*J. Biol. Macromol.**3** (2) 69-74 (2003)*

Articles

ATP-binding proteins of spinach chloroplast membranes

Keiko Kishimoto, Sumio Ishijima and Masatake Ohnishi
Graduate School of Agriculture, The Prefecture University of Kyoto
(Shimogamo, Sakyo-ku, Kyoto 606-8522, Japan)

Received June 3, 2003; Accepted July 11, 2003

The chloroplast membrane proteins from spinach were solubilized by various detergents, and adsorbed to ATP-agarose gels. Triton X-100 was most useful for solubilization, and most proteins were adsorbed to the ATP-agarose gel that had matrix attachment of N-6 of ATP. About 10 chloroplast membrane proteins were bound to ATP-agarose gels, which included H^+-ATPase β subunit. Addition of ATP disturbed the adsorption of the proteins to the ATP-agarose gel, and the adsorbed proteins were eluted by ATP from the ATP-agarose gel.

Key words: ATP-agarose; Detergent; ATPase; Chloroplast; Spinach

Mg^{2+} works as a key factor on photosynthesis in plant chloroplasts. We have a final purpose to make the Mg^{2+} transport proteins clear in chloroplasts. In the preceding paper[1], we characterized ATPase activities of spinach chloroplast thylakoid and envelope membranes, but however, we could not identify "Mg^{2+}-dependent" ATPase. As another approach for obtaining ATP-binding proteins and purifying the Mg^{2+} transport proteins of chloroplast membranes, we here solubilized chloroplast membrane proteins by various detergents, and adsorbed the solubilized proteins to ATP-agarose gels.

Materials and methods

Materials

Detergents and ATP-agarose gels were purchased from Sigma, and 5'-ATP, 2Na was obtained from Yamasa.

Solubilization of chloroplast membranes by detergents

Spinach chloroplast membranes were obtained as described previously[1]. Membranes were incubated for 1 h at 4°C in solubilization medium (0.2 M

Abbreviation: PVDF, polyvinylidene difluoride.

sucrose, 10 mM Tricine-KOH (pH 7.5), 2 mM EDTA, 5 mM mercaptoethanol) containing 1% (v/w) detergent as indicated. After incubation, the mixture was centrifuged at 60,000×g for 90 min. The supernatant was again centrifuged at 60,000×g for 1 h. The supernatant was applied to an ATP-agarose gel.

Binding of proteins to ATP-agarose gel

Three kinds of ATP-agarose gels were used (Table 1) in a 1.5 ml of sampling tube. The solubilized chloroplast membranes were added to the gel that was equilibrated with an equilibration medium (40 mM Tricine-KOH (pH 7.5), 5 mM $MgCl_2$, 100 mM NaCl). After incubation at 4℃ for 5-15 min, the tubes were centrifuged at 100×g for 2 min. After the supernatant was removed, 100 µl of the equilibration medium was added and the suspension was centrifuged at 100×g for 2 min. This gel-washing procedure was repeated twice.

The proteins adsorbed to the ATP-agarose gel were eluted with the SDS sample buffer (10% glycerol, 64 mM Tris-HCl (pH6.8), 87 mM SDS, 0.64 M mercaptoethanol), and analyzed with SDS-polyacrylamide gel electrophoresis (PAGE).

N-Terminal amino acid sequence analysis

The proteins were separated by SDS-PAGE and then blotted to PVDF membrane. The proteins were stained with Coomassie brilliant blue R-250. The protein bands were cut and applied to a Shimadzu PPSQ21 sequencer.

Table 1. ATP-agarose gels used.

