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Subunit III of the chloroplast ATP-synthase can form a Ca^{2+}-binding site on the lumenal side of the thylakoid membrane

Stanislav D. Zakharov**, Robert G. Ewy, Richard A. Dilley*

Department of Biological Sciences, Purdue University, West Lafayette, IN 47907-1392, USA

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Subunit III, the 8 kDa component of the chloroplast CF_0 H^+ channel, was isolated and purified from pea thylakoids for the purpose of studying its Ca^{2+}-binding properties. After *n*-butanol extraction and ether precipitation, HPLC purification was accomplished using a poly(styrene-divinylbenzene) column which removes lipid and protein contaminations. The main components of protein contamination were two hydrophobic proteins of near 4 kDa molecular mass, the *psa*I and *psb*K gene products associated with PSI and PSII reaction centers, respectively. Purified subunit III as well as the unfractionated organic-solvent soluble preparation were used in a $^{45}Ca^{2+}$-ligand blot assay known to detect high affinity Ca^{2+}-binding sites in proteins. Polypeptides were separated with SDS-PAGE and were transferred onto PVDF membranes. Treatment of the membrane with $^{45}CaCl_2$ in the presence of 10-fold excess of $MgCl_2$ and 200-fold excess KCl led to the labeling of only the 8 kDa polypeptide. The Ca^{2+} binding was inhibited after derivatizing aqueously exposed carboxyl groups with a water soluble carbodiimide plus a nucleophile, after de-formylation of the N-terminal methionine, or with a subsequent treatment with La^{3+}. Ca^{2+} binding was maximum at pH 7.5–8.5 and was greatly decreased at acidic pH. Dicyclohexylcarbodiimide treatment (no nucleophile was added) of thylakoid membranes, which derivativizes the hydrophobically located Glu-61, decreased the electrophoretical mobility of isolated subunit III but did not inhibit the Ca^{2+} binding. The data indicate that the carbonyl group of the formylated N-terminal Met-1 and probably the carboxyl group of the subunit III C-terminal Val-81 provide some of seven essential oxygen ligands normally required for defining a Ca^{2+}-binding site in proteins. It is probable, but not yet established that an oligomeric form of subunit III polypeptides is essential for forming the Ca^{2+}-binding site. Based on the accepted models for the hairpin conformation of the subunit III, it does seem clear that the Ca^{2+}-binding site can form on the lumenal side of the membrane in the functional CF_0 structure.

Ca^{2+}-binding protein; CF_0 subunit III; Thylakoid membrane protein

1. INTRODUCTION

Calcium ions are an important component of higher plant cells, being implicated in diverse signal transduction processes and physiological responses [1]. Chloroplasts and the thylakoids within contain large amounts of Ca^{2+}, mostly bound to membranes and macromolecules (total Ca^{2+} in the organelle is near 20 mM with free Ca^{2+} about 5–10 μM [2]). Recent work has led us to suggest a role for thylakoid Ca^{2+} in regulating the flow of protons into the CF_0 H^+ channel part of the energy coupling complex [3,4]. In that work, there was no direct measurement of calcium binding, rather the Ca^{2+}-dependent photoaffinity labeling of [^{3}H]chlorpromazine (a well-known probe for high affinity Ca^{2+}-binding sites in proteins [5] was used to identify thylakoid proteins whose differential labeling correlated with Ca^{2+} effects on the energy coupling reactions [3]. As shown in [3], the 8 kDa subunit III of the CF_0 complex and an unidentified protein near 6 kDa molecular mass were photoaffinity-labeled by chlorpromazine.

*Corresponding author.

**On leave from the Institute of Soil Science and Photosynthesis, Russian Academy of Sciences, Pushchino, Russia.

This report deals with direct Ca^{2+}-binding assays in an attempt to determine which, if any, of the protein(s) that are implied as interacting with Ca^{2+} actually have Ca^{2+}-binding properties. The assay used here is the $^{45}Ca^{2+}$ overlay technique, wherein polypeptides separated on SDS-PAGE gels are electroblotted onto polyvinyl difluoride (PVDF) membranes and then washed with salt regimes including $^{45}Ca^{2+}$ ions. The technique is widely used to detect proteins having high affinity Ca^{2+}-binding sites, such as calmodulin, troponin C and other proteins including thylakoid membrane proteins [6,7].

