

XpsE oligomerization triggered by ATP binding, not hydrolysis, leads to its association with XpsL

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GspE belongs to a secretion NTPase superfamily, members of which are involved in type II/IV secretion, type IV pilus biogenesis and DNA transport in conjugation or natural transformation. Predicted to be a cytoplasmic protein, GspE has nonetheless been shown to be membrane-associated by interacting with the N-terminal cytoplasmic domain of GspL. By taking biochemical and genetic approaches, we observed that ATP binding triggers oligomerization of *Xanthomonas campestris* XpsE (a GspE homolog) as well as its association with the N-terminal domain of XpsL (a GspL homolog). While isolated XpsE exhibits very low intrinsic ATPase activity, association with XpsL appears to stimulate ATP hydrolysis. Mutation at a conserved lysine residue in the XpsE Walker A motif causes reduction in its ATPase activity without significantly influencing its interaction with XpsL, congruent with the notion that XpsE–XpsL association precedes ATP hydrolysis. For the first time, functional significance of ATP binding to GspE in type II secretion system is clearly demonstrated. The implications may also be applicable to type IV pilus biogenesis.

The EMBO Journal (2006) 25, 1426–1435. doi:10.1038/sj.emboj.7601036; Published online 9 March 2006

Subject Categories: membranes & transport

Keywords: ATP binding; GspE–GspL interaction; GspE oligomerization; type II secretion system; *Xanthomonas campestris*

Introduction

Protein effectors, toxins or hydrolytic enzymes of various kinds are secreted by pathogenic bacteria as ammunition in attacking host cell be it of animal or plant origin. In Gram-negative bacteria, proteins have to traverse two layers of membrane before reaching extracellular environment. As resolutions, a variety of multiprotein machineries have been evolved. In type II secretion pathway, exoproteins are

secreted in two steps. While the Sec or Tat apparatus is utilized in the first step for translocation across cytoplasmic membrane, a specific apparatus T2SS constituted of 12–15 Gsp protein components is required for transporting proteins from periplasm to the milieu (Sandkvist, 2001; Filloux, 2004). Despite their involvement in protein transport across the outer membrane, these proteins appear to form a trans-envelope complex spanning both membranes.

The secretion pore located in outer membrane is constituted of 12–14 subunits of GspD (with or without assistance of GspS for stable GspD protein assembly) (Nouwen *et al*, 1999, 2000). Spanning between outer membrane and cytoplasmic membrane is a pilus-like structure composed of one major, GspG protein, and possibly four minor (GspH, -I, -J, -K) pseudopilins (Lory, 1992; Blevess *et al*, 1998; Nunn, 1999; Sauvonnnet *et al*, 2000; Hu *et al*, 2002). Of the four integral cytoplasmic membrane proteins, three (GspL, -M, -C) have been demonstrated to form a hierarchically structured ternary complex (Tsai *et al*, 2002). The ternary complex appears to connect the secretion pore and the GspE protein component that is located in cytoplasm. The GspC protein was shown to interact with GspD protein, whereas the GspL protein interacts, through its N-terminal cytoplasmic domain, with GspE protein (Sandkvist *et al*, 1995; Py *et al*, 1999; Lee *et al*, 2000; Possot *et al*, 2000). In addition, interaction between the major pseudopilin GspG with the GspD protein and the GspC protein was recently demonstrated with cross-linking results (Lee *et al*, 2005). The same study also showed that the GspC protein is required for crosslinking between GspG and GspD.

The cytoplasmic component EpsE protein of *Vibrio cholerae* T2SS was demonstrated to associate with the cytoplasmic membrane through its interaction with the N-terminal domain of a bitopic cytoplasmic membrane protein component EpsL (Sandkvist *et al*, 1995). Interaction of *Erwinia chrysanthemi* OutE with the N-terminal cytoplasmic domain of OutL, but not with its C-terminal periplasmic domain, was also suggested from yeast two-hybrid studies (Py *et al*, 1999). Although it is well documented that the Walker A motif of GspE is indispensable for its normal functioning in T2SS (Turner *et al*, 1993; Possot and Pugsley, 1994; Sandkvist *et al*, 1995; Py *et al*, 1999), the mechanistic role of Walker A motif has remained obscure. Yeast two-hybrid studies revealed that Walker A mutations in OutE did not influence its interaction with OutL (Py *et al*, 1999). Nor was OutL conformational change induced by OutE affected, implied from limited proteolysis studies. Given that these scattered observations provide little functional relevance regarding the highly conserved Walker A motif, we wondered what roles ATP binding and ATP hydrolysis may play in T2SS.

Size exclusion chromatography of EpsE disclosed it primarily exists as monomers. Yet, albeit being low in abundance, oligomeric EpsE was detected as being active in ATP hydrolysis (Camberg and Sandkvist, 2005). Oligomerization of *E. chrysanthemi* OutE was also implicated from

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Received: 20 September 2005; accepted: 14 February 2006; published online: 9 March 2006

yeast two-hybrid studies (Py *et al*, 1999). These observations indicate that the functional unit of GspE may be oligomeric, but what controls the oligomerization state of GspE remains to be solved. Crystal structure of a truncated form of the *V. cholerae* EpsE (EpsE Δ 90) protein was determined (Robien *et al*, 2003). However, no major differences were observed between AMPPNP-bound and unliganded forms.

Being the only member in T2SS that possesses a nucleotide-binding motif, the Walker A motif, GspE was proposed to be the molecular motor in T2SS. Not until recently, weak ATPase activity was detected in oligomeric EpsE of *V. cholerae* (Camberg and Sandkvist, 2005). However, it is still unclear how GspE couples energy from ATP hydrolysis to exoprotein secretion through the secretion pore, whose opening is presumably under tight control. Recently, the ATPase involved in Tfp assembly in enteropathogenic *Escherichia coli* (EPEC) BfpD (a likely GspE/PilB functional homolog) was also shown to display ATP hydrolysis activity (Crowther *et al*, 2005). Its ATPase activity was markedly stimulated by its interactive partners BfpC (a likely GspL functional homolog) and BfpE (a likely GspF functional homolog), suggesting possible involvement of protein-protein interaction in activating other BfpD-like ATPases.

To determine the functional role of ATP binding and hydrolysis in T2SS, we chose to work on XpsE, the GspE protein of a plant pathogen *Xanthomonas campestris* pv. *campestris*. Monomeric XpsE was shown to form oligomers only when preincubated with Mg-AMPPNP, but not with Mg-ADP. Pull-down assay revealed that association of monomeric, but not oligomeric, XpsE with the N-terminal domain of XpsL (XpsL_N) requires Mg-AMPPNP. A double-mutant XpsE mutated at K331 and R504, whose equivalents were shown in the crystal structure of EpsE Δ 90 to be involved in ATP binding (Robien *et al*, 2003), was constructed. With significantly weakened ATP binding affinity, it no longer oligomerized in the presence of Mg-AMPPNP, nor did it associate with XpsL_N. Its membrane association was barely detectable. In contrast, while K331M single mutation in

Walker A motif reduced the ATPase activity by 17-fold and caused XpsE(K331M) non-unctional, it remained membrane-bound as well as the wild-type XpsE.

