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Phospholipid requirement and pH optimum for the in vitro enzymatic activity of the *E. coli* P-type ATPase ZntA

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Abstract

Detergent solubilization and purification of the *E. coli* heavy metal P-type ATPase ZntA yields an enzyme with reduced hydrolytic activity in vitro. Here, it is shown that the in vitro hydrolytic activity of detergent solubilized ZntA is increased in the presence of negatively charged phospholipids and at slightly acidic pH. The protein–lipid interaction of ZntA was characterized by enzyme-coupled ATPase assays and fluorescence spectroscopy. Among the most abundant naturally occurring phospholipids, only phosphatidyl-glycerol lipids (PG) enhance the in vitro enzymatic ATPase activity of ZntA. Re-lipidation of detergent purified ZntA with 1,2-dioleoylphosphatidyl-glycerol (DOPG) increases the ATPase activity four-fold compared to the purified state. All other *E. coli* phospholipids fail to activate the ATPase. Among the phosphatidyl-glycerol family, highest activity was observed for 1,2-dioleoyl-PG followed by 1,2-dimyristoyl-PG, 1,2-dipalmitoyl-PG and 1,2-distearoyl-PG. Increasing intrinsic Trp fluorescence quantum yield upon relipidation of ZntA was used to determine a pH maximum for lipid binding at pH 6.7. The pH dependence of the lipid binding was confirmed by pH-dependent ATPase assays showing maximum activity at pH 6.7. The biophysical characterization of detergent solubilized membrane proteins crucially relies on the conformational stability and functional integrity of the protein under investigation. The present study describes how the *E. coli* ZntA P-type ATPase can be stabilized and functionally activated in a detergent solubilized system.

Keywords: ATPase assay; Lipid activation; P-type ATPase; pH optimum

1. Introduction

Zinc and other heavy metals can have deleterious effects on cell growth. Gram-negative bacteria have developed an elaborate system to control the intracellular Zn^{2+} concentration which, if it were all free, has been estimated to be on the order of 0.6 mM in the cytoplasm [1]. High extracellular Zn^{2+} concentrations trigger the expression of efflux systems, such as the P-type heavy metal ATPase ZntA, the CzcD cation diffusion facilitator and the inner and outer membrane spanning Czc ABC complex [2,3].

ZntA belongs to the soft heavy metal transporting P-type ATPases, which translocates Zn^{2+}, Pb^{2+} and Cd^{2+} across the *E. coli* inner cell membrane [4–6]. It contains an N-terminally located, cysteine-rich heavy metal binding domain. Removal of this domain does not abolish the hydrolytic activity; however, the overall ATPase activity decreases by about 50–70% [7]. A conserved Cys–Pro–Cys sequence is found in transmembrane helix 6. These amino acids are believed to contribute to a metal binding site at the center of the lipid bilayer [6].

P-type transport ATPases were among the first integral membrane proteins identified to exhibit a strong lipid dependence of enzyme activity [8,9]. Phospholipids (PL) consist of a hydrophilic "head group" and hydrophobic fatty acids esterified via a glycerol group. Among the P-type ATPase superfamily, examples have been found for phospholipid "head group" as well as fatty acid "side chain" specific protein–lipid interactions [10–18]. However, the relative specificity of

Abbreviations: a.u.; arbitrary units; C12E8; dodecyl octaethylene glycol monoether; DTT; dithiothreitol; HEPES; N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; MES; 2-(morpholino)-ethanesulfonic acid; MRC; Medical Research Council, Trishydroxymethyl-aminomethane

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protein–lipid interactions observed for P-type ATPases as well as for a variety of other integral membrane enzymes appears to be weak [13,14,19–21]. About 30–40 PL molecules can be found in the lipid annulus surrounding a transmembrane protein of average size [22] with an average exchange rate with bulk PL on the order of 10^6 to 10^7 per second [23,24].

Biochemical and structural work on membrane proteins often involves their detergent solubilization. Therefore, weak protein–lipid interactions are likely to be disrupted, leading to a loss of enzyme activity or structural stability. In some cases, however, reversible inactivation following detergent solubilization and subsequent restoration of the enzymatic activity upon relipidation in a detergent solubilized system has been reported. Examples include P-type ATPases, cation diffusion facilitators and ion channels [8,25–27]. It must be stressed, however, that the presence of detergent in a relipidation experiment may cause a variety of different effects due to detergent–lipid, detergent–protein and lipid–protein interactions [25].

The availability of higher resolution membrane protein crystal structures enables us to study protein–lipid interactions from a structural point of view [28,29]. However, as the majority of membrane protein crystal structures is derived from detergent solubilized systems, loosely bound lipids are either lost during the purification procedure or appear positionally disordered in the crystallographic analysis. Nevertheless, tightly bound lipid molecules can be found in some of the higher resolution X-ray structures, e.g., the bacterial potassium channel KcsA [30], the rotor ring of F- and V-type ATPases [31,32] and bacteriorhodopsin [33].