As a preparatory step, the 8 kDa subunit III protein was purified from other hydrophobic proteins present in the butanol extract using an improvement on the HPLC method similar to that reported earlier [3].

2. MATERIALS AND METHODS

2.1. *Isolation and purification of the ATP-synthase subunit III*

Thylakoid membranes were prepared from pea plants as described earlier [3]. The 8 kDa subunit III of the ATP-synthetase was extracted from thylakoids with *n*-butanol according to the procedure of Nelson et al. [8] with some modifications. Thylakoids (10–12 ml) containing 8–12 mg/ml Chl were injected into 250 ml *n*-butanol (0°C) and stirred vigorously for 45–60 min. The precipitate was removed by centrifugation for 10 min at 16,000 × *g* followed by the filtration through a

0.2 μm mesh nylon filter. The filtrate was added with stirring to 1250 ml ethylether precooled to −20°C. After 2.5–3 h the precipitate was collected by centrifugation for 20 min at 10,000 × *g* (−20°C) and dissolved in $CHCl_3$/MeOH (v/v; 2:1). Undissolved material was removed by centrifugation. The solvent was evaporated and the residue was dissolved in 5% SDS.

To reduce lipid contamination the recovered protein was washed in cold acetone (−15°C) by taking 0.3 ml of the protein in 5% SDS and stirring with 30 ml acetone followed by centrifugation for 30 min at 8,000 × *g* (−15°C). The pellet was dissolved and kept in $CHCl_3$/MeOH (v/v/; 2:1) or 5% SDS.

Further purification of subunit III was accomplished as in the earlier work [3] using reverse-phase HPLC on a poly(styrene-divinylbenzene) column of size 5 cm × 0.41 cm (i.d.) packed with the column material having 20 μm particle size and 6,000–8,000 Å through-pores connected by 800–1,500 Å pores (obtained from Perceptive Inc., Boston, MA). Protein samples (100–200 μg) in 5% SDS with 0.1% TFA added were injected into the column and reversed-phase HPLC was performed on a Waters Model 680 gradient controller, with a Model M-45 solvent delivery system and a Model 480 LC spectrophotometer using a detection wavelength of 220 or 280 nm. The column was eluted with a linear gradient of acetonitrile in water, 20–100% (v/v) with 0.1% TFA. The 8 kDa subunit III was eluted at approximately 60% acetonitrile.

Protein concentration was determined by Peterson's modification of the Lowry method [9] with BSA as a standard.

2.2. *SDS-electrophoresis, electroblot polypeptide transfer onto PVDF membranes and amino acid sequence determination*

SDS-electrophoresis in 16.5% polyacrylamide gels was run according to Schägger and von Jagow [10]. After electrophoresis the gel was stained with Coomassie R with subsequent additional staining (where shown) with Silver, or it was soaked 10–15 min in buffer used for electroblotting as in [7] using 17 mM H_3BO_3-Tris, pH 8.4, 20% methanol, 0.03% SDS). Semi-dry electroblotting of polypeptides from the gel onto the PVDF membrane (Bio-Rad) was accomplished using a semidry electroblotter (SemiPhor TE 70, Hoefer Sci. Instr.) for 2 h at a current of 130 mA and an upper voltage limit of 13 V.

Protein electroeluted onto the PVDF paper was subjected to amino acid sequence analysis by the Purdue Biochemistry Department Protein Sequencing facility, using a gas phase Applied Biosystems Model 470A sequencer.

2.3. *$^{45}Ca^{2+}$ binding to polypeptides on the PVDF membrane*

Polypeptides able to bind $^{45}Ca^{2+}$ were detected according to Maruyama and Nomomura [11]. The blot was rinsed with water, washed once in 5 mM EGTA, pH 7.0, and washed three times in 60 mM KCl, 3 mM $MgCl_2$, 5 mM imidazole. The blot was then incubated for 15 min in the same buffer with 0.3 mM $^{45}CaCl_2$ (5 μCi ^{45}Ca/ml), and washed 3 times with 50% ethanol (2 min each), dried, and exposed to Kodak XAR-5 film for 1–2 days. For experiments on the pH dependence of $^{45}Ca^{2+}$ binding, 5 mM MES and 5 mM Tris were used in place of imidazole.