Results

XpsE oligomerization caused by AMPPNP, but not by ADP

When analyzed by size exclusion chromatography, the affinity column-purified *Strep*-tagged XpsE appeared to exist in several forms covering a wide range of molecular sizes, besides a major monomeric form (data not shown), implying that oligomerization is an intrinsic property of XpsE protein. A highly conserved nucleotide-binding motif (Walker A) present in all members of the GspE protein family had been shown to be essential for their normal functioning in type II secretion (Turner *et al*, 1993; Possot and Pugsley, 1994; Sandkvist *et al*, 1995; Py *et al*, 1999). ATPase activity, albeit weak, was recently demonstrated for the recombinant EpsE of *V. cholerae* (Camberg and Sandkvist, 2005). However, specific role of ATP hydrolysis in the secretion process remains unknown. Moreover, significance of ATP binding to GspE in its protein-protein interaction has never been examined. In order to distinguish the effect caused by ATP binding from that by ATP hydrolysis, we analyzed the effect of ATP binding on XpsE oligomerization by making use of the non-hydrolyzable ATP analog AMPPNP.

The monomeric XpsE purified from the first size exclusion column was analyzed for oligomerization upon a second column, with or without preincubation with AMPPNP and MgCl₂, and followed by immunoblotting. For comparison, a sample preincubated with Mg-ADP was analyzed in parallel. While ADP has no effect on the elution profile of XpsE, preincubation with Mg-AMPPNP clearly caused XpsE to oligomerize (Figure 1). The monomeric XpsE (with predicted molecular weight of 62 kDa) fractionated at fractions 24–25, and the major oligomeric form appearing at fractions 15–17 was roughly estimated to be hexameric. This result implied

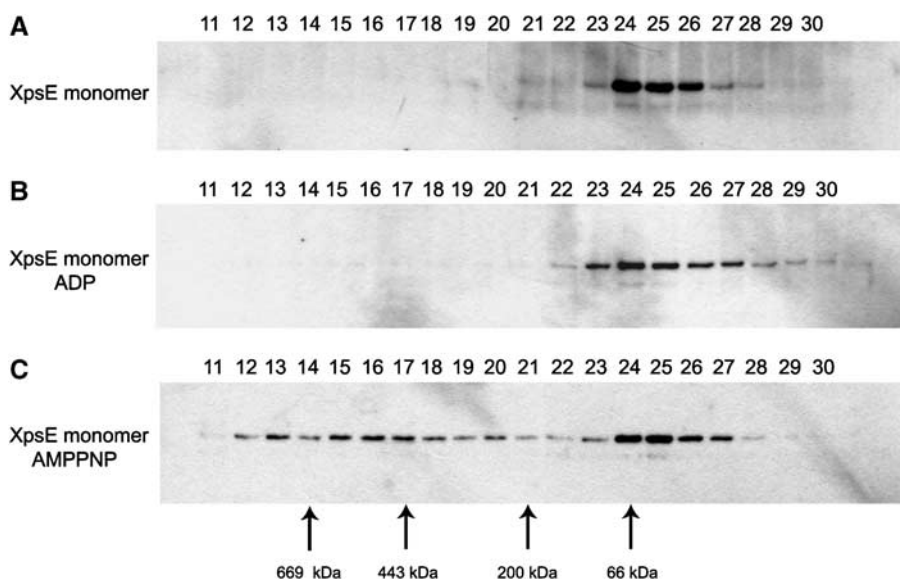


Figure 1 Size distribution of monomeric XpsE-*Strep*, preincubated at 4°C for 30 min with (A) no addition, (B) 1 mM ADP and 10 mM MgCl₂ or (C) 1 mM AMPPNP and 10 mM MgCl₂, analyzed upon size exclusion chromatography, followed by immunoblotting with antiserum against XpsE visualized with chemiluminescence.

that ATP binding alone is adequate to drive oligomerization of XpsE.

To characterize the AMPPNP-dependent XpsE oligomerization in greater detail, we further examined the effect of Mg-AMPPNP on molecular size of XpsE by performing sedimentation velocity experiments. Congruent with the results from size exclusion chromatography, the presence of Mg-AMPPNP indeed caused significant increase in the proportion of oligomers (Figure 2A and B; Table I). However, to our surprise, the sedimentation coefficient of the major oligomeric form suggested a trimeric XpsE (7.2 S) (Figure 2A), about half the size of what was estimated from size exclusion chromatography (Figure 1C). The formation of distinct XpsE oligomers may be caused by differences in AMPPNP concentration used in the two experiments. Due to its high molar extinction coefficient at 280 nm, AMPPNP was included in sedimentation analysis at a lower concentra-

tion (0.2 mM) than in size exclusion chromatography (1 mM). Perhaps, this lowered AMPPNP concentration is not sufficient for driving hexamer formation. A minor portion (11%) of the wild-type XpsE, without preincubating with AMPPNP, was also detected as trimers (7.8 S) (Figure 2B; Table I), which might have arisen from ATP binding to XpsE before or during its purification. Such a notion will be further scrutinized with mutant analysis in forthcoming section.

Monomeric XpsE associates with the XpsL_N in the presence of AMPPNP, not ADP

In the secretion-competent strain, GspE is distributed in two subcellular fractions, one in the cytoplasm and the other being associated with the cytoplasmic membrane (Sandkvist *et al*, 1995). It has been well documented that membrane association of GspE is mediated through its interaction with the N-terminal domain of a bitopic cytoplasmic membrane

