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Oligomerization of H^+-pyrophosphatase and its structural and functional consequences

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Abstract

The H^+-pyrophosphatase (H^+-PPase) consists of a single polypeptide, containing 16 or 17 transmembrane domains. To determine the higher order oligomeric state of *Streptomyces coelicolor* H^+-PPase, we constructed a series of cysteine substitution mutants and expressed them in *Escherichia coli*. Firstly, we analyzed the formation of disulfide bonds, promoted by copper, in mutants with single cysteine substitutions. 28 of 39 mutants formed disulfide bonds, including S545C, a substitution at the periplasmic side. The formation of intermolecular disulfide bonds suppressed the enzyme activity of several, where the substituted residues were located in the cytosol. Creating disulfide links in the cytosol may interfere with the enzyme's catalytic function. Secondly, we prepared double mutants by introducing second cysteine substitutions into the S545C mutant. These double-cysteine mutants produced cross-linked complexes, estimated to be at least tetramers and possibly hexamers. Thirdly, we co-expressed epitope-tagged, wild type, and inactive mutant H^+-PPases in *E. coli* and confirmed the formation of oligomers by co-purifying one subunit using the epitope tag used to label the other. The enzyme activity of these oligomers was markedly suppressed. We propose that H^+-PPase is present as an oligomer made up of at least two or three sets of dimers.

Keywords: Disulfide bond; H^+-pyrophosphatase; Oligomerization; Proton pump

1. Introduction

H^+-translocating inorganic pyrophosphatase (H^+-PPase) uses inorganic pyrophosphate (PP_i) as an energy source to transport protons across biomembranes, generating electrochemical proton gradients for active secondary transport systems [1]. H^+-PPases, which consist of single polypeptides of about 80 kDa, form a unique protein family distinct from P-, F-, and V-type H^+-ATPases [2–4]. Their primary sequences differ from other H^+-pumps and soluble PPases, although they share a few functional motifs with the soluble PPases and P-type ATPases [2,4,5].

H^+-PPases are found in higher plants [4], parasitic and free-living protozoa [6,7], some eubacteria [8–10], and archaebacteria [3,11]. In these organisms, H^+-PPase acidifies intracellular organelles, such as vacuoles in plants [4], and acidocalcisomes in protozoa [12] and bacteria [13], together with V-ATPase. Recently, H^+-PPase has been suggested to exist in the yolk granule membrane of the insect, *Rhodnius prolixu* [14]. One of the physiological roles of H^+-PPase is to compensate for V-ATPase under conditions of energy stress [1]. In the photosynthetic bacterium *Rhodospirillum rubrum*, the transcription of H^+-PPase has been shown to increase in anaerobic conditions and increasing salt stress but decrease in aerobic conditions, under the control of its promoter [15]. Analysis of a null mutant in *R. rubrum* showed that this enzyme is not essential, but plays an important role in growth under low energy conditions such as low light intensity [16]. In plants, salt and osmotic stress are known to enhance the transcription of H^+-PPase [17] and its over-expression confers

Abbreviations: H^+-PPase, H^+-translocating pyrophosphatase; PP_i, inorganic pyrophosphate; ScPP, *S. coelicolor* H^+-PPase; BM, 3-(*N*-maleimidylpropionyl)biocytin; CuPh, Cu(II)-(1,10-phenanthroline)$_3$; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; ECL, enhanced chemiluminescence

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salt tolerance in a salt-sensitive yeast mutant [18] and tolerance to salt and drought in transgenic *Arabidopsis* plants [19]. Another function of this enzyme is to scavenge cytoplasmic PP_i, which is produced as a byproduct of various metabolic processes [1].

As well as the physiological role of H^+-PPase, the coupling mechanism between PP_i hydrolysis and active H^+ transport has also been studied. H^+-PPases strictly require Mg^{2+}, both for enzyme function and structural stabilization [20,21], but have been subdivided into two groups according to their K^+ requirements and their amino acid sequences [3]. Type I enzymes require at least 30 mM K^+ to be active [1] whereas Type II enzymes are fully active in the absence of K^+ [11,22]. Mutagenesis and antibody studies have identified functional residues and motifs in both types of H^+-PPase [5,23–30]. The presence of lysine rather than alanine residues in the conserved GNTT(K/A) motif has been proposed as a useful criterion to distinguish Type I and II enzymes, respectively [31]. Recently, Glu^{197} and Glu^{202} in *R. rubrum* H^+-PPase were shown to be involved in substrate binding, and Glu^{550} and Glu^{649} were demonstrated to be important for the correct folding of the polypeptide [32].