In this study, the protein–lipid interaction of the N-terminally truncated heavy metal transport P-type ATPase ZntA from *E. coli* was investigated. Expression, detergent solubilization and purification of the Zn^{2+} ATPase yielded an enzyme whose hydrolytic activity was increased upon the addition of detergent solubilized *E. coli* total lipid extract. Among the various *E. coli* phospholipids tested, phosphatidyl-glycerols were identified as the strongest activating membrane component. Phosphatidyl-ethanolamine and cardiolipin, the other major *E. coli* membrane lipids, failed to restore the ATPase activity. Increasing intrinsic Trp fluorescence of ZntA upon relipidation was used to determine the pH dependency of the protein–lipid interaction. Fluorescence data, together with ATPase activity analysis, identified a pH optimum for protein–lipid interaction at approximately 6.7.

The biophysical characterization of membrane integrated enzymes requires their purification in a conformationally stable and catalytically active state. The data presented highlight the pH-dependent protein–lipid interaction of ZntA and the in vitro activation of the enzyme by PG phospholipids. This analysis was performed in a detergent solubilized system leading to reaction conditions possibly involving lipid–detergent, detergent–protein and lipid–protein interactions. Nevertheless, the information obtained highlights a general approach to stabilize and maintain functionally active ZntA and presumably other P-type ATPases in a detergent solubilized state. This might help to characterize other members of this important class of membrane transporter in more detail.

2. Experimental procedures

2.1. Materials

The *E. coli* strain Top10 was obtained from Invitrogen, Groningen, The Netherlands. Phospholipids 1,2-dioleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (DOPG), 1,2-distearoyl-*sn*-glycero-3[phospho-rac-(1-glycerol)] (DSPG), 1,2-dipalmitoyl-*sn*-glycero-3[phospho-rac-(1-glycerol)] (DPPG), 1,2-dimyristoyl-*sn*-glycero-3[phospho-rac-(1-glycerol)] (DMPG), cardiolipin (heart, bovine) and *E. coli* total lipid extract were purchased from Avanti Polar Lipids, Alabama. Detergents decyl-β,D-maltopyranoside (DM) and C12E8 were obtained from Anatrace, Maumee, OH. All other reagents were of highest commercial grade.

2.2. Methods

2.2.1. Expression and purification of ZntA107

The gene for the N-terminally truncated version of ZntA was PCR amplified and cloned into the pBAD expression vector (Invitrogen) as described [7]. The construct was sequenced to confirm the integrity of the gene. Transformed Top10 cells were grown in Luria Bertani medium at 37 °C until the optical density reached 1.0. Protein expression was induced by addition of 0.02% arabinose and cells were harvested 4 h after induction. A 1 liter cell culture pellet was resuspended in 20 ml Resuspension Buffer (25 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM $ZnCl_2$, 20% glycerol) and broken open using a high pressure homogenizer (EmulsiFlex-C5, Avestin). Protease inhibitors such as PMSF, leupeptin, pepstatin and benzamidine were added at 1 mM concentration. Cell debris and aggregates were spun down for 30 min at 10,000 rpm and 4 °C (Eppendorf, 5804R). The membrane fraction was pelleted for 1 h at 150,000×*g* in a Beckman Ultracentrifuge (Beckman, L8-M). The membrane pellet was resuspended in 20 ml Solubilization Buffer (Resuspension Buffer supplemented with 30 mM DM). Membranes were solubilized for 2 h at 4 °C. The C-terminally His-tagged protein was bound to a Co^{2+} metal affinity column (Clontech). The column was washed with approximately 10 times the column volume of Wash Buffer I (25 mM Tris–HCl pH 7.5, 50 mM NaCl, 20% glycerol, 1 mM $ZnCl_2$, 1 mM C12E8) and 25 ml Wash Buffer II (Wash Buffer I containing 500 mM NaCl). The bound protein was eluted in Elution Buffer (Wash Buffer I supplemented with 300 mM imidazole). The eluted protein was further purified by gel filtration chromatography (S200, Pharmacia). The column was equilibrated in 25 mM Tris–HCl pH 7.5, 50 mM NaCl, 20% glycerol, 1 mM $ZnCl_2$, 3 mM β-mercaptoethanol, 1 mM C12E8. The final protein concentration was determined by UV absorbance and amino acid analysis (MRC facility, Oxford University). The protein was stored as a 50% glycerol stock at − 20 °C.

2.2.2. Preparation of lipid stock solutions

Lipid solubilized in chloroform was dried in an argon stream followed by incubation in a dessicator for 3–4 h to remove residual solvent. The dried lipid was subsequently solubilized in 40 mM C12E8 detergent at a final lipid concentration of 5–10 mg/ml. Lipid solubilization was usually overnight at 37 °C in a fast shaking incubator followed by sonication at room temperature for 30–60 min if necessary. Lipid stock solutions were stored at −20 °C under argon.

