixture which contained 1.25 mM ATP; 0.62 mM [^{14}C]ADP, 10^5 cpm; 5 mM tricine–NaOH buffer pH 8.0 and 20 to 150 μg of protein. After 4 min at 37°C in the dark, a few drops of ethanol were added immediately and the mixture placed in a boiling water bath for 2–3 min. Nucleotides were separated by chromatography on PEI-cellulose precoated TLC plates (Merck). The solvent used was 1.2 M LiCl. The reaction rates were calculated according to Kahn [17].

Chlorophylls were determined spectrophotometrically in 80% acetone extracts of chloroplasts [18]. Protein content was determined by the method of Lowry et al. [19].

3. Results

Chlorophylls and trypsin-activated Ca^{2+}-dependent ATPase of the coupling factor I were markers for the

Table 1
Specific activities of marker enzymes, total chlorophyll content and galactolipids synthesis in the fraction obtained after disruption of intact chloroplasts by hypotonic treatment

Fraction	Chlorophylls	Trypsin-activated Ca^{2+}-dependent ATPase	Fructose 1,6-diphosphatase	Galactolipids synthesis
	μg/mg protein	μmol P_i formed/hr/mg protein		pmol of galactose incorporated/min/mg protein
Thylakoids	150	71	1	47
Stroma	0	0.4	15.5	4
Envelope	0.2[a]	0.3	0.1	3750[b]

[a] The small amounts of chlorophyll found in this fraction was entirely due to a contamination of the envelope preparation by small fragments of thylakoids: the bulk of vesicles deriving from the envelope do not produce the typical red fluorescence of the chlorophyll when observed under UV light.
[b] All the galactolipids (mono-, di-, tri- and tetragalactosyldiglyceride) were synthesized.

thylakoid membranes fraction [1]; fructose 1,6-diphosphatase was the marker for the stroma [1]; galactolipids synthesis was the marker for the envelope [16]. The marker activities were distributed among the fractions as anticipated, thus validating the fractionation procedure used (table 1). In addition, the envelope differed strikingly from the mitochondrial and microsomal subfractions in that it exhibited no NAD(P)H: Cytochrome *c* oxidoreductase activity [15].

Table 2 clearly indicates that the Mg^{2+}-dependent ATPase of chloroplast envelope was also Mn^{2+}-dependent. Other cations were tested: Co^{2+}, Fe^{2+}, Ca^{2+}, Zn^{2+} slightly activated the ATPase, Cu^{2+}, Ni^{2+}, Hg^{2+}, K^+ and $*Na^+$ were without effect. In the absence of cation in the incubation medium, less than 0.2 μmol P_i was formed/hr/mg protein. This ATPase was able to hydrolyse UTP, GTP and CTP to about the same extent as ATP. However ADP, AMP and pyrophosphate were not hydrolysed. Lack of activity with β-sodium glycerophosphate (10 mM) ruled out the possibility that this ATPase was an unspecific phosphatase.

Electron micrographs [21] showed that the envelope fraction consisted of double membrane vesicles. The effect of Triton X-100 on the envelope was tested to determine if such a structure limited ATPase activity [22]. Furthermore, it is well established that ATP is unable to pass through the inner membrane of the envelope [23,24]. While very low concentrations [22] of Triton X-100 were without effect, higher concentration inhibited activity (table 3). This experiment clearly indicates that in our conditions the envelope bound ATPase is freely accessible to ATP.

Table 2
Mg^{2+}- and Mn^{2+}-dependent ATPase activity in envelope obtained after disruption of intact chloroplasts by hypotonic treatment

ATPase	Specific activity μmol P_i formed/hr/mg protein
Mg^{2+}-dependent	6.6 ± 0.8 [29]
Mn^{2+}-dependent	5.6 ± 1.1 [21]

Mg^{2+}- or Mn^{2+}-dependent ATPase was assayed in a 1 ml reaction mixture which contained 40 mM tricine–NaOH buffer, 10 mM ATP, 10 mM $MgCl_2$ (pH 7.8) or $MnCl_2$ (pH 7.6) and 120 μg of protein. After 30 min at 37°C, 1 ml of 20% trichloroacetic acid was added. After centrifugation, the supernatant solution was analysed for P_i [20].
Higher values were obtained in spring and autumn (8–12 μmol P_i formed/hr/mg protein) and lower ones during winter and summer ($\simeq \cdot 4$ μmol P_i formed/hr/mg protein). The number of experiments is between brackets and the standard deviation for the mean is given for 95%.