3. RESULTS

3.1. *Analysis of the polypeptides extracted from thylakoids by* n-*butanol*

The *n*-butanol extraction method of Nelson et al. [8] gives the 8 kDa subunit III, as expected, and two additional hydrophobic proteins, with M_r near 4 kDa and faint bands near 6 kDa, as indicated in Fig. 1. After acetone washing to remove lipids – an important step for obtaining good HPLC and SDS-PAGE separations – the protein fraction was run on HPLC using the poly(styrene-divinylbenzene) column with a gradient of

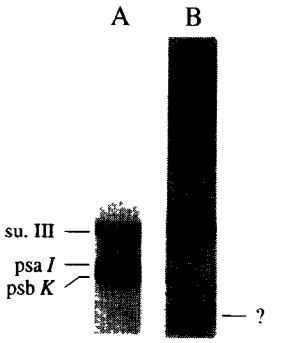

Fig. 1. SDS-PAGE separation and identification of calcium-binding polypeptides in the *n*-butanol extract of thylakoid membranes after transfer to a PVDF membrane. (A) Coomassie-stained gel of the acetone washed *n*-butanol extract of thylakoids (40 μg). (B) Autoradiograph of the polypeptides after transferring onto a PVDF membrane and performing the $^{45}Ca^{2+}$ ligand blot assay as described in section 2.

acetonitrile. The elution pattern, shown in Fig. 2A, shows several peaks which were collected and subjected to SDS-PAGE, and Silver staining (Fig. 2B). Fractions 1 and 2 were electroeluted and protein sequencing revealed their identities as the *psa*I and *psb*K gene products, respectively.

The *psa*I gene product protein sequence was analyzed at the 70–90 pmol level for residues 3 to 5 with the expected attenuation in later cycles. Contaminating residues were observed, but usually at <50% of amount of the principal residue. The observed sequence was, starting at position 3:

N L P S L F V P L V S L L F P A V A M R S L F
3 10 * 20 * 25

The comparison to the literature value shows 91% positive and 86% identical amino acid sequence as reported in [12]. At position 13 we found Ser (Gly is the reported residue) and at 22 Arg instead of Ala.

The *psb*K gene product was the main constituent of fraction 2 and after electroblotting it sequenced with a yield near 20–25 pmol in the early cycles with very low contamination. The observed sequence was:

K L P E A Y A F L N P I V D F M P V I P L L
1 10 * 20

giving 95% identity to the reported sequence [12,13]. At position 15 Phe occurred instead of Ile. Both *psa*I and *psb*K gene products have been found in PSI and PSII reaction center preparations, respectively [12,13].

3.2. *Ca^{2+}-binding assays using $^{45}Ca^{2+}$*

The Ca^{2+}-binding capability of the four polypeptides mentioned above was tested using the method essentially as reported by Maruyama and Nomomura [11]. The *n*-butanol extract polypeptides (not subjected to HPLC) were fractionated by SDS-PAGE and were transferred onto PVDF membranes and incubated with $^{45}CaCl_2$ in the present of 10-fold access of $MgCl_2$ and