2.2.3. ATPase assay

The ATPase activity of ZntA107 was assayed by the pyruvate kinase (PK) and lactate dehydrogenase (LDH) coupled enzyme assay. The indicated amount of phospholipid was incubated with 1.8 μg ZntA107 for 30 min at RT prior to assays. Phospholipids were dissolved in 40 mM C12E8 and added to the reaction mix in a total volume of 10 μl. If no lipid was added, 10 μl of 40 mM C12E8 was added to maintain a constant detergent concentration. The reaction mix contained 100 mM MES pH 6.8, 50 mM NaCl, 5 mM $MgSO_4$, 4 mM C12E8, 0.5 mM $ZnCl_2$, 5 mM DTT, 1 u (PK), 1 u LDH, 1.25 mM phosphoenolpyruvate, 0.25 mM NADH, 5 mM ATP. ATPase assays were performed at 21 °C and the NADH absorbance was measured at 340 nm using a

Agilent 8453 spectrophotometer. For pH titrations, MES in the reaction buffer was replaced by 100 mM Na_2HPO_4/NaH_2PO_4 buffer and, if necessary, the correct pH was adjusted manually using a micro-pH electrode. All ATPase assays were repeated at least once with the exception of the pH titration data.

2.2.4. Fluorescence spectroscopy

If not otherwise stated, fluorescence spectroscopy was performed in buffer containing 10 mM MES pH 6.8, 50 mM NaCl, 5 mM $MgSO_4$, 1 mM $ZnCl_2$, 4 mM C12E8, 5 mM DTT at 21 °C. Phospholipid was added in a total volume of 10 µl 40 mM C12E8. If no lipid was added, 10 µl of 40 mM C12E8 was added. The displacement of DOPG by DSPG from the surface of ZntA was performed by titrating DSPG to a preformed ZntA107–DOPG complex. The ZntA107–DOPG complex was formed by incubating 1.8 µg ZntA107 with 0.1 mg/ml DOPG for 30 min at 21 °C prior to addition of DSPG. Fluorescence emission spectra were recorded from 290 to 450 nm after excitation at 280 nm using a quartz cuvette (HELLMA, 10 mm excitation, 2 mm emission pathlength). Data were collected on a Perkin Elmer LS50B fluorescence spectrometer. Slits for excitation and emission were 10 × 15 mm, with a scan speed of 150 nm/min. For all lipid concentrations, samples lacking ZntA107 were measured as background and subtracted.

2.2.5. Data analysis

All fluorescence spectra were corrected for the attenuation of the signal intensity due to absorption. Absorbance spectra of all samples were measured in a wavelength range from 290 to 450 nm in an Agilent 8453 spectrometer in 1 nm wavelength intervals at 21 °C. The fluorescence intensity attenuation was calculated according to the Lambert–Beer Law. The sample transmission, t, was calculated over half the excitation and emission pathlength of the quartz cuvette according to

$$t = 10^{-(0.5\times X_{\mathrm{Ex}}+0.1\times X_{\mathrm{Em}})},$$

where X_{Ex} and X_{Em} represent sample absorbance at excitation and emission wavelength and 0.5 and 0.1 is half the excitation and emission pathlength in cm, respectively. The measured fluorescence emission spectra were divided by the calculated sample transmission to obtain transmission spectra.

ATPase activity as well as fluorescence data were fit according to

$$y = yo + \frac{(y_{\max} - y_o)\times x^n}{(K+x)^n},$$

where y_0, $y_{\max}$, K and n were fit independently. $y_{\max}$ represents the maximum response, $v_{\max}$ or $I_{\max}$, and y_0 is the enzyme activity or fluorescence intensity at zero lipid concentration. K is the equilibrium dissociation constant and n the cooperativity factor. n was defined as 1 (simple binding mode) if the initial fitting process resulted in $n=1\pm0.2$. The equation was derived from reference [34], associating the measured effect only with a fully relipidated enzyme. Data fitting was performed as a method of comparison and not as an accurate description of the lipid binding process to the ATPase. Dissociation constants were calculated with a DOPG molecular weight of 797.04 g/mol.

pH titration data of the Trp fluorescence of relipidated ZntA107 were fit according to the Henderson–Hasselbach Equation. Both transitions were assumed to have different total intensities, $I_{\max}(1)$ and $I_{\max}(2)$, and can display different cooperativity in lipid interaction (coop1 and coop2):

$$I = \frac{I_{\max}(1)}{1+\left(10^{(coop1*(\mathrm{p}K_{a1}-pH))}\right)} + \frac{I_{\max}(2)}{1+\left(10^{(coop2*(\mathrm{p}K_{a2}-\mathrm{pH}))}\right)}.$$

Data analysis was carried out using the *Origin* data analysis software [35].

3. Results

3.1. Phosphatidyl-glycerol restores the ATPase activity of delipidated ZntA

Detergent solubilized and purified ZntA107 was used to determine the lipid dependence of its in vitro ATPase activity. ATPase assays at saturating ATP concentrations [5] were performed in the presence of increasing concentrations of detergent solubilized *E. coli* total lipid extract. Upon re-addition of at least 1.5 mg/ml *E. coli* lipid, the ATPase activity increased approximately three-fold compared to the activity of the delipidated enzyme (Fig. 1). The data were fit according to a multiple binding mode with a $v_{\max}$ of 116 nmol/(mg min) and a cooperativity factor, n, of 3.4.