Matrix attachment	Matrix spacer (atoms)	Matrix activation	Matrix	ATP(nmol)/gel(µl)
N-6	11	cyanogen bromide	cross-linked 4% beaded agarose	1.3
C-8	9	cyanogen bromide	cross-linked 4% beaded agarose	3.1
ribose hydroxyls	11	cyanogen bromide	cross-linked 4% beaded agarose	2.9

Results and Discussion

Binding of chloroplast membrane proteins to ATP-agarose gel

The chloroplast membrane proteins were solubilized by detergents such as Triton X-100, octylglucoside, CHAPS, and cholate. The proteins adsorbed to the ATP-agarose gels were eluted with the SDS sample buffer, and analyzed with SDS- PAGE (Fig. 1). Similar but

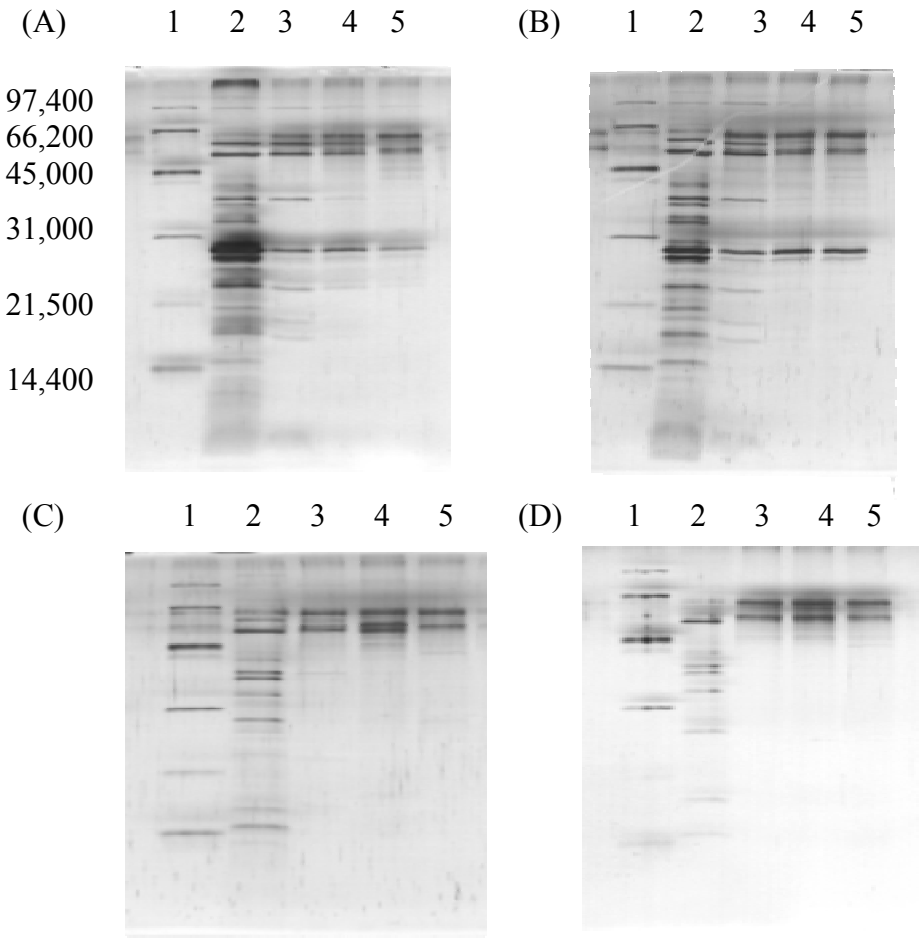

Fig. 1. Adsorption of chloroplast membrane proteins to ATP-agarose gel. The chloroplast membrane proteins were solubilized with 1% Triton X-100 (A), octylglucoside (B), CHAPS (C), and cholate (D), and the solubilized proteins were adsorbed to the ATP-agarose gels (Table 1). The proteins adsorbed to the gel were eluted with the SDS sample buffer, and applied to SDS-PAGE. Lane 1, molecular weight standards: Phosphorylase b, 97,400; serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; trypsin inhibitor, 21,500; lysozyme, 14,400. Lane 2, the solubilized protein fraction. Lanes 3-5, the protein fractions adsorbed to the ATP-agarose gels that had matrix attachment of N-6 (lane 3), C-8 (lane 4), and ribose hydroxyls (lane 5) of ATP.