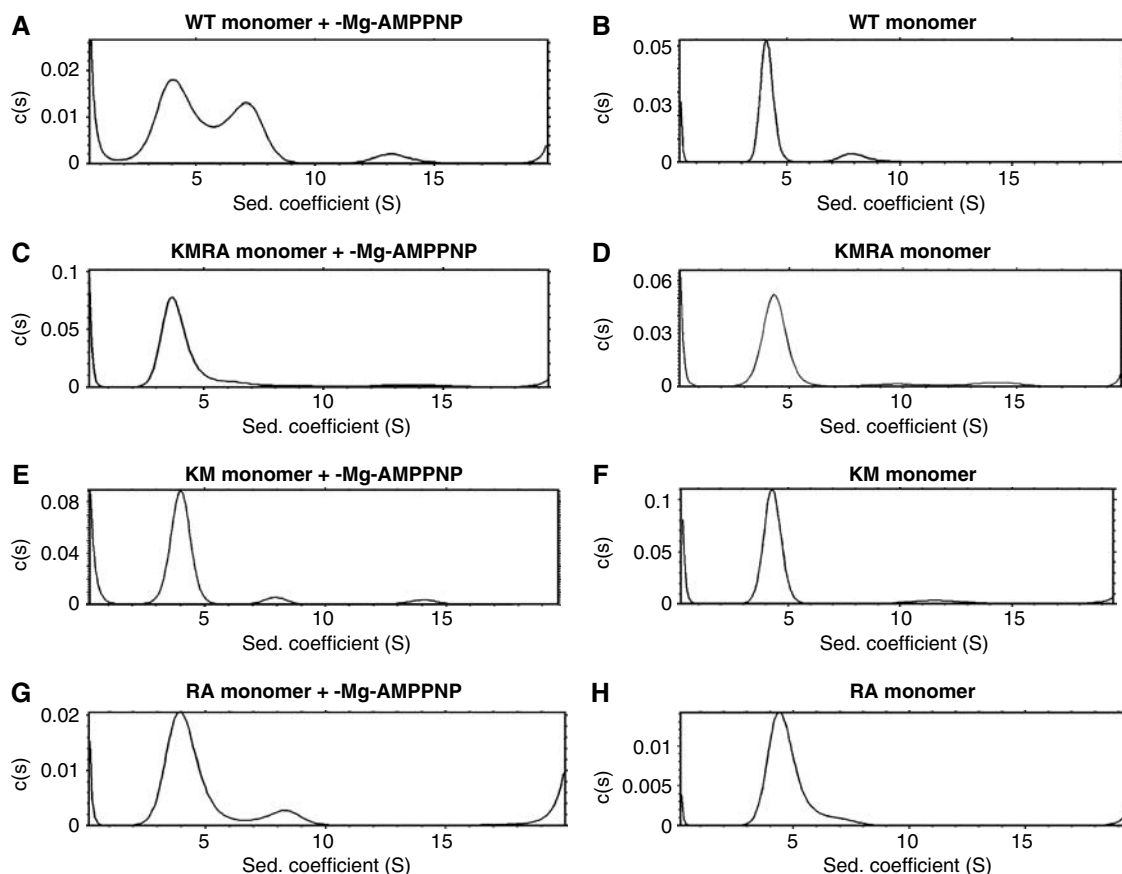


Figure 2 Sedimentation coefficient distributions of wild-type XpsE and its variants, with (A, C, E, G) or without (B, D, F, H) preincubation with Mg-AMPPNP. Monomeric XpsE isolated from size exclusion chromatography was analyzed via sedimentation velocity experiments. WT: wild-type XpsE (A, B); KMRA: XpsE(K331M, R504A) (C, D); KM: XpsE (K331M) (E, F); RA: XpsE(R504A) (G, H).

Table I Sedimentation coefficient distribution of XpsE and its variants

	+ AMPPNP	-AMPPNP
Wild-type XpsE	4.1 S (51%), 7.2 S (41%), 13 S (8%)	4.1 S (89%) ^a , 7.8 S (11%)
XpsE(K331M, R504A)	3.5 S (100%)	4.5 S (100%)
XpsE(K331M)	4 S (92%), 8 S (8%)	4.3 S (100%)
XpsE(R504A)	4 S (87%), 8.2 S (13%)	4.3 S (100%)

^aNumbers in parenthesis represent percentage in total.

protein GspL (Sandkvist *et al*, 1995). To analyze whether XpsE oligomerization has an effect on its interaction with the XpsL_N, we constructed a clone expressing MBP-XpsL_N fusion protein. The pull-down assay was then performed by immobilizing MBP-XpsL_N to amylose resin.

By incubating the monomeric XpsE, isolated from size exclusion chromatography, with the amylose resin-bound MBP-XpsL_N, we observed that almost all XpsE was present in unbound fraction (Figure 3). When AMPPNP (with MgCl₂) was included, XpsE was detected in bound fraction. In contrast, XpsE was hardly detectable in bound fraction when ADP was included, implicating XpsE-XpsL association attainable by ATP binding may be disrupted upon ATP hydrolysis.

These results also suggested that XpsE, probably by binding with ATP, becomes competent in associating with the XpsL_N. Given that Mg-AMPPNP also promoted oligomerization of XpsE, we speculated that XpsE oligomerization triggered by ATP binding may have made its association with XpsL_N possible. By collecting hexameric XpsE (fraction 16) and monomeric XpsE (fraction 24) separately from the second size exclusion column (as shown in Figure 1C), we analyzed their respective association with MBP-XpsL_N without preincubating with Mg-AMPPNP. As expected, only hexameric, not monomeric, XpsE could be detected in bound fraction (Figure 4), suggesting XpsE oligomerization leads to XpsE-XpsL association.

***XpsE(K331M, R504)* double-mutant severely weakens ATP binding, and abolishes the oligomerization and XpsL-binding capability of XpsE**

The results shown in previous sections illustrate a potential correlation between XpsE oligomerization and its association with XpsL_N in their enhancement by Mg-AMPPNP, but not by Mg-ADP. Thus, the role of ATP binding to XpsE in triggering these two observations is suggested. To confirm such an implication, we looked for a mutant XpsE that is defective in ATP binding. By examining the crystal structure of EpsEΔ90 of *V. cholerae* (Robien *et al*, 2003), we

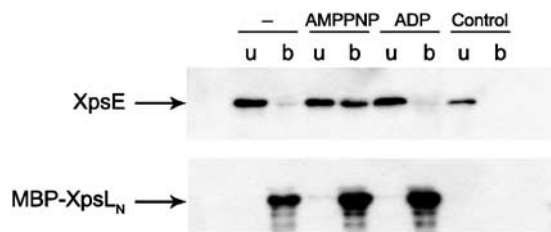


Figure 3 MBP pull-down assay of association of monomeric XpsE with MBP-XpsL_N. Monomeric XpsE obtained from size exclusion column was divided into four equal fractions. Preincubation was conducted at 4°C for 30 min without any addition (-), with the addition of 1 mM AMPPNP and 10 mM MgCl₂ (labeled 'AMPPNP'), or 1 mM ADP and 10 mM MgCl₂ (labeled 'ADP'), before mixing at 4°C for 1.5 h with 0.6 μM MBP-XpsL_N immobilized on amylose resin and washed thoroughly with Buffer A. In control experiment (labeled 'Control'), XpsE preincubated with Mg-AMPPNP was mixed with blank amylose resin. The supernatant collected from centrifugation at 2800 g for 15 min was saved as unbound (u) fraction. The resin-bound protein (b) washed three times with Buffer A was analyzed, along with unbound protein, in SDS-polyacrylamide gel followed by immunoblotting with antiserum against XpsE or MBP.

discovered, in addition to the highly conserved lysine residue in Walker A motif (K331 in XpsE) that is required for ATP hydrolysis (Sakai *et al*, 2001), an arginine residue R441 (equivalent to R504 in XpsE) also interacts extensively with the bound AMPPNP. Like K331, R504 is highly conserved in all GspE members as well. Using [α -³²P]ATP in an ATP binding assay, we found the dissociation constant ($K_{d_{ATP}}$) of the single mutant XpsE(K331M) to be $2.35 \pm 0.36 \mu\text{M}$, weakened only by four-fold compared to the wild-type $0.64 \pm 0.06 \mu\text{M}$ (Table II). We therefore further constructed the XpsE(K331M, R504A) double mutant and measured its $K_{d_{ATP}}$. Indeed, the double-mutant XpsE exhibited ATP binding ability significantly weaker than the wild-type protein ($K_{d_{ATP}}$ of $13.62 \pm 2.89 \mu\text{M}$, Table II). To examine if the R504A mutation is epistatic to the K331M mutation in ATP binding, we determined dissociation constant of the single mutant XpsE(R504A) and found its $K_{d_{ATP}}$ ($3.86 \pm 0.70 \mu\text{M}$, Table II) within the same order of magnitude as that of the single mutant XpsE(K331M). Such results indicated that the significantly weaker ATP binding affinity exhibited by the double-mutant XpsE(K331M, R504A) was due to a synergistic effect from both mutations.