Although many functional residues have been reported, information about the tertiary structure of H^+-PPase is limited by the general difficulty of crystallizing membrane proteins. Most H^+-PPases contain 16 transmembrane domains with conserved motifs in their cytoplasmic loops [33]. Several groups have proposed that the functional unit for H^+-PPases is a dimer [1], on the basis of chemical cross-linking studies [34], gel filtration chromatography [35,36], radiation inactivation [35–37], inactivation by high hydrostatic pressure [38], and thermo-inactivation [39] using plant enzymes.

To determine which residues in the hydrophilic loops are involved in oligomerization, we have constructed a series of mutant enzymes, expressed them in *E. coli* and analyzed oligomer formation by cross-linking cysteine residues. In this study, we have focused on *S. coelicolor* H^+-PPase (ScPP), which can be functionally expressed in *E. coli.* We produced a series of site-directed mutants, substituting cysteines for single residues within the ScPP protein, and looked at the formation of disulfide bonds between the subunits. Studies using site-directed thiol cross-linking have provided insights into the structure of several other membrane proteins including the Na^+/H^+ exchanger [40,41], the metal-tetracycline/H^+ antiporter [42], lactose permease [43], mannitol permease [44], the multidrug transporter [45], and P-glycoprotein [46]. We have applied this method to ScPP to identify neighboring residues within oligomeric forms and to evaluate the biochemical implications of oligomerization. In addition, we constructed two epitope-tagged forms of ScPP, an active wild-type ScPP and an inactive ScPP mutant. Purification of these hetero-oligomeric enzyme complexes allowed us to confirm the association of multiple polypeptides in the bacterial membrane and to study the effect of oligomerization on enzyme activity. This study provides insights into the functional oligomerization and the structure–function relationship of H^+-PPases in membranes.

2. Materials and methods

2.1. Plasmid construction and site-directed mutagenesis

Substitution mutants of ScPP, in which individual cysteine residues or all cysteine residues were changed, were generated from a ScPP gene (sScPP), synthesized previously [33]. The sScPP gene was identical to the native enzyme (DDBJ/GenBank™/EBI accession number AB180905) with the exception of the substitution of Phe for Ser^{282}. ScPP proteins were expressed in plasmid pYN309 which was constructed from pET23b (Novagen) by modifying the *Pst*I site. A His_6-tagged cysteine-less ScPP (C-less-His) was generated from the expression plasmid pYN316, which encodes a His_6-tagged ScPP (ScPP-His), by substituting the four endogenous cysteine residues as follows: C178S, C179S, C253A, and C621V. Single cysteine substitutions were generated from a plasmid containing C-less-His (pHM17). Double cysteine mutants were generated from a plasmid that already contained the S545C substitution (pHM45). The plasmid vectors pET-Duet-1 and pCDFDuet-1 (Novagen) were used to co-express the native ScPP gene (nScPP) and sScPP. The original tags in these vectors were exchanged for a His_6-tag (pDuet-M2-His_6) and a FLAG epitope (pCDF-M2-FLAG), respectively, by PCR. The nScPP gene was amplified from the chromosomal DNA of a laboratory strain of *S. coelicolor* by PCR with KOD Dash DNA polymerase (Toyobo Co., Osaka, Japan) and inserted into the *Nde*I and *Xho*I sites of pCDF-M2-FLAG, to construct the expression plasmid (pHM99) for FLAG-tagged ScPP (ScPP-FLAG). sScPP was transferred from pYN316 to the *Nde*I and *Xho*I sites of pDuet-M2-His_6 to prepare a plasmid (pHM93) expressing ScPP-His. The pHM93 plasmid was used as a template to generate four mutants of ScPP-His ($S263C_w$, $S402C_w$, $S609C_w$, and $S694C_w$). Mutagenesis was carried out using a QuickChange site-directed mutagenesis kit (Stratagene) using the method of Kirsch and Joly [47]. All PCR-amplified products were sequenced to confirm the correct mutations were present.

2.2. Protein expression in E. coli and crude membrane preparation

Proteins were expressed in *E. coli* and crude membranes were prepared as previously described [33], but with slight modifications. *ScPP* constructs derived from plasmid pYN309 were introduced into the *E. coli* strain BLR(DE3)-pLysS K128I [33]. Transformants were selected in Luria–Bertani plates containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. Expression vectors derived from pDuet-M2-His_6 and pCDF-M2-FLAG were introduced into the