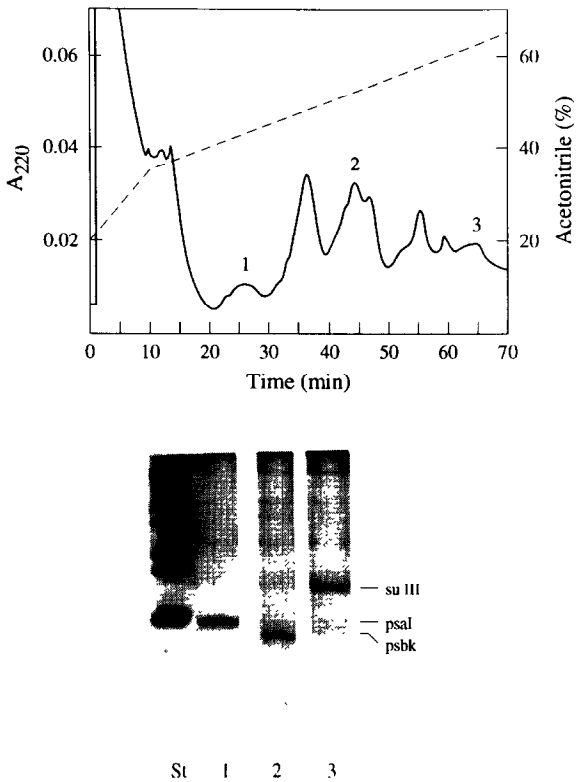

Fig. 2. Reverse-phase HPLC on a poly(styrene-divinylbenzene) column of the *n*-butanol extracted polypeptides of thylakoid membranes. 200 μg of protein was loaded on the column and eluted at 2 ml/min with the indicated step gradient (---) as described in section 2; absorbance was monitored at 220 nm. Collected fractions were lyophilized and dissolved in 5% SDS. SDS-PAGE of the indicated fractions was accomplished followed by silver staining of the gel. Standards (st) were myoglobin fragments: 17.0, 14.4, 8.2, 6.2, 2.5 kDa.

200-fold access of KCl. Following subsequent washings of the membrane with 50% ethanol, polypeptides able to retain $^{45}Ca^{2+}$ were visualized by autoradiography (Fig. 1B). Only the 8 kDa subunit III gave Ca^{2+} binding. Some Ca^{2+}-binding material was observed near the dye front (lower than the 2 kDa polypeptide standard). Microsequence analysis showed there were no peptides there.

3.3. *Effect of amino acid modification on Ca^{2+} binding to subunit III*

The protein ligands participating in Ca^{2+} binding are virtually always oxygen atoms [14] and in subunit III these could be the side chain carboxyl groups of glutamic acid, the α-carboxyl group of C-terminal amino acid, main chain peptide bond carbonyl groups, and the carbonyl groups of the formyl function on the N-terminal Met and the carbonyl oxygens of asparagine or glutamine; for example, that of Asn-2. The treatment of subunit III with acid methanol to deformylate the N-terminal methionine suppressed $^{45}Ca^{2+}$ labeling of subunit III (Fig. 3B). The treatment slightly decreased the SDS-PAGE mobility of the protein (Fig. 3A). That result suggests that the Ca^{2+}- binding site requires the carbonyl oxygen of the formyl group on the N-terminal Met.

The incubation of purified subunit III with the water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) in the presence of the nucleophile glycinemethyl ester (GME) eliminated $^{45}Ca^{2+}$ labeling of the polypeptide (Fig. 4A,B). Four or five carboxyl groups could be derivatized by that treatment, Glu-37, -44 and -46 (and perhaps -61) and the C-terminal COO^- of Val-81. However, treatment with dicyclohexyl carbodiimide (DCCD), expected to derivatize only the Glu-61 carboxyl in subunit III, did not block the $^{45}Ca^{2+}$ binding whether the DCCD was given to thylakoids (Fig. 3) or to an SDS solution of the purified subunit III (Fig. 4). When thylakoid membranes were incubated with 1 mM DCCD (at a chlorophyll concentration of 0.4 mg/ml) for 15 h (4°C) to reach maximal modification of subunit III at position Glu-61, the *n*-butanol extracted polypeptide showed a decreased electrophoretical mobility (Fig. 3A). It should be noted that commercial low molecular weight protein standards are not suited for the estimation of very hydrophobic polypeptides such as the 8 kDa subunit III and give low values of apparent molecular weight (Figs. 2,3). The in situ DCCD-modified subunit III gave two bands, near 10 and 15 kDa, and both gave $^{45}Ca^{2+}$ binding (Fig. 3, compare lane 2 (control) and lane 3 (DCCD-treated)). It has been previously shown that the DCCD derivati-