The double-mutant XpsE(K331M, R504A) protein isolated from size exclusion chromatography as described in previous section was subsequently analyzed for oligomerization by passing through a second size exclusion column, with or without preincubation with AMPPNP and MgCl₂. In both cases, the double-mutant protein was eluted as monomers (Figure 5A). Apparently, preincubation with Mg-AMPPNP no

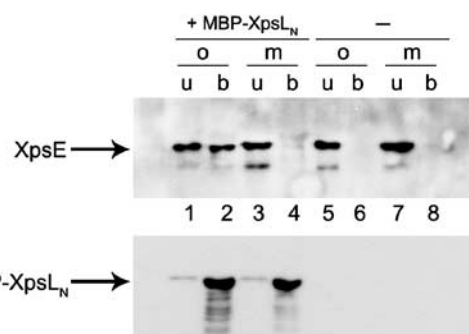


Figure 4 MBP pull-down assay of oligomeric XpsE opposed to monomeric XpsE for their association with MBP-XpsL_N in the absence of Mg-AMPPNP. Oligomeric (designated as 'o') and monomeric (designated as 'm') XpsE were collected as fractions 16 and 24, respectively, from the size exclusion column as shown in Figure 1C. Each was mixed with 0.6 μM MBP-XpsL_N immobilized on amylose resin (lanes 1–4) or blank amylose resin (lanes 5–8). MBP pull-down assay and immunoblotting were performed as described in Figure 3 legend.

Table II $K_{d_{ATP}}$ and ATPase activity of XpsE and its variants

	$K_{d_{ATP}}$ μM	ATPase activity units (pmol Pi released/min/mg protein)
Wild-type XpsE	0.64 ± 0.06	318.1 ± 30.3
XpsE(K331M)	2.35 ± 0.36	18.5 ± 1.5
XpsE(R504A)	3.86 ± 0.70	168.3 ± 24.6
XpsE(K331M, R504A)	13.62 ± 2.89	18.9 ± 2.2

longer stimulated oligomerization of the double-mutant XpsE. Such a finding adheres to the results from sedimentation velocity experiments (Figure 2C; Table I) and demonstrates clearly that ATP binding is required for XpsE oligomerization.

To analyze interaction of the double-mutant XpsE protein with XpsL, we performed MBP-XpsL_N pull-down assay as described above. While nearly half of the wild-type XpsE appeared in bound fraction by preincubating with Mg-AMPPNP, barely any double-mutant XpsE(K331M, R504A)

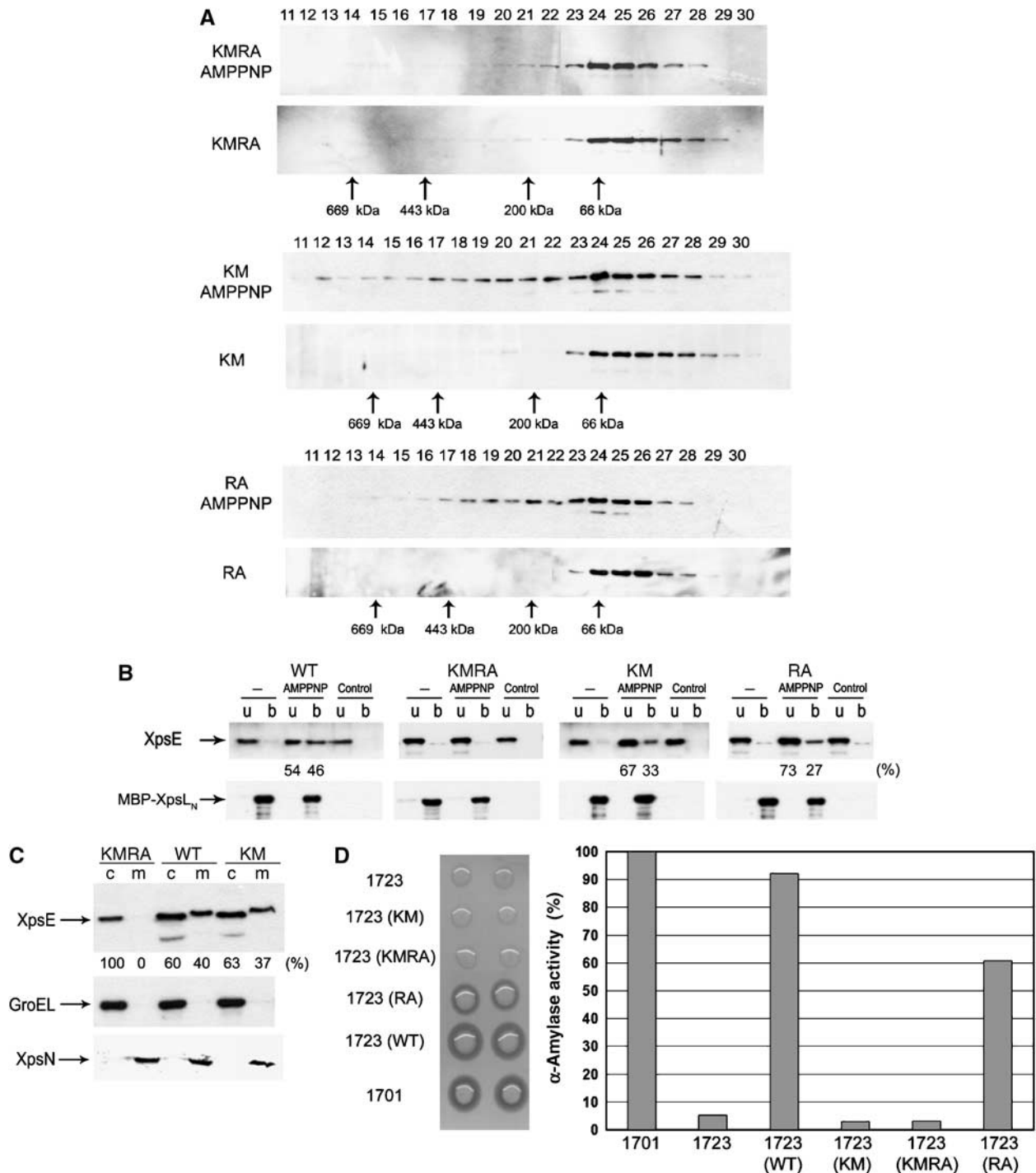


Figure 5 Analyses of the mutants XpsE(K331M, R504A), XpsE(K331M) and XpsE(R504A). KMRA represents the mutant XpsE(K331M, R504A); KM, the mutant XpsE(K331M); RA, the mutant XpsE(R504A); WT, the wild-type XpsE. (A) Effect of Mg-AMPPNP on mutant XpsE oligomerization analyzed upon size exclusion chromatography. (B) Effect of Mg-AMPPNP on XpsE association with XpsL_N analyzed by MBP pull-down assay. 'u' designates unbound fraction; 'b' designates bound fraction. Controls were as described in Figure 3 legend. (C) Subcellular distribution of mutant XpsE. 'c' designates cytoplasmic fraction; 'm', membrane fraction. The numbers below XpsE immunoblot depict the signal intensity of each fraction as percentage of the sum of signal intensities in both fractions. Immunoblottings of GroEL and XpsN serve as markers for cytoplasmic and membrane fraction, respectively. (D) α-Amylase secretion assayed on starch plate (left panel) and quantified by assaying α-amylase activity in extracellular fraction (right panel).

was detected as bound form, with or without Mg-AMPPNP (Figure 5B). We further analyzed subcellular distribution of the double-mutant XpsE(K331M, R504A) in the genetic background of XC1723 in parallel with the wild-type XpsE. While the double-mutant XpsE was hardly detectable in the Triton X-100-extracted membrane fraction, 40% of the wild-type XpsE appeared membrane-bound (Figure 5C). Distribution of the cytoplasmic protein GroEL and the cytoplasmic membrane protein XpsN were as expected in all cases.