All naturally occurring *E. coli* PL species were tested for their ability to restore the ZntA107 ATPase activity. To limit the number of variables and because of its general availability, the fatty acid component was chosen to be oleic acid for all synthetic PL tested. To ensure homogenous solubilization, all lipids were solubilized in 40 mM C12E8 detergent and added to the reaction mix such that a constant detergent concentration was maintained for all samples measured. Fig. 1 shows strong activation of the hydrolytic activity of ZntA107 by DOPG only. DOPE, DOPA and cardiolipin failed to increase the enzymatic activity of ZntA107. DOPG increased the hydrolytic activity of ZntA107 approximately four-fold compared to the initial state. The DOPG titration data were fit to a simple binding mode with a $v_{\max}$ of 167 nmol/(mg min) and an apparent K_D of 3.7 µM.

3.2. Relipidation of ZntA107 increases the Trp fluorescence quantum yield

Fluorescence spectroscopy was used to investigate further the protein–lipid interaction observed. Sequence alignments of heavy metal P-type ATPases and hydrophobicity plots revealed that four out of the five Trp residues of ZntA107 are located at the beginning of transmembrane (TM) helices on the periplasmic side of the membrane (TM helix 1: W142; TM helix 6: W377, W380; TM helix 8: W688). The fifth Trp residue is located close to the intracellular side of TM helix 4 (W219).

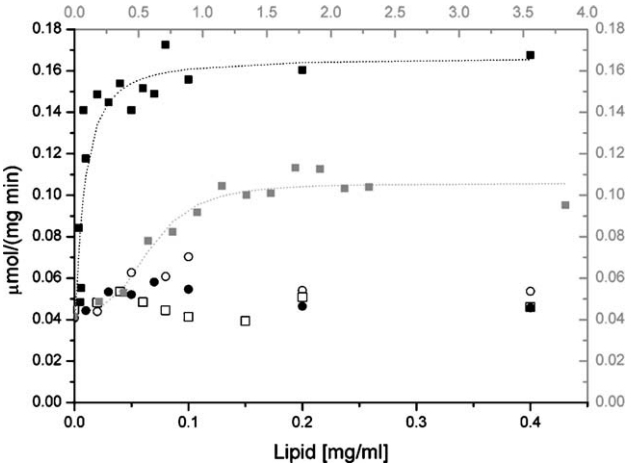

Fig. 1. Identification of endogenous lipid that enhances ZntA's in vitro hydrolytic activity. (A) Detergent purified ZntA107 was incubated with an increasing concentration of detergent solubilized *E. coli* total lipid extract. The ATPase activity was measured 30 min after pre-equilibration of enzyme and lipid in the presence of 5 mM ATP at 21 °C and pH 6.8 (■). Detergent solubilized 1,2-dioleoyl-phospholipids and cardiolipin were tested for their ability to activate the Zn^{2+} ATPase. (■): DOPG, (○): DOPE, (●): cardiolipin and (□): DOPA titration.

Due to their close proximity to the water lipid interface, possible changes in Trp fluorescence upon relipidation of the ATPase were investigated. Either detergent solubilized ZntA107 or ZntA107 supplemented with an increasing concentration of DOPG was excited at 280 nm and fluorescence emission spectra (290–450 nm) were recorded. Fig. 2 A shows that re-lipidation of purified ZntA107 increases the intrinsic Trp fluorescence by approximately 40%. DOPG titration data of the ZntA107 Trp fluorescence were best fit to a multiple binding mode equation with a cooperativity factor, n, of 1.9, a maximum fluorescence intensity, I_{max}, of 246 (a.u.) and a DOPG concentration at 50% total response of 72 μM (Fig. 2B).

Further experiments aimed at characterizing the specificity of the ZntA107–lipid interaction. With respect to the fatty acid selectivity, the fully saturated stearic, palmitic and myristic acid forms of 1,2 diacyl-phosphatidyl-glycerol (DSPG, DPPG, DMPG) were tested for their ability to increase the intrinsic Trp fluorescence of ZntA107. These synthetic PL are commercially available and represent the most abundant fatty acids found in *E. coli* native membranes with the exception of