different patterns of protein bands were observed: many protein bands were observed with solubilization with Triton X-100 and octylglucoside. Among 3 kinds of ATP-agarose gels tested, the gel that had matrix attachment of N-6 of ATP (the N-6 gel) adsorbed many kinds of proteins. Then, the chloroplast membrane proteins were solubilized by Triton X-100, and adsorbed to the N-6 gel. ATP was added during or after incubation of the solubilized proteins with the N-6 gel. Addition of ATP during incubation of the solubilized proteins with the ATP-agarose gel disturbed the adsorption of the proteins to the ATP-agarose gel (Fig. 2). When 50-fold molar amount of ATP·Mg was added to the ATP-agarose gel after the solubilized proteins were adsorbed to the gel, the adsorbed proteins were eluted from the gel to the supernatant (Fig. 3). These results indicated that the proteins adsorbed to the ATP-binding gel did adsorb to the ATP moiety.

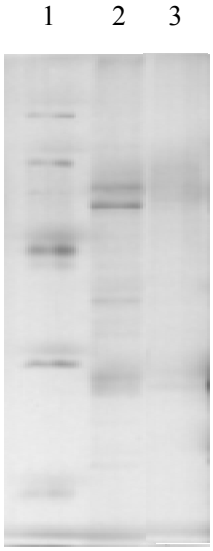

Fig.2. ATP disturbs the adsorption of the proteins to ATP-agarose gel. The chloroplast membrane proteins were solubilized with 1% Triton X-100, and the solubilized proteins were incubated with the ATP-agarose gel that had matrix attachment of N-6 of ATP in the absence (lane 2) and presence (lane 3) of 3.5 mM ATP. Lane 1, molecular weight standards (see Fig. 1).

N-Terminal amino acid sequence of ATP binding proteins

The chloroplast membrane proteins solubilized by Triton X-100 and adsorbed to the ATP-agarose (N-6) gel were applied to N-terminal sequence analysis. The proteins were separated by SDS-PAGE and blotted to PVDF membrane, and usually observed 8 protein bands were applied to N-terminal amino acid sequence analysis (Fig. 4). Only when the solubilized proteins were adsorbed to another ATP-agarose gel that had matrix attachment of ribose hydroxyl groups of ATP, 40 kDa protein band was observed. This protein was also applied to N-terminal sequence analysis. Figure 4B shows N-terminal sequences of the proteins. Unfortunately, N-terminal ends of 6 proteins were blocked, but N-terminal amino acid residues of the