Both starch plate assay and semi-quantitative assay for extracellular α -amylase indicated that the double-mutant XpsE(K331M, R504A) was deficient in restoring type II secretion in the *xpsE* mutant strain XC1723 (Figure 5D). So was the single mutant XpsE(K331M). In contrast, the single mutant XpsE(R504A) remained partially functional.

Examinations of the single mutant, either XpsE(K331M) or XpsE(R504A), suggested that each oligomerized upon preincubation with Mg-AMPPNP, despite to a significant lesser extent (Figure 2E and G; Table I). Deviation in oligomer sizes from those of the wild-type XpsE was also noticed upon size exclusion chromatography. While the wild-type XpsE formed hexamers appearing in fractions 15–17 (Figure 1C), oligomers of both single mutants were widely distributed in fractions 17–23 (Figure 5A). Given the slightly lowered ATP binding affinity of the single mutants, it appeared that the stability of XpsE hexamer was sensitive to its association with nucleotide, and that XpsE oligomerization was indeed initiated by ATP binding.

Regardless of the difference in their oligomeric distribution in size exclusion column, the single mutant XpsE(K331M) and XpsE(R504A) became XpsL_N-bound upon preincubation with Mg-AMPPNP at similar levels as that of the wild-type XpsE (Figure 5B). Furthermore, subcellular distribution of the single mutant XpsE(K331M) is strikingly similar to that of the wild-type XpsE (Figure 5C). Perhaps the trimeric form binds XpsL as well as the hexamer. Alternatively, albeit being the rare species, hexameric form of the single mutants may be stabilized by its association with XpsL. The slightly slower migration of the wild-type XpsE and the XpsE(K331M) mutant proteins in membrane fraction ('m' in Figure 5C) than those in cytoplasmic fraction ('c' in Figure 5C) was probably due to the presence of Triton X-100 in membrane fraction.

XpsE ATPase activity is abolished by K331M mutation

All phenotypes exhibited by the double-mutant XpsE(K331M, R504A) are consistent with the notion that ATP binding triggers XpsE oligomerization as well as its association with XpsL. On the other hand, despite being entirely nonfunctional in secretion, the properties displayed by the single mutant XpsE(K331M) appeared surprisingly similar to the wild-type XpsE and differs significantly from the double mutant. In particular, its distribution in membrane fraction was hardly distinguishable from that of the wild-type XpsE. The K331-equivalent lysine residue in Walker A motif had been demonstrated to be essential for ATP hydrolysis in the R64 PilQ, an ATPase involved in Tfp assembly (Sakai *et al*, 2001). Possibly what causes the XpsE(K331M) mutant protein nonfunctional is the abolishment of its ATPase activity.

Our analysis revealed that the wild-type XpsE hydrolyzes ATP with a specific activity of 318.1 ± 30.3 U (Table II). Indeed, the single mutant XpsE(K331M) exhibited a specific activity of 18.5 ± 1.5 U, reduced by 17-fold from that of the

wild-type XpsE. This result supports the notion that the detected ATP hydrolysis activity is specific to XpsE. In contrast, single mutation R504A caused much less reduction in XpsE ATPase activity than the K331M mutation. Specific activity of the mutant protein XpsE(R504A), 168.3 ± 24.6 U, is approximately half of the wild-type XpsE and about 10 times higher than that of the XpsE(K331M) mutant (Table II). The residual ATPase activity explains why the XpsE(R504A) mutant protein, albeit exhibiting reduced ATP binding affinity to similar extent as the XpsE(K331M) mutant protein, remains partially functional. Interestingly, the double-mutant XpsE(K331M, R504A), having lost the ability to bind ATP, displayed almost identical specific activity, 18.9 ± 2.2 U (Table II), as that of the single mutant XpsE(K331M), agreeing with the view that the conserved lysine residue in Walker A motif being the key player in ATP hydrolysis.

XpsE ATPase activity is stimulated by MBP-XpsL_N

Since ATP binding alone is adequate for XpsE–XpsL association, could such a protein–protein interaction be involved in stimulating the ATPase activity of XpsE? In previous section, we observed XpsE, while at ATP-bound state, could associate with XpsL_N. We thus questioned if XpsL_N is stimulatory to the XpsE ATPase activity. Because the XpsL_N could not be recovered intact from the MBP-XpsL_N fusion, we analyzed the fusion protein instead of the XpsL_N itself for effect on the XpsE ATPase activity. All proteins included in ATP hydrolysis assay were washed extensively with buffer containing ATP in order to remove the major contaminating ATPase DnaK. Its elimination was substantiated with Western blotting (data not shown).

When MBP-XpsL_N was present, specific activity of the wild-type XpsE ATPase was raised to 639.3 ± 37.9 U, implying a two-fold stimulation. Addition of MBP alone made no difference to XpsE ATPase activity, ruling out the possibility that the stimulation may have been caused by the MBP moiety. This observation was congruent with the idea that XpsE–XpsL association precedes ATP hydrolysis by XpsE.

Discussion

XpsE oligomerization triggered by ATP binding leads to its association with XpsL

In the present study, by utilizing AMPPNP, the non-hydrolyzable analog of ATP, we demonstrated for the first time in T2SS that oligomerization of XpsE protein, as well as its association with XpsL_N, is achieved by ATP binding to XpsE. Without preincubating with Mg-AMPPNP, only oligomeric, not monomeric, XpsE is capable of associating with XpsL_N. We thus postulate that XpsE oligomerizes upon ATP binding, which in turn makes XpsE competent in associating with XpsL (Figure 6), as well as with cytoplasmic membrane. In agreement, the double-mutant XpsE(K331M, R504A), which is defective in ATP binding, was refractory to oligomerization even when Mg-AMPPNP was present. As expected, its association *in vitro* with the cytoplasmic domain of XpsL, as well as its association with membrane *in vivo* was significantly diminished.