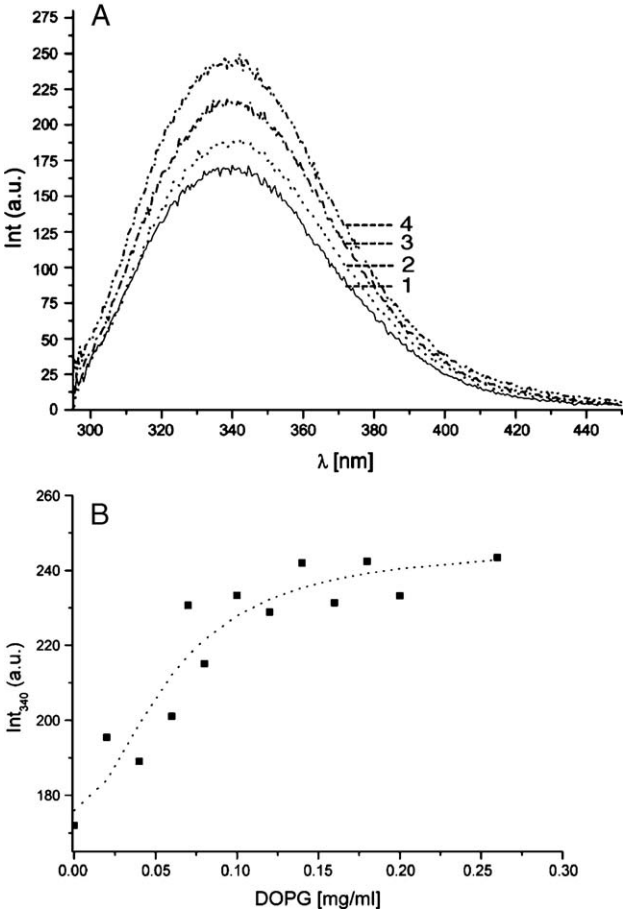

Fig. 2. Increasing Trp fluorescence quantum yield of ZntA107 with increasing concentrations of DOPG. (A) ZntA107 was incubated with an increasing concentration of detergent solubilized DOPG at 21 °C and pH 6.8 and fluorescence emission spectra were recorded after excitation at 280 nm. ZntA107 in the presence of no (1), 0.04 mg/ml, (2), 0.08 mg/ml (3), or 0.26 mg/ml (4) DOPG. (B) 340 nm fluorescence intensity data were collected at the indicated DOPG concentrations.

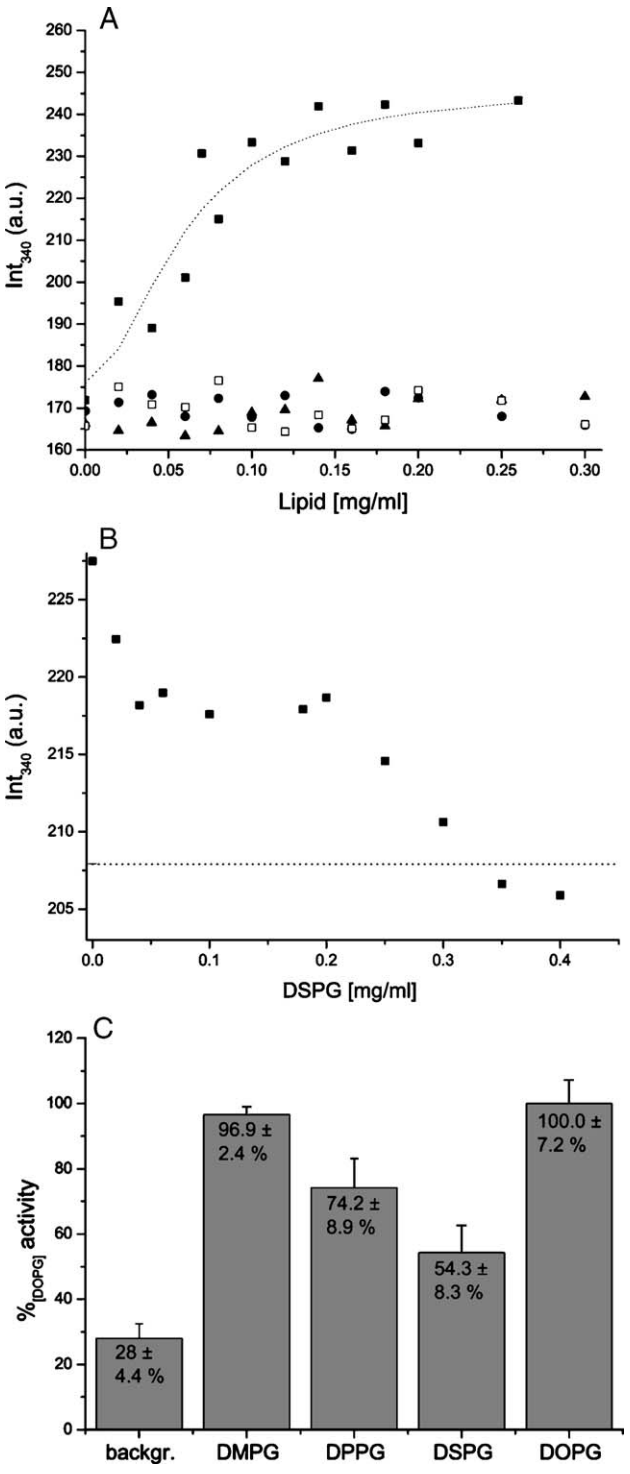

Fig. 3. Ability of PG derivatives to increase ZntA's Trp fluorescence. (A) Fluorescence emission intensity of ZntA107 at 340 nm after relipidation with either 1,2 distearoyl- (□), 1,2 diplamitoyl- (●) or 1,2 dimyristoyl (▲)-phosphatidyl-glycerol at 21 °C and pH 6.8. Data obtained are compared to the DOPG data introduced in Fig. 2 (■). (B) An increasing concentration of DSPG was added to a pre-formed ZntA107–DOPG complex and the Trp fluorescence intensity was monitored. Protein fluorescence in the absence of any additional lipid was measured as baseline (- - -). C: The ATPase activity of ZntA107 in the presence of 0.2 mg/ml DOPG was defined as 100% and the activities in the presence of 0.2 mg/ml DMPG, DPPG and DSPG are expressed relative to it. The background enzymatic activity was measured in the absence of any additional PL. Data represent the means of three independent measurements at 21 °C and pH 6.8.