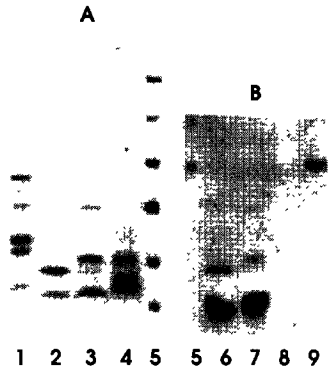

Fig. 3. Effect of N-terminal Met-1 deformylation and DCCD modification of Glu-61 on calcium binding capacity of subunit III. (A) Coomassie-stained gel of *n*-butanol extracted polypeptides of thylakoids. (B) Autoradiograph of the polypeptides after the transfer onto a PVDF membrane and performing the $^{45}Ca^{2+}$ ligand blot assay as described in section 2. The *n*-butanol fraction of thylakoids dissolved in chloroform/methanol (v/v; 2:1) was lyophilized and suspended in MeOH/con. HCl (v/v; 23:1). After 24 h incubation at room temperature, the solvent was evaporated and the polypeptides were solubilized in SDS-PAGE buffer. Lanes 4,8 = 20 μg de-formylated polypeptides; lanes 3,7 = the polypeptides of DCCD-treated thylakoids (18 μg). Thylakoids (0.4 mg Chl/ml) were incubated in 1 mM DCCD 14 h at 4°C. After sedimentation, the DCCD-treated thylakoids were used for *n*-butanol extraction as described in section 2. Lanes 2,6 = the polypeptides of untreated thylakoids (20 μg); lane 1 = myoglobin fragments; lanes 5,9 = prestained myoglobin fragments. Note that prestained polypeptides sharply change their electrophoretic mobility in comparison with the unstained material (lane 1).

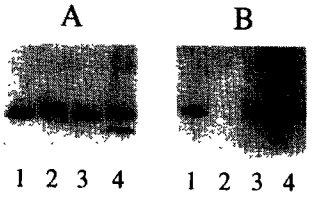

Fig. 4. Effects of cyanogen bromide cleavage and EDAC (plus GME) and DCCD modification of carboxyl groups on calcium binding with the purified subunit III of the ATP-synthase. The polypeptides after SDS-PAGE were electrotransferred onto a PVDF membrane, and the membrane was then used in the $^{45}Ca^{2+}$ ligand blot assay as described in section 2, autoradiographed (B), then stained with Coomassie R (A). Note that Coomassie R staining removes $^{45}Ca^{2+}$. Purified subunit III in 5% SDS was incubated for 16 h at room temperature with 10 mM EDAC in the presence of 25 mM GME (lane 2) or with 1 mM DCCD (lane 3) and diluted with SDS-PAGE buffer. Lane 1 = unmodified subunit III. Cyanogen bromide cleavage was performed as described [11]. After several solvent evaporation steps, the residue was dissolved in SDS-PAGE buffer.

zation of the subunit III causes retardation of its mobility on DEAE columns [15] and in its electrophoretic mobility in SDS-PAGE [16].

Cyanogen bromide cleavage of subunit III results in the expected formation of a 6 kDa fragment (Fig. 4A) and it did not show $^{45}Ca^{2+}$ binding (Fig. 4B). The predicted 2 kDa fragment was not observed in the stained SDS-PAGE gel, nor did any Ca^{2+} binding appear in that molecular mass region (Fig. 4B).

The $^{45}Ca^{2+}$ binding to subunit III has a broad pH optimum above pH 7.0 (data not shown), with binding absent at pH 4.5. Such data are consistent with the notion that deprotonated carboxyl groups participate in the $^{45}Ca^{2+}$ binding, and that the pK_a values are shifted to a significantly higher range than expected for a normal carboxyl group. Moody et al. [19] have observed that the carboxyl groups of *E. coli* subunit III in chloroform/methanol have abnormally high pK_a values.

An important question is the stoichiometry of Ca^{2+} binding with subunit III. Only a rather rough estimate is available at this time and it gives the stoichiometry as 1 Ca^{2+} per 5 subunit III for $^{45}Ca^{2+}$ binding at pH 7.0, but this is likely an underestimation. The incubation of the blot with 0.1 mM $LaCl_3$ following the $^{45}Ca^{2+}$ ligand blot assay completely removed bound Ca^{2+} of subunit III (data not shown).