* ATPase activity with Na^+ was measured in Tris–HCl or Tris–maleate buffers.

Table 3
Effect of Triton X-100 on Mg^{2+}-dependent ATPase activity in envelope obtained after disruption of intact chloroplasts by hypotonic treatment

Triton X-100 % (w/v)	0	0.005	0.01	0.02	0.05	0.1
ATPase[a] μmol P_i/hr/mg protein	9.3	9.3	10.2	8.9	6.1	1.9

The reaction mixture was the same as described in table 2.
[a] The error for each value is about 20%.

Fig.1 shows the pH dependence of Mg^{2+}- and Mn^{2+}-activated ATPase in the envelope. ATPase activity increases with either cation in the slight alkaline region, maximal values were obtained between 7.5 and 7.8. The decrease in activity observed with Mn^{2+} above pH 7.8 could be attributable to the precipitation of $Mn(OH)_2$ in alkaline buffers (up to pH 8.0) [25].

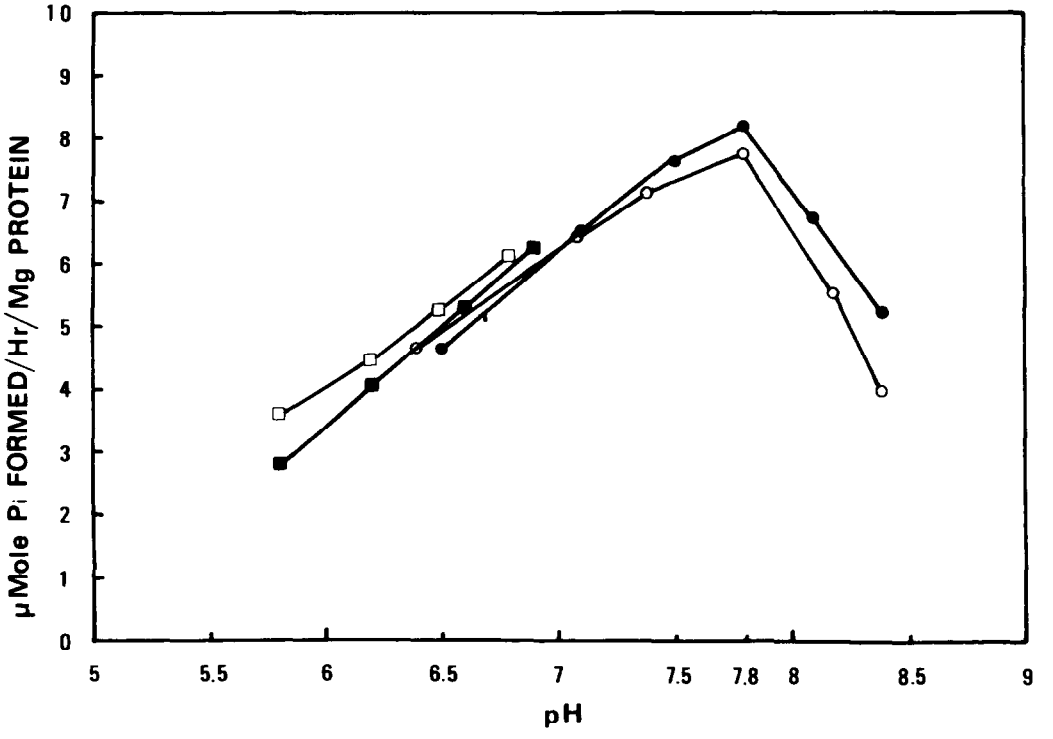
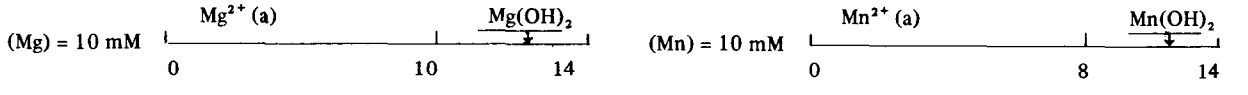


Fig.1. Effect of pH on Mg^{2+}- and Mn^{2+}-dependent ATPase activity in envelope obtained after disruption of intact chloroplasts by hypotonic treatment. Mg^{2+}: (●–●–●) tricine NaOH buffer; (■–■–■) Tris–maleate NaOH buffer. Mn^{2+}: (○–○–○) tricine NaOH buffer; (□–□–□) Tris–maleate NaOH buffer. The reaction mixture was the same as described in table 2. The chemical forms of manganese and magnesium are related to pH [25]:



(a) Manganese and magnesium are in fact in hydrated form: $Mg(H_2O)_6^{2+}$ and $Mn(H_2O)_6^{2+}$ [35].