Table 1
Enzymatic and fluorescence characteristics of ZntA107 as obtained from ATPase assays and fluorescence spectroscopy

Lipid	v_{max} [nmol/(mg min)]	K [μM]	Cooperativity (n)
Hydrolytic Activity			
E. coli total lipid extract	116±10		3.4
DOPG	167±8.5	3.7±1.7	1
DMPG	161±4		
DPPG	124±14.9		
DSPG	90.7±13.3		
Fluorescence characteristics			
DOPG		72±48	1.9
DMPG		no effect	
DPPG		no effect	
DSPG		no effect	

The ATPase activity of ZntA107 in the presence of DMPG, DPPG and DSPG was determined in three independent ATPase assays at 0.2 mg/ml lipid concentration. The activity in the presence of DOPG was determined after fitting titration data. The DOPG fluorescence intensity maximum, I_{max}, was determined to be 246 (a.u.).

palmitoleic acid (cis Δ^9 16:1, not tested) [36,37]. Fig. 3A compares the DOPG induced shift in Trp fluorescence of ZntA107 with the fluorescence intensity data obtained when DSPG, DPPG or DMPG was used for relipidation. None of these "DOPG-derivatives" reproduced the fluorescent signal observed for DOPG.

3.3. Phosphatidyl-glycerols bind to equivalent sites on ZntA107

In order to discriminate whether the DOPG molecule occupies a unique binding site on the membrane spanning surface of ZntA107, the ability of DSPG, a "DOPG-derivative" failing to enhance the Trp fluorescence, was tested to replace DOPG at its binding site on the ATPase. DSPG was added in increasing concentrations to a pre-formed ZntA107–DOPG complex. Fig. 3B shows that the high Trp fluorescent ZntA–DOPG complex was readily converted into a low Trp fluorescent ZntA–DSPG complex. This result indicates that DOPG and DSPG utilize the same binding site on ZntA.

The previous results indicate that the DOPG molecule, among all other phosphatidyl-glycerols tested, has a unique effect on the intrinsic Trp fluorescence of ZntA107. Next, the same phosphatidyl-glycerols were examined for their ability to increase the hydrolytic activity of ZntA107. Fig. 3C shows the means of 3 independent ATPase activity measurements in the presence of 0.2 mg/ml DMPG, DPPG, DSPG or DOPG. The