ATP-dependent oligomerization was also observed in BfpD (a likely GspE/PilB functional homolog) that is required for Tfp biogenesis in EPEC (Crowther *et al*, 2005). Moreover, ATP-dependent oligomerization of *V. cholerae* EpsE could be

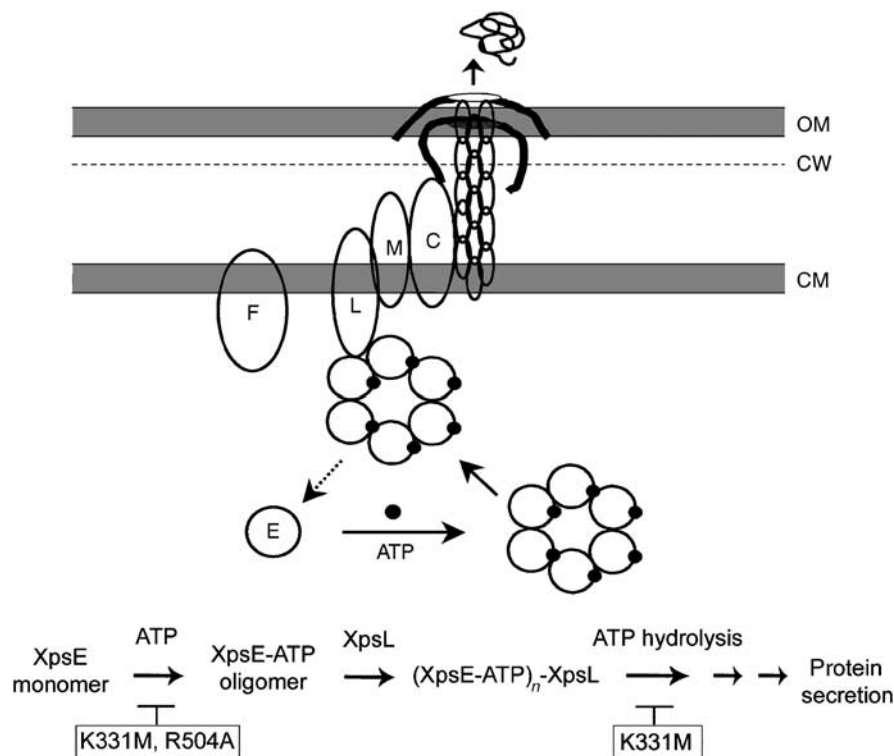


Figure 6 A model depicting sequence of events in T2SS initiated by ATP binding to XpsE inferred from this study. The inverted ‘saucer and cup’-like structure in outer membrane (OM) depicts the secretion pore constituted of GspD (secretin) (Chami *et al*, 2005). The structure bridging between OM and cytoplasmic membrane (CM) represents the pseudopilus constituted of pseudopilins (Hu *et al*, 2002). The L protein in association with the hexameric E is tentatively represented as part of the L–M–C complex (Tsai *et al*, 2002). Stoichiometry of all components, except E, is simplified. E oligomer depicted as hexamer is inferred from what has been determined for other GspE–VirB11 protein members (Yeo *et al*, 2000; Crowther *et al*, 2005). Filled circles depict ATP. Steps blocked by mutant XpsE are indicated by mutated residue(s) in boxes.

implicated from the observation that it was active in ATP hydrolysis only when in oligomeric form (Camberg and Sandkvist, 2005). Conversely, some members of the GspE–VirB11 superfamily exhibited ATP-independent oligomerization. One is involved in T4SS (HP0525 of *Helicobacter pylori*) (Savvides *et al*, 2003) and the other in conjugation (TrbB of RP4) (Krause *et al*, 2000). Two branches were revealed in GspE–VirB11 phylogenetic tree: a T4SS (VirB11) and a T2SS (GspE) subfamily (Planet *et al*, 2001). Interestingly, both HP0525 and TrbB belong to the T4SS subfamily, whereas EpsE, like XpsE, is a member of the T2SS subfamily. Despite its absence from the study, BfpD could very well fit in the T2SS subfamily due to its functional similarity to PilB (belonging to T2SS subfamily) in Tfp biogenesis (Turner *et al*, 1993). Such analyses suggest that a fundamental difference may exist between the two subfamily members in ATP utilization. In T2SS, and probably in Tfp biogenesis as well, ATP binding to the GspE/PilB proteins may set the machineries ready for subsequent steps by triggering their oligomerization and permitting them to associate with their interactive partner(s). ATP hydrolysis is probably not switched on until then. On the other hand, although they share significant sequence similarity with T2SS members, T4SS members may operate differently.

Members of the GspE–VirB11 protein family have been demonstrated to form hexamer as supported by 3D structure (Savvides *et al*, 2003) and results from size exclusion chromatography, sedimentation analysis and electron microscopy (Camberg and Sandkvist, 2005; Crowther *et al*, 2005). In this

study, while examining the effect of ATP binding on XpsE oligomerization by sedimentation velocity experiments, we accidentally discovered a trimeric form of XpsE (sedimentation coefficient of around 8 S), in addition to hexamer (sedimentation coefficient of about 13 S). The trimeric XpsE was detected either when Mg-AMPPNP concentration was reduced (Figure 2A) or when certain mutations that weaken ATP binding affinity was introduced into XpsE protein (Figure 5A). Both conditions are unfavorable to the assembly of hexamer, implicating the assembly of hexamer may involve at least an additional intermediate state.

The hexameric XpsE was apparently capable of XpsL_N binding, since when collected from size exclusion chromatography (fractions 15, 16), it remained binding competent (Figure 4). However, we were unable to perform the same type of experiment on the trimeric form (fractions 20, 21) due to its instability, as suggested by the elution profile from a second size exclusion chromatography. While almost all hexamer remained fractionating in fractions 15, 16, more than half of the trimeric XpsE (fractionating in fractions 20, 21) dissociated into monomers (data not shown). The partially functional mutant XpsE(R504A) formed trimer and can interact with XpsL_N in the presence of Mg-AMPPNP, suggesting trimeric XpsE may be able to bind XpsL. Nonetheless, it remains possible that the presence of XpsL_N may facilitate hexamer formation or that XpsE(R504A) may form a transient hexamer. Further studies are required to clarify whether or not the trimeric XpsE is able to associate with XpsL.

XpsE hydrolysis activity involved in steps downstream of XpsE–XpsL association

Unlike the XpsE(K331M, R504A) double-mutant, single mutation in XpsE at K331 in Walker A motif, albeit causing 17-fold reduction in ATP hydrolysis activity, did not affect its membrane-association. At the same time, the mutant XpsE(K331M) exhibited an ATP dissociation constant of $2.35 \pm 0.36 \mu\text{M}$, which was intermediate between that of the wild-type XpsE ($0.64 \pm 0.06 \mu\text{M}$) and that of the double mutant ($13.62 \pm 2.89 \mu\text{M}$), indicating an ATP binding affinity weaker than the wild-type XpsE by roughly four-fold, but stronger than the double mutant by nearly six-fold. Perhaps, local concentration of ATP around the T2SS machinery *in vivo* was sufficient for ATP binding to the mutant XpsE(K331M), which permitted its oligomerization and association with XpsL, but this concentration was not enough for significant ATP binding to the double-mutant, nor for its membrane association.

However, the XpsE(K331M) protein was not functional in α -amylase secretion, suggesting ATP hydrolysis by XpsE was absolutely essential for its normal function in secretion, likely to be involved in steps downstream of XpsE–XpsL association (Figure 6), for instance, XpsE–XpsL dissociation. Consistent with this view, despite the lack of ATPase activity data, mutations in Walker A motif of *E. chrysanthemi* OutE protein did not affect its interaction with OutL_N in yeast two-hybrid system (Py *et al*, 1999). Our observation that preincubation of monomeric XpsE with Mg-ADP did not lead to XpsE association with XpsL_N also agrees with such a prediction. Unlike AMPPNP, which is not hydrolyzable, ADP is the product of ATP hydrolysis. The presence of ADP disfavoring XpsE–XpsL association implicates ATP hydrolysis may have elicited XpsE–XpsL dissociation.