The apparent Michaelis constants, K_m, for ATP and the apparent activator constants, K_A, for both cations, were lower for Mn^{2+} than for Mg^{2+} (figs.2 and 3). This result might suggest a greater affinity of the chloroplast envelope ATPase for Mn^{2+} than for Mg^{2+}. However, it has to be pointed out that the stability constants observed for small molecule complexes of these two metal ions, for instance ATP-Mg and ATP-Mn, are higher for manganese [26,27].

Up to now, all of our attempts to stimulate this ATPase have not been very successful. In contrast with the other membrane-bound ATPase of plants [22,28–30], the Mg^{2+}- or Mn^{2+}-dependent ATPase of the envelope was not salt stimulated. For instance, KCl, NaCl, divalent cations and anions such as pyruvate, citrate, succinate, malate and oxaloacetate** were without effect. Bicarbonate [31] stimulates the Mg^{2+}- or Mn^{2+}-dependent ATPase to an insignificant extent.

Finally, the considerable ADP–ATP exchange present in the spinach chloroplasts [17] was found to be associated closely with the stroma, but not with the envelope (table 4). Hence this exchange, different from the light-triggered thioldependent ADP–ATP exchange detected in washed broken chloroplasts [32] is not a partial reaction of the Mg^{2+}-dependent ATPase found in the envelope.

** ATPase activity with oxaloacetate was measured at pH 6.0 instead of 7.8.

4. Discussion

The data reported in this paper strongly suggest that the ATPase bound to the chloroplast envelope is a Mn^{2+}-dependent ATPase, which is not salt stimulated. Mg^{2+}-dependent ATPases are not necessarily activated by manganese. For instance, this cation is without effect upon the ATPase of turnip microsomal membra-

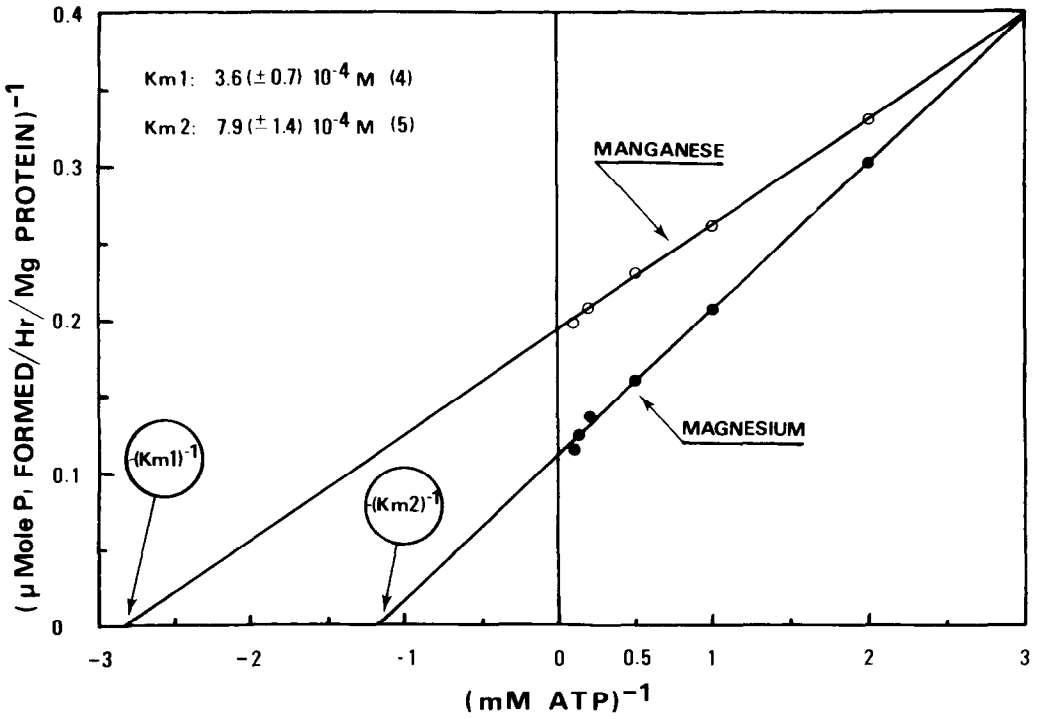


Fig.2. Effect of (ATP) on Mg^{2+}- and Mn^{2+}-dependent ATPase activity in envelope obtained after disruption of intact chloroplasts by hypotonic treatment. Lineweaver and Burk representation. The reaction mixture was the same as described in table 2. The number of experiments is between brackets.