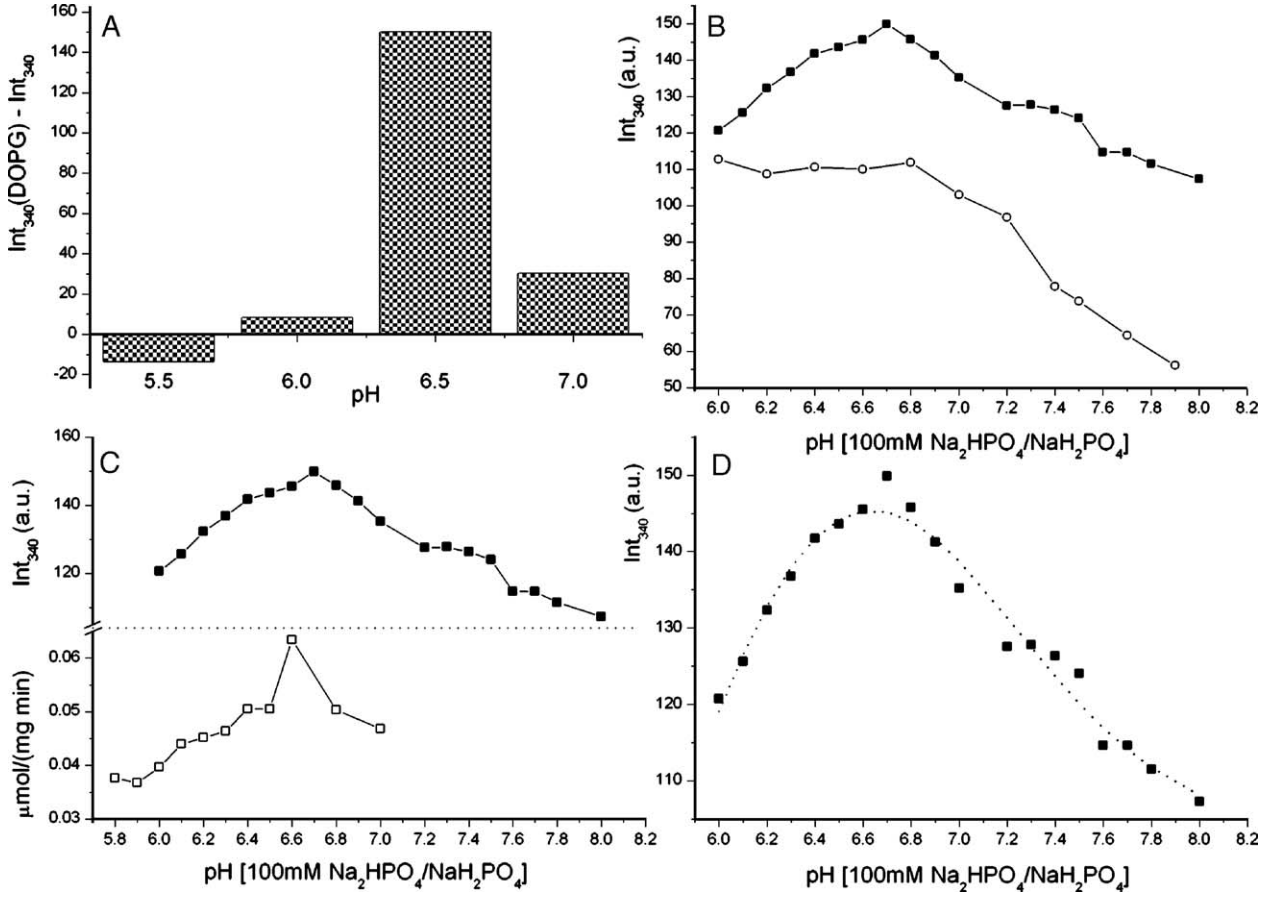

Fig. 4. pH dependence of protein–lipid interaction and ATPase activity. (A) Broad pH screen, the differences in Trp fluorescence intensity of the ZntA107–DOPG complex and purified ZntA107 are compared in a pH range from 5.5 to 7.0. (B) Refinement of the DOPG pH titration. Samples were measured in 100 mM Na_2HPO_4/NaH_2PO_4 buffer at 0.1 pH unit increments, (■): ZntA107 + DOPG, (○): ZntA107 only. (C) Comparison of the fluorescence data and the ATPase activity of ZntA107. Protein + DOPG data shown in B, (■), and pH-dependent ATPase activity in the presence of 0.2 mg/ml DOPG and 100 mM Na_2HPO_4/NaH_2PO_4, (□). (D) Henderson–Hasselbach analysis of ZntA107 + DOPG titration data shown in B, pK_{a1}=5.7, pK_{a2}=7.1. All experiments were performed at 21 °C.

activating efficiency of the PL tested follows the order DOPG > DMPG > DPPG > DSPG assuming equal solubilization of all PL tested in the C12E8 detergent. The enzymatic and fluorescence properties of ZntA107 are summarized in Table 1.

3.4. ZntA107–lipid interaction reveals a pH optimum close to neutrality

The pH dependence of lipid binding to the ZntA107 ATPase was determined by fluorescence spectroscopy. A pH region from 5.5 to 8.5 was investigated, a higher pH was not possible due to protein instability. Fig. 4A shows the difference in intrinsic Trp fluorescence of ZntA107 between the lipidated and detergent purified state in the pH range from 5.5 to 7.0. Within this broad pH range a maximum fluorescence increase is observed at pH 6.5. The region between pH 6.0 and 8.0 was refined in 0.1 pH unit increments. The data shown in Fig. 4B suggest a pH optimum for ZntA107–DOPG interaction of approximately pH 6.7. The pH dependence of the fluorescence data was compared with the hydrolytic activity of ZntA107 within the same pH region. ATPase assays performed in a pH range from 5.8 to 7.0 show a peak activity at pH 6.6 (Fig. 4C). Analysis of the refined pH-dependent Trp fluorescence data according to the Henderson–Hasselbach Equation fit pK_a values for the first and second transition of 5.7 and 7.1, with an $I_{max}(1)$ of 186.33, $I_{max}(2)$ of −84.41 and cooperativity factors close to unity (0.96 and 1.11) (Fig. 4D).

4. Discussion

In this study, the lipid dependence of the *E. coli* heavy metal transport ATPase ZntA was characterized. Detergent solubilization of integral membrane proteins often leads to the loss of essential protein–lipid interactions, which can be deleterious for the long-term stabilization of the solubilized protein. If, however, the lipids essential for enzymatic function and/or protein stability are known, the possibility exists to supplement in vitro systems with these essential components to achieve long-term stabilization of the detergent solubilized protein.

Among the most abundant *E. coli* phospholipids, PE, PG and CL, phosphatidyl-glycerols were identified as the only PL component able to increase the hydrolytic activity of detergent purified ZntA. Supplementation of the ZntA ATPase with DOPG increased the in vitro ATPase activity approximately four-fold. All other major *E. coli* PL failed to reproduce the effect observed for DOPG.