EPEC Tfp biogenesis ATPase BfpD was stimulated by the cytoplasmic domain of BfpC (a putative GspL functional homolog) by 1000-fold (Crowther *et al*, 2005). Inclusion of a peptide covering a region in the N-terminal cytoplasmic domain of BfpE (a putative GspF functional homolog) raised ATPase activity by an additional 200-fold. In present study, XpsE ATP hydrolysis activity promoted by the N-terminal cytoplasmic domain of XpsL reached a mere two-fold stimulation. Possibly, another cytoplasmic membrane protein XpsF, whose C-terminal cytoplasmic domain has been shown to interact with XpsE in yeast two-hybrid system (unpublished results), may also be required for optimal stimulation of XpsE ATPase activity. Alternatively, T2SS ATPases may only be observed during transportation of extracellular proteins, supporting the idea that T2SS is strictly gated. XpsE exhibits very low ATPase activity in the absence of exoproteins to prevent futile ATP hydrolysis.

GspE as ‘molecular motor’ in T2SS

Like GspE, *E. coli* DNA replication initiator DnaA is also a Walker-type ATPase. While ATP-bound DnaA is active in initiating DNA replication by associating with the replication origin *oriC*, ADP-bound DnaA is not. Hydrolysis of DNA-bound ATP, promoted by interacting with the *E. coli* DNA polymerase III subunit β clamp and a third factor, produces the ADP-bound DnaA preventing further rounds of initiation (Katayama *et al*, 1998). Likewise, ATP-bound GspE may be active in eliciting pore opening by associating with GspL, whereas ADP-bound GspE may not be. Hydrolysis of GspE-

bound ATP, prompted by interacting with GspL, and probably requiring GspF, may yield GspE in ADP-bound or nucleotide-free form keeping the secretion pore from constantly opened. Furthermore, unidirectional transport of exoprotein may be assured. A similar situation was observed in *E. coli* DNA polymerase III holoenzyme, where the β clamp is loaded on primed DNA with assistance from a clamp-loading complex in an ATP-dependent manner. However, clamp loading is achieved upon ATP binding to the γ/τ subunit, whereas ATP hydrolysis is utilized to eject the clamp-loading complex from β -DNA complex, assuring β loading on to DNA, but not unloading (Turner *et al*, 1999).

Conformational change in *E. chrysanthemi* OutL at its C-terminal periplasmic domain was observed, when co-expressed with OutE. However, yeast two-hybrid studies suggested that only the N-terminal cytoplasmic domain of OutL was involved in direct interaction with OutE (Py *et al*, 1999). These observations imply that association of GspE with GspL at its cytoplasmic domain may set off, on the periplasmic side of membrane, a series of protein conformational changes, since GspL, -M and -C proteins had been shown to form a hierarchically structured ternary complex in cytoplasmic membrane (Lee *et al*, 2001). Furthermore, it was recently illustrated in *X. campestris* pv. *campestris* that interaction between major constituent of the pilus-like structure (XpsG) and that of the secretion pore (XpsD) was reliant on GspC (XpsN) (Lee *et al*, 2005), implying GspC may mediate interaction between the pilus-like structure and the secretion pore. One likely outcome of GspE association with, or dissociation from, GspL could be assembly of pilus-like structure from pseudopilins. In agreement, GspE of both *Klebsiella oxytoca* and *Pseudomonas aeruginosa* T2SS was demonstrated to be essential for appearance of pilus-like structure on cell surface (Sauvonnnet *et al*, 2000; Durand *et al*, 2003). Presumably, the pilus-like structure, while being assembled, may act as a piston pushing exoprotein through the strictly gated secretion pore.

Materials and methods

Bacterial strains, plasmids and antisera

C-terminally *Strep*-tagged XpsE, its variants and MBP-XpsL_N are overexpressed in *E. coli* BL21(DE3)(pLysS). The plasmid pMT37, which overexpresses XpsE-*Strep* from T7 promoter, was constructed in two steps. First, a *Strep*-tag linker encoding SNWSPQFEK was subcloned in pET29a at *EcoRI*, *XhoI* sites, with an inframe stop codon upstream of *XhoI* site, generating the plasmid pMT31. The plasmid pMT37 was obtained by cloning a 1.7-kb PCR fragment encoding wild-type XpsE in pMT31 at *NdeI*, *EcoRI* sites. Mutants XpsE(K331M), XpsE(R504A) and XpsE(K331M, R502A) were expressed, as C-terminally *Strep*-tagged proteins, from the plasmid pCH13, pKD2 and pCH17, respectively. Each was obtained from site-specific mutagenesis of the *xpsE* gene cloned in pMT37 using double-stranded DNA template and selecting mutants with *DpnI* (Sambrook and Russell, 2001). The *Strep*-tag, which binds to *Strep*-Tactin[®] (IBA GmbH) Sepharose specifically, when attached at its C-terminus, did not affect normal function of XpsE (data not shown). The plasmid pMT38 expressing *Strep*-tagged XpsE from a broad host-range vector was constructed by subcloning the *Strep*-tagged *xpsE* gene from pMT37 into pCPP30 (a kind gift from Dr D Bauer). Similarly constructed are plasmids pCH18, pKM11 and pKD12 for expressing, respectively, the *Strep*-tagged mutant proteins XpsE(K331M, R504A), XpsE(K331M) and XpsE(R504A) in *X. campestris* pv. *campestris*. The plasmid pCY4 overexpressing MBP-XpsL_N from *tac* promoter was constructed by cloning a PCR fragment encoding residues 1–215 of XpsL (designated as XpsL_N) in pMAL-c2X.

XC1701 is the parental strain of *X. campestris* pv. *campestris*, arising as a spontaneous rifampicin-resistant mutant from the natural isolate XC17 (Hu *et al*, 1992a). XC1723, a chromosomal nonpolar mutant with in-frame deletion in *xpsE* gene, was constructed by following a two-step selection procedure (Kamoun *et al*, 1992). In brief, the deleted *xpsE* gene with its flanking sequences cloned in a suicidal plasmid pUCD4121 was introduced into XC1701 chromosome by selecting for kanamycin-resistant colonies that are sensitive to 5% sucrose. The chosen colony was grown in LB at 28°C for 4 h before plating on plates containing 5% sucrose and rifampicin, selecting for strains that have lost the plasmid. The chromosomal DNA was then examined via PCR for the presence of deleted *xpsE* gene.

Antiserum against XpsE was elicited in rabbit by injecting partially purified His₆-tagged XpsE overproduced in *E. coli* BL21 (DE3). Antiserum against XpsN raised in rabbit was obtained from previous studies (Lee *et al*, 2000). Antiserum against maltose-binding protein (MBP) was purchased from New England Biolab. Antiserum against *Bacillus subtilis* GroEL, which crossreacts with the GroEL of *X. campestris* pv. *campestris*, was a kind gift from Professor B-Y Chang. Antiserum against DnaK was purchased from Stressgen.