4. DISCUSSION

In this work, using a $^{45}Ca^{2+}$ ligand blot assay, we have shown that a Ca^{2+}-binding site is formed by the purified 8 kDa subunit III of the ATP-synthase after the polypeptide was transferred to PVDF paper. The relevance of this procedure for discerning high affinity Ca^{2+}-binding proteins has been established previously [6,7,11]. Ca^{2+} binding with subunit III was selective, as indicated by the fact that a ten-fold excess of Mg^{2+} and 200-fold excess of K^+ did not prevent the Ca^{2+} binding. Subunit III was the only polypeptide of four present in the *n*-butanol extract which showed $^{45}Ca^{2+}$ binding in the assay (Fig. 1B). Comparing the earlier work using the Ca^{2+} dependence of photoaffinity labeling of [^{3}H]chlorpromazine, which implicated both the 8 kDa subunit III protein and a poorly-defined polypeptide with M_r near 6 kDa as possible Ca^{2+}-binding proteins [3,4], it seems clear that only the subunit III protein shows $^{45}Ca^{2+}$ binding using the present method. Neither the still-unidentified 6 kDa protein, nor the *psa*I and *psb*K gene product proteins have the type of high affinity Ca^{2+}-binding site revealed by the $^{45}Ca^{2+}$ overlay technique.

This work does not completely define the subunit III binding site for Ca^{2+}, and it is not clear whether a single polypeptide provides the site or whether several 8 kDa proteins must interact as a complex. However, some indications as to the location of the binding site can be deduced. The location very likely is on the lumen side of the membrane, indicated mainly by the observation that removing the formyl carbonyl group on the N-terminal Met causes the loss of Ca^{2+} binding (Fig. 3). The evidence to date indicates that the subunit III has a 'hairpin' structure with both N- and C-termini on the lumen side, with the two transmembrane helices close together [17]. If that is so, then the C- and N-termini would be close to each other, possibly allowing both the formyl group –C=O and the C-terminal Val-81 carboxyl group to contribute oxygen ligands to the Ca^{2+}-binding site. That is consistent with carboxyl modification by EDAC and GME (Fig. 4), also causing loss of Ca^{2+} binding. However, it cannot be ruled out that modifying the Val-81 carboxyl and some or all of the carboxyl groups in the hydrophilic loop (Glu-37, Glu-44 and Glu-46) [18] could disrupt the oligomeric structure which the subunit III appears to favor [17].

It does seem clear, and quite interesting as well, that the Glu-61, located in the hydrophobic second transmembrane helix [18] and expected to be blocked by DCCD, does not contribute oxygen ligands to the Ca^{2+}-binding site (Figs. 3 and 4).

The assignment of a group near the N- and C-termini as providing the Ca^{2+}-binding site is supported by NMR data showing that the C-terminal Ala of the *E. coli* subunit *c* (III) has a high affinity for lanthanide ions [19], a clear indication of the Ala carboxyl group having a propensity to interact with Ca^{2+}. In data to be published separately, we have shown that the *E. coli* subunit *c*, kindly provided by Prof. R. Fillingame, Univ. of Wisconsin, has Ca^{2+}-binding properties similar to that of the pea thylakoid subunit III, including effects of removing the N-terminal Met formyl group.

The low Ca^{2+} per subunit III stoichiometry indicated in these results (about 1 Ca^{2+} per 5 subunit III polypeptides) could be owing to either the polypeptide on the PVDF paper not having the optimal conformation or, more interestingly, it may be that the Ca^{2+}-binding site is formed by an oligomer of subunit III proteins. Oli-

gomerization of subunit III on the PVDF paper is possible upon lowering the SDS concentration in the blot procedure. It appears from other data that the subunit III can maintain an oligomeric structure in SDS on PAGE gels [20] and in chloroform/methanol [17]. The native CF_o is believed to function with 10–12 copies of subunit III, based on analogy to the *E. coli* system [17]. The loss of Ca^{2+} binding to the 6 kDa cyanogen bromide cleavage product (Fig. 4) could be owing to the loss of oligomerization or alteration in the predicted close approach of the C- and N-termini, but this remains to be determined.

More work is necessary to define better the structural factors giving rise to Ca^{2+} binding by subunit III. It is of physiological interest that the work leading to these experiments suggested that Ca^{2+} interacts with the CF_o H^+ channel such as to provide a gating action on H^+ flux across the lumenal part of the CF_o by Ca^{2+} binding/debinding ([3,4]; cf. [21] for a review). The gating action can block ATP formation driven by lumenally derived H^+ efflux [3,4] but it does not block that component of ATP formation attributable to H^+ efflux from membrane-localized domains [3].

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