Many integral membrane proteins require particular lipids for proper function. For example the KcsA K^+ channel requires negatively charged PL for proper ion conduction [30] and P-glycoprotein activity requires cholesterol [38]. The presence of the specific lipid either exerts its influence through a bulk effect, altering the properties of the surrounding lipid membrane, or by binding directly to the protein, supporting and/or restraining transmembrane helical movements.

The ATPase activity of the ZntA protein exhibits a strong dependence on phosphatidyl-glycerol lipids. Even though the PL "head group" appears to be most important for the observed effect, the chemical nature of the fatty acid acyl chains can also modulate the enzyme's activity in vitro. All of the "PG-derivatives" tested were able to activate the hydrolytic activity of the ATPase above baseline but did so to differing degrees.

Comparing the effects of different PL exerted on the ATPase activity as well as the fluorescence properties of ZntA are based on the assumption that all PL tested are homogenously solubilized in the C12E8 detergent. Despite our best efforts to ensure complete solubilization of all lipid stock solutions we cannot rule out the possibility of aggregation of some of the lipids tested, in particular in the presence of divalent cations such as Mg^{2+} and Zn^{2+} (see reference [39] for a discussion of this effect on lipid bilayers). This might lead to aggregation of, e.g., DOPA and cardiolipin, thereby lower their effective concentration.

The observed changes in Trp fluorescence intensity upon re-lipidation of ZntA exhibit a striking dependence on the fatty acid composition of the phosphatidyl-glycerol lipid. Diacyl-phosphatidyl-glycerols carrying fully saturated fatty acids such as myristic, palmitic and stearic acid failed to reproduce the effect observed for DOPG (Fig. 3A). This suggests a unique effect of DOPG, which is altered if oleic acid is replaced by fully saturated fatty acids. Interestingly, the non-natural phospholipid DOPC (DOPG with a choline rather than a glycerol head group) stimulates the ATPase activity of ZntA as well as DOPG does and also increases the Trp fluorescence of ZntA upon relipidation (data not shown). This suggests that the mono-unsaturated fatty acids of DOPG and DOPC are required to increase the Trp fluorescence quantum yield of ZntA.

However, it is unlikely that DOPG occupies a specific binding site on the membrane spanning surface of ZntA, as the DOPG molecule of the ZntA-DOPG complex is readily replaced by DSPG. It is conceivable that DOPG binding induces conformational readjustments altering the microenvironment of Trp residues responsible for the observed effect. This is in contrast to the lack of effect seen for all other "DOPG derivatives" tested.

An alternative, less likely, explanation for the lack of effect observed for DSPG, DPPG and DMPG is the possibility that these lipids differ in their solubility in the C12E8 detergent. Insufficient lipid solubilization could lead to a separate gel phase of the lipid next to a detergent solubilized state. The presence of such a two-state system under assay conditions could influence the results since protein–lipid and lipid–lipid interaction phenomena might overlap.

The mechanism by which the PG lipids enhance ZntA's ATPase activity is unknown. However, considering the spatial separation of the transmembrane spanning region and the ATP binding site of P-type ATPases [40,41], it is likely that the increasing in vitro hydrolytic activity of ZntA following relipidation is an indirect consequence of stabilizing the membrane spanning region by the added lipid. The pH titration of the hydrolytic activity and Trp fluorescence of ZntA in the presence of DOPG lipids identified a pH optimum for the ZntA–DOPG interaction at pH 6.7 in accordance with earlier findings [5]. Most likely, this pH dependence arises from pH-

dependent lipid binding and a corresponding increase or decrease of ZntA's ATPase activity. However, it is also conceivable that pH variations affect the complex formation of Mg^{2+} or Zn^{2+} with ATP or alter the protonation state of the γ-phosphate of ATP, factors potentially capable of affecting the overall ATPase activity.

The fluorescence properties of ZntA in its purified and DOPG supplemented state indicate the perturbation of the local environment of a Trp residue close to a TM helix terminus. pK_a values for DOPG binding of 5.7 and 7.1 indicate the contribution of Glu or Asp and His residues to the lipid binding process [42]. Accordingly, these functional groups have been shown to quench indol fluorescence [43], supporting the idea of an intramolecular Trp fluorescence quenching process in the purified state of the ZntA ATPase.

The structural understanding of the function of P-type ATPases has dramatically increased over the last years. So far, most of the structural studies have been performed on the rabbit SERCA Ca^{2+}-ATPase purified from source. The functional over-expression and subsequent purification of P-type ATPases remains a major obstacle in the structural investigation of this important class of transporters. This study describes the biochemical characterization of an over-expressed P-type bacterial heavy metal ATPase. The enzymatic activity and stability of the enzyme in C12E8 detergent is strongly pH and lipid dependent. On the basis of the data presented, an optimized purification protocol for ZntA can be derived to purify and maintain crystallographic amounts of catalytically active enzyme. A detailed characterization of the biochemical buffer conditions required to yield high-quality protein material is a prerequisite for a successful biophysical analysis.

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