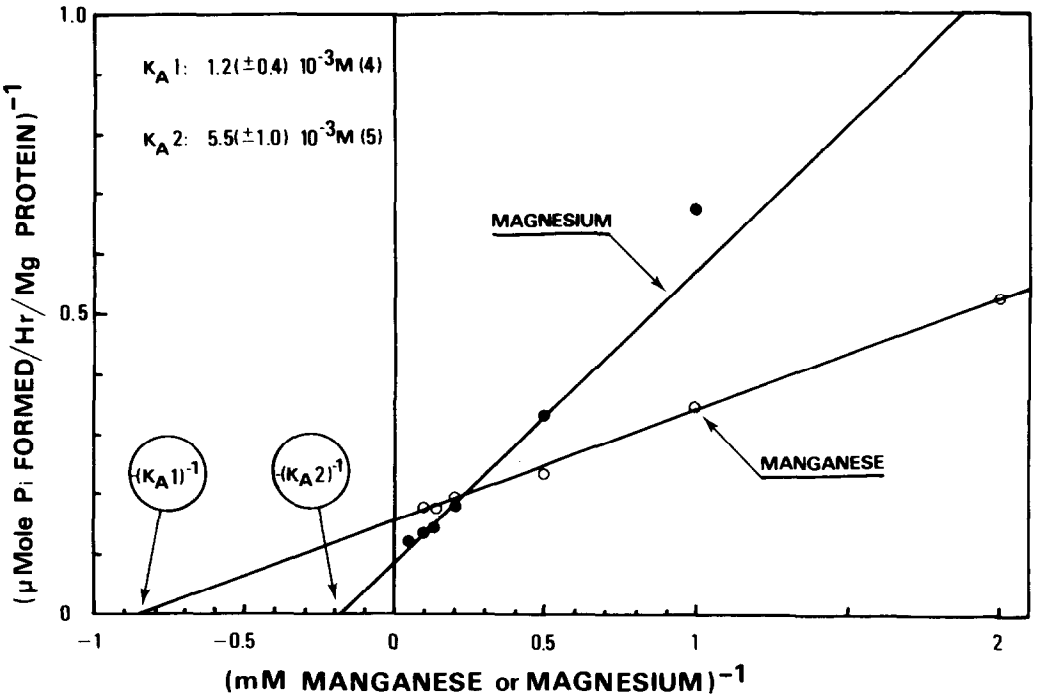


Fig.3. Effect of Mg^{2+} and Mn^{2+} on ATPase activity in envelope obtained after disruption of intact chloroplasts by hypotonic treatment. Lineweaver and Burk representation. The reaction mixture was the same as described in table 2. The number of experiments is between brackets.

nes [22] while it can easily replace magnesium in activation of the plasma membrane ATPase of oat roots [29].

Our results agree with a possible role for the envelope ATPase in the cation transport into the chloroplast, especially the transport of manganese. Such an ATPase exists in the plasma membrane of yeast cells where relations between Mn^{2+}-dependent ATPase and Mn^{2+} transport into the cells have been established [33]

Manganese permeability of the envelope is suggested by many results. Using radioactive tracers, Possinghan and Spencer [4], Homann [5], have shown incorpora-

Table 4
Mg^{2+}-dependent ATPase activity and ADP–ATP exchange in fractions obtained after disruption of intact chloroplasts by hypotonic treatment

Fractions	Mg^{2+}-dependent ATPase μmol formed/hr/mg protein	ADP–ATP exchange μmol exchanged/min/mg protein
Thylakoids	0.3	0.06
Stroma	0.3	2.5[a]
Envelope	6.4	0.1

[a] This exchange is catalysed by the nucleoside diphosphate kinase present in large amounts in the soluble fraction of the chloroplast (Douce, unpublished observations).

tion of Mn^{2+} in chloroplasts of starved plants. Brief illumination of dark grown chlorella [6], greening of etiolated plastids [7], transformation from proplastids to chloroplasts and illumination of dark adapted mosses protonemas [8,9], result in the incorporation of manganese into the plastids, especially in oxygen evolving centers. Unfortunately, attempts to stimulate the envelope ATPase with light have been unsuccessful. Furthermore the ATPase activity was not affected when a mixture of Mg^{2+} and Mn^{2+} was used instead of Mn^{2+} or Mg^{2+} alone.