Protein purification

XpsE protein and its variants, C-terminally fused with *Strep*-tag, were purified from the *Strep*-Tactin[®] Sepharose column by following the recommended procedures of the supplier. In brief, broken cell lysates were mixed with *Strep*-Tactin[®] Sepharose, which has been prewashed with Buffer A (20 mM Tris-HCl pH 8.0, 200 mM NaCl), at 4°C for 1 h, before being packed into column. After washing with buffer B (buffer A plus 1 mM PMSF, 5 mM DTT), resin-bound proteins were eluted with buffer C (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin). In order to keep XpsE proteins ATP-free, we added 0.1 µg/ml hexokinase, 1 mM MgCl₂ and 1 mM glucose (Motojima and Yoshida, 2003) to the eluted fractions. MBP-XpsL_N protein was purified from amylose resin. Batch binding and washings were performed as described for XpsE-*Strep* purification. Elution was instead achieved using buffer D (20 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM DTT, 1 mM maltose).

To remove contaminating DnaK from XpsE-*Strep* or MBP-XpsL_N protein preparation, elution was preceded with excess washings (100 and 150 ml, respectively) with buffer B containing 5 mM ATP and 5 mM MgCl₂. DnaK elimination was confirmed with immunoblotting with antiserum against DnaK detected with ECL chemiluminescence.

Size exclusion chromatography

To separate oligomers from monomeric form, affinity-purified XpsE-*Strep* was separated on the Superdex HR200 size exclusion column that has been preequilibrated with Buffer A. Proteins were eluted with the same buffer at a flow rate of 0.3 ml/min. Eluted fractions collected at 0.5 ml/fraction were separated in sodium dodecyl sulfate (SDS)-polyacrylamide gel followed by Coomassie blue staining. Monomeric XpsE-*Strep* collected from gel filtration was further analyzed for the effect of preincubation with 1 mM AMPPNP or ADP, plus 10 mM MgCl₂, on its oligomerization by running through a second size exclusion column as before, followed by SDS-PAGE and immunoblotting with antiserum against XpsE.

Analytical ultracentrifugation

Size distribution analysis was conducted using Beckman-Coulter XL-A analytical ultracentrifuge in an An-50 Ti rotor. Sedimentation velocity experiments were carried out at 30 000 r.p.m. at 20°C in double sector centerpieces filled with Buffer A with or without *Strep*-tagged XpsE or its variants (at a final protein concentration of 0.1 mg/ml) that was isolated from size exclusion chromatography as monomer. When applicable, XpsE was preincubated with 0.2 mM AMPPNP and 10 mM MgCl₂ on ice overnight before ultracentrifugation. Data were collected at 280 nm in a continuous scan mode with a time interval of 480 s and a step size of 0.002 cm without averaging. Data analysis was carried out using SEDFIT (Schuck *et al*, 2002), which is based on Lamm equation, to determine the sedimentation coefficient distribution.

MBP pull-down assay

MBP-XpsL_N immobilized on amylose resin after thorough washing with Buffer A was used in assaying association of XpsE-*Strep* with

XpsL_N. XpsE-*Strep* or its variants (at a final protein concentration of 0.6 µM) was incubated with or without 1 mM AMPPNP and 10 mM MgCl₂ on ice for 30 min followed by mixing with resin-bound MBP-XpsL_N or blank resin (not prebound with MBP-XpsL_N) at 4°C for 1.5 h. Nonspecific binding of XpsE with MBP has been ruled out by using MBP-bound amylose resin as control (data not shown). Subsequently, amylose resin with bound proteins separated from unbound proteins by centrifugation at 2800 g for 15 min was washed three times with Buffer A before analyzed in SDS-polyacrylamide gel followed by immunoblotting with antiserum against XpsE or MBP.

Subcellular fractionation

The *xpsE* mutant XC1723 of *X. campestris* pv. *campestris* expressing *xpsE* or its variants were grown in 200 ml LB to OD₆₀₀ 1.0. Following centrifugation, cells were resuspended in 5 ml Buffer A and broken by passing through French press. The membranes were sedimented by centrifugation at 195 000 g, for 45 min at 4°C, the supernatant was saved as the cytoplasmic fraction, and the pellets were treated with 5 ml Buffer E (Buffer A plus 1% Triton X-100, 1 mM PMSF) overnight and centrifuged at 195 000 g, for 45 min at 4°C. The supernatant was collected as the membrane fraction. Samples were then analyzed in SDS-polyacrylamide gel followed by immunoblotting with antiserum against XpsE, GroEL (cytoplasmic protein marker), or XpsN (membrane protein marker).

ATPase assay

ATP hydrolysis activity was assayed in 20 µl reaction buffer (Buffer A plus 1 mM DTT and 2.5 mM MgCl₂) containing 500 µM [γ -³²P]ATP. Assays in triplicates were initiated by adding 0.8 µM XpsE or its variants followed by incubating at 28°C for 20 min. While assaying the effect of MBP-XpsL_N on XpsE ATPase activity, 1 µM MBP-XpsL_N (or MBP) was added to the reaction mixture containing 12 µM XpsE and 42 µM [γ -³²P]ATP. Aliquots (0.5 µl) of the reaction mixture were spotted onto thin layer chromatography (TLC) sheet coated with polyethyleneimine (PEI-Cellulose F, Merck) and developed in 0.5 M LiCl and 0.5 M formic acid for 70 min. Released ³²P-labeled inorganic phosphate (Pi), which migrates faster than ATP, was quantified using Typhoon[™] imaging system (Typhoon 9200 Variable Mode Imager, Amersham Biosciences) and TINA (2.09e) software. Quantification of released ³²P-labeled Pi was extrapolated from the standard curve drawn by using a series of diluted [γ -³²P]ATP with known concentrations.

ATP binding assay

ATP binding assay was performed in 50 µl reaction buffer (Buffer A plus 1 mM DTT and 1 mM MgCl₂) containing 0.01–30 µM [α -³²P]ATP. Assays were initiated by adding 1 µg XpsE or its variants followed by incubating at 4°C for 15 min. At the end of incubation, reaction mixtures were transferred to ProbeQuant G-50 Micro Column (Amersham) and spun at 2800 g for 1 min to remove free ATP. Protein-bound ATP collected as flow-through from spin column was quantified by analyzing aliquots on TLC following procedures as described for quantifying released Pi in ATPase assay.

Assays for α -amylase secretion

In vivo function of XpsE or its variants was determined by introducing plasmid expressing each protein into the *xpsE* mutant XC1723 of *X. campestris* pv. *campestris* and assaying for α -amylase secretion on starch plate. Either starch plate assay (Hu *et al*, 1992b) or semiquantitative assay (Lee *et al*, 2004) for starch-hydrolyzing activity in extracellular fraction was performed.

Acknowledgements

We thank Dr Gu-Gang Chang, Dr Hui-Chih Hung and Chi-Yuan Chou for kind assistance in sedimentation coefficient analysis, Mei-Ting Wu for constructing the plasmids pMT31 and pMT37, Chu-Chi Lin for constructing the *xpsE* mutant strain XC1723 and raising antibody against XpsE, Yeh Chen for constructing the plasmid pCY4, Kun-Da Wu for constructing the plasmids pKD2 and pKD12. This work was supported by grants from Republic of China: NSC93-2311-B-005-003 from National Science Council and E-91-B-FA05-1-4 from Ministry of Education.

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