Positive co-operative activity and dimerization of the isolated ABC ATPase domain of HlyB from *Escherichia coli*

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The ATPase activity of the ABC (ATP-binding cassette) ATPase domain of the HlyB (haemolysin B) transporter is required for secretion of *Escherichia coli* haemolysin via the type I pathway. Although ABC transporters are generally presumed to function as dimers, the precise role of dimerization remains unclear. In the present study, we have analysed the HlyB ABC domain, purified separately from the membrane domain, with respect to its activity and capacity to form physically detectable dimers. The ATPase activity of the isolated ABC domain clearly demonstrated positive co-operativity, with a Hill coefficient of 1.7. Furthermore, the activity is (reversibly) inhibited by salt concentrations in the physiological range accompanied by proportionately decreased binding of 8-azido-ATP. Inhibition of activity with increasing salt concentration resulted in a change in flexibility as detected by intrinsic tryptophan fluorescence. Finally, ATPase activity was sensitive

towards orthovanadate, with an IC_{50} of 16 μ M, consistent with the presence of transient dimers during ATP hydrolysis. Nevertheless, over a wide range of protein or of NaCl or KCl concentrations, the ABC ATPase was only detected as a monomer, as measured by ultracentrifugation or gel filtration. In contrast, in the absence of salt, the sedimentation velocity determined by analytical ultracentrifugation suggested a rapid equilibrium between monomers and dimers. Small amounts of dimers, but apparently only when stabilized by 8-azido-ATP, were also detected by gel filtration, even in the presence of salt. These data are consistent with the fact that monomers can interact at least transiently and are the important species during ATP hydrolysis.

Key words: ATP-binding cassette transporter (ABC transporter), ATPase, *Escherichia coli*, haemolysin (Hly), secretion.

INTRODUCTION

ABC (ATP-binding cassette) proteins constitute a specific class of ATPases that are concerned with a wide variety of transport processes, including both inward (import) and outward (export or secretion), the latter being conserved from bacteria to humans [1]. Many ABC transporters in prokaryotes (such as HisP) are required for the uptake of nutrients and involve separately encoded ABC ATPases and transport (membrane) domains [2,3]. A smaller subgroup of transporters concerned in particular with the export of proteins are composed of a membrane domain fused to a C-terminal ABC domain. The most studied of these transporters are key components of the Type I secretion pathway in Gram-negative bacteria, including HlyB (haemolysin B), essential for secretion of HlyA (haemolysin A) from *Escherichia coli* [4].

The mechanism of action of ABC transporters is usually predated on the supposition that dimerization of the ABC domain is essential for some parts of the catalytic and transport cycle. However, while the stoichiometry of the *in vivo* or reconstituted histidine and maltose uptake systems indicates the presence of two molecules of the separately encoded HisP or MalK ABC ATPases [5,6], direct evidence for stable dimerization of individual ABC NBDs (nucleotide-binding domains) in purified form, even in the presence of nucleotides, has been difficult, if not impossible, to obtain [7–11]. On the other hand, OpuAA, required for uptake of glycine betaine in *Bacillus subtilis*, does form dimers, independently of ATP [11]. These studies indicate that the precise conditions for formation of a stable ABC dimer are unclear.

The ABC superfamily is characterized by some major, highly conserved structural motifs in the NBD domains. However, other studies now indicate an unexpected variation in some details of the structure of different ABC domains. For example, we re-

cently described the high-resolution structure of the HlyB NBD monomer that forms the characteristic fold for the ABC ATPases, but also displayed an unusual relative arrangement of the Walker A and B motifs [12]. Moreover, in a comparative analysis of the available ABC structures, we also identified a structurally diverse region of approx. 30 residues in the helical domain. This region, sandwiched between the highly conserved Q-loop and C-loop sequences, appears so far to be structurally unique to each ABC NBD. Minor, but significant, additional differences between different ABC domain structures have also been noted by others [13–15]. All these data suggest that, although highly conserved overall, the NBDs of ABC transporters may be more variable than supposed, having adapted to the requirements for coupling the activity of the membrane and ABC domains for the transport of cognate allocrites, that are all unique to each type of transporter.

In the present study, we show that it is possible to purify large amounts of the ABC ATPase domain of HlyB in soluble form, expressed separately from the membrane domain. In line with the structural variations between ABC NBDs in general, the ATPase activity of purified HlyB NBD displayed a number of unusual features: orthovanadate sensitivity, co-operativity consistent with dimerization and, uniquely, sensitivity to salt. Under most conditions, only the monomer form could be detected, indicating that dimers were very unstable.

EXPERIMENTAL

Construction of the overexpressing plasmid pPSG122

The ABC HlyB DNA fragment encoding residues D⁴⁶⁷–D⁷⁰⁷ from HlyB and completely lacking the membrane domain was generated as an NdeI/EcoRI fragment using PCR. This fragment was

Abbreviations used: ABC, ATP-binding cassette; Hly, haemolysin; NBD, nucleotide-binding domain.

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cloned under the control of an arabinose-inducible promoter in the expression vector pBAD18 [16]. The template DNA was plasmid pLG570 '*hlyCABD*' [17]. A hexahistidine tag was engineered into the N-terminus of the ABC fragment. Primers for the PCR were: upstream, 5'-ATATCATATGCATCACCATCACCATCACGATATCACTTTTCGTAATATCCGG-3' (with the NdeI site in bold, and the His₆ tag underlined) and downstream, 5'-ATATGAATTC-TTAGTCTGACTGTAAGTATATAAG-3' (with the EcoRI site in bold).

Production and purification of the HlyB ABC domain

E. coli strain DH5 α was transformed with plasmid pPSG122 encoding the His-tagged ABC domain. Cultures in 2 litres of LB (Luria–Bertani) broth were grown at 25 °C until the A₆₀₀ reached 2, induced by the addition of arabinose (final concentration of 4 mM) and were grown for a further 3 h. Cells were harvested by centrifugation, the cell pellet was resuspended in 35 ml of buffer A (10 mM Tris/HCl, pH 8, 100 mM KCl and 10 % glycerol), and lysed by four passages through a French pressure cell at 10 000 psi (~68 900 kPa). The cytoplasmic fraction, containing 50–60 % of the HlyB ABC protein in a stable, soluble form was collected after centrifugation at 10 000 *g* for 30 min and loaded on to a 16/20 column containing Ni²⁺ immobilized on fast-flow chelating Sepharose (Amersham Biosciences). The column was washed (8 column vol.) with buffer A₁ (Buffer A plus 10 mM imidazole), and protein was eluted by a linear gradient of imidazole (10–350 mM). The fractions containing the HlyB ABC protein, eluted between 180 and 250 mM imidazole, were pooled, concentrated and exchanged with a suitable buffer using the Bio-Rad Econo-Pac 10DG desalting column. The samples, in buffer A or phosphate buffer (50 mM phosphate buffer, pH 8, 50 mM KCl and 10 % glycerol) were stored with a protein concentration up to 15 mg/ml. These could be kept for at least 2 days at 4 °C without loss of activity, or indefinitely at –80 °C. At 25 °C, concentrated solutions of the ABC domain lost 20 % activity through precipitation over 24 h. The total yield of the soluble ABC domain was 15–20 mg from 1 litre.

ATPase activity assay

The purified HlyB ABC domain was assayed for ATP hydrolysis using the meta-arsenite colorimetric method, which depends upon released P_i [18]. Activity was also assayed using the Malachite Green method [19,20]. In both assays, the amount of P_i liberated was determined by a colorimetric assay (at A