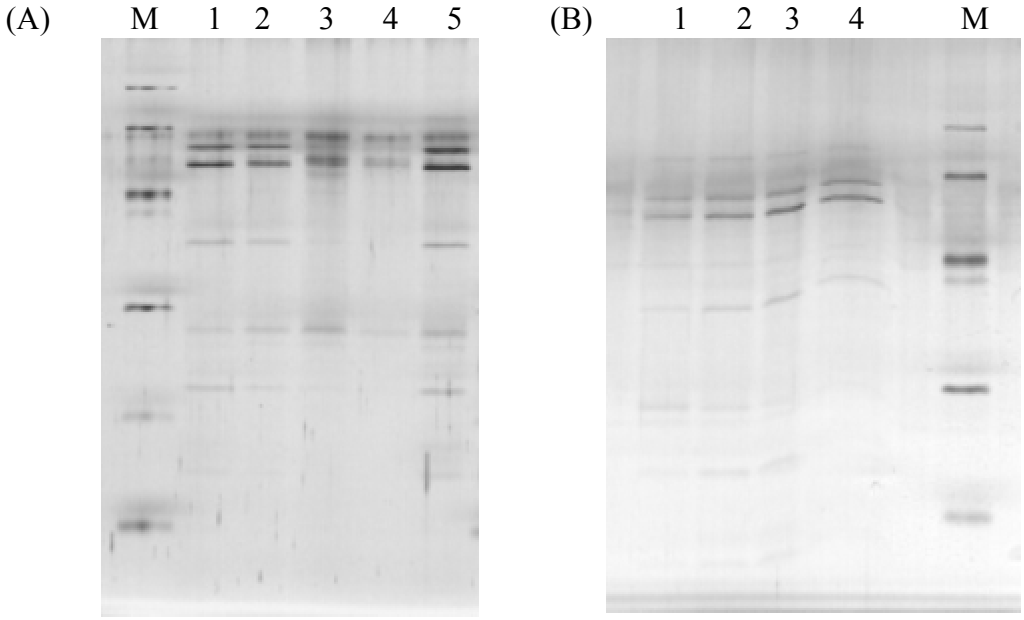

Fig. 3. The proteins adsorbed to the ATP agarose gel are eluted by ATP. Equal (3.5 mM, lane 1), 5-fold (17.5 mM, lane 2), 10-fold (35 mM, lane 3), and 50-fold (175 mM, lane 4) molar amount of ATP were added to the ATP-agarose (N-6) gel, to which the proteins solubilized with 1% Triton X-100 were adsorbed. After incubation at 4℃ for 15 min, the gel suspensions were centrifuged at 1,000×g for 3 min, and the proteins that remained to be adsorbed to the gel (A) and were eluted to the supernatant (B) were applied to SDS-PAGE. Lane M, molecular weight standards (see Fig. 1); lane 5 in (A), the proteins adsorbed to the ATP-agarose gel without addition of ATP.

other proteins were sequenced. Computer database search with the FASTA program revealed that the indicated proteins have same or homologous N-terminal amino acid sequences.

The 51 kDa protein was identified to be H^+-ATPase β subunit. Chloroplast H^+-ATPase (CF_1) is composed of 5 subunits, and the β subunit binds ATP[2]. These results indicate validity of this method for obtaining ATP-binding proteins, but now no other ATPase was identified, maybe due to the blocking of their N-terminal ends. Amino acid sequence analysis of the internal peptides of the proteins whose N-terminal ends are blocked are now in progress.

Conclusion

About 10 chloroplast membrane proteins from spinach were bound to ATP-agarose gels, which included H^+-ATPase β subunit. The adsorbed proteins and their amounts were different depending upon the detergents used for

(A) (B)

Protein band	N-Terminal sequence	Identified protein
94k	blocked	
77k	blocked	
55k	blocked	
51k	MRINP	H^+-ATPase β subunit
37k	blocked	
28k	blocked	
27k	blocked	
26k	GNDAW	Chlorophyll binding protein
	AYGEAYNVFGKPY(K)XN	Oxygen-evolving enhancer protein 2
40k*	AKKSVGDLTSAD	Phosphoglycerate kinase

*Obtained with the ATP-agarose gel that had matrix attachment of ribose hydroxyls of ATP

Fig. 4. N-Terminal amino acid sequences of the proteins adsorbed to the ATP-agarose gel. The proteins adsorbed to the ATP-agarose gel that had matrix attachment of N-6 (otherwise indicated) or ribose hydroxyls of ATP were separated by SDS-PAGE (A), and applied to N-terminal amino acid sequence analysis (B). Computer database search with the FASTA program revealed that the proteins indicated as "identified" have same or homologous N-terminal amino acid sequences.

solubilization and the ATP-agarose gels that had different matrix attachment of ATP. Triton X-100 was most useful for solubilization, and most proteins were adsorbed to the ATP-agarose gel that had matrix attachment of N-6 of ATP.

In the preceding paper[1], we solubilized ATPase activities of spinach chloroplast membranes by detergents, but however, we could not identify Mg^{2+}-dependent ATPase. Studies on ATPase activities of the preparations purified here by the ATP-agarose gel are now in progress.

Acknowledgments

This study was supported in part by Grant-in-Aid for Scientific Research (C) (15580096) from the Ministry of Education, Culture, Sports, Science & Technology of Japan.

References

1) K. Kishimoto, S. Ishijima, and M. Ohnishi: *J. Biol. Macromol.*, **3**, 61-68 (2003).

2) A. Blair, L. Ngo, J. Park, I.T. Paulsen, and M.H. Saier: *Microbiology*, **142**, 17-32 (1996).

Communicated by Hayakawa Kiyoshi.