The location of this ATPase still remains to be found. The outer membrane being freely permeable to metabolites [34], this enzyme is more likely to be on the inner membrane of the envelope.

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References

[1] Douce, R., Holtz, R. B. and Benson, A. A. (1973) J. Biol. Chem. 248 (29), 7215–7222.
[2] Lin, P. C. and Nobel, P. S. (1971) Arch. Biochem. Biophys. 145, 622–632.
[3] Duval, D. and Duranton, J. (1974) Photosynthetica 8 (1), 1–8.
[4] Possingham, J. V. and Spencer, D. (1962) Aust. J. Biol. Sci. 15, 58–58.
[5] Homann, P. H. (1967) Plant Physiol. 42, 997–1007.
[6] Cheniae, G. M. and Martin, I. F. (1973) Photochem. Photobiol. 17 (6), 441–449.
[7] Joyard, J. and Fourcy, A. (1971) C. R. Acad. Sci. série D 273, 572–575.
[8] Chevallier, D. (1973) Physiol. Vég. 11 (8), 461–473.
[9] Chevallier, D. (1973) Physiol. Vég. 11 (3), 475–486.
[10] Walker, D. A. (1971) Methods in Enzymology 23, 211–220.
[11] Hall, D. O. (1971) Nature 235, 125–126.
[12] Leech, R. M. (1964) Biochim. Biophys. Acta 79, 637–639.
[13] Vambutas, V. K. and Racker, E. (1965) J. Biol. Chem. 240, 2660–2667.
[14] App, A. A. (1966) Methods in Enzymology 9, 636–642.
[15] Douce, R., Manella, C. A. and Bonner, W. D. (1973) Biochim. Biophys. Acta 292, 105–116.
[16] Douce, R. (1974) Science 183, 852–853.
[17] Kahn, J. S. (1971) Methods in Enzymology 23, 561–565.
[18] Strain, H. H., Cope, B. T. and Svec, W. A. (1971) Methods in Enzymology 23, 452–476.
[19] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[20] Taussky, H. and Shorr, E. (1953) J. Biol. Chem. 202, 675–685.
[21] Mackender, R. O. and Leech, R. M. (1974) Plant Physiol. 53, 496–502.
[22] Rungie, J. M. and Wiskich, J. T. (1973) Plant Physiol. 51, 1064–1068.
[23] Walker, D. A. (1975) MTI Int. Rev. Sci. Biochem. Sec., vol. 11 in press.
[24] Heber, U. (1974) Ann. Rev. Plant. Physiol. 25, 393–421.
[25] Charlot, G. (1957) in: L'analyse qualitative et les réactions en solution, pp. 149–153, and pp. 206–210, Masson, Paris.
[26] Malmstrom, B. G. and Rosenberg, A. (1959) Adv. Enzymol. 21, 131–136.
[27] Tu, A. T. and Heller, M. J. (1974) in: Metal ions in Biological systems (Sigel, H., ed.), Vol. I, pp. 2–45, Dekker, New-York.
[28] Leonard, R. T., Hansen, D. and Hodges, T. K. (1973) Plant Physiol. 51, 749–754.
[29] Leonard, T. R. and Hodge, T. K. (1973) Plant Physiol. 52, 6–12.
[30] Hansson, G. and Kylin, A. (1969) Z. Pflanzenphysiol. 60, 270–275.
[31] Grisolia, S. and Menselson, J. (1974) Biochem. Biophys. Res. Comm. 58, 968–973.
[32] Stewart, B. W. and Rienits, K. G. (1968) Biochim. Biophys. Acta 153, 907–909.
[33] Furhmann, G. F., Werhli, E. and Boehm, C. (1974) Biochim. Biophys. Acta 363, 295–310.
[34] Heldt, H. W. and Sauer, F. (1971) Biochim. Biophys. Acta 234, 83–91.
[35] Burlamacchi, L. (1971) J. Chem. Phys. 55 (3), 1205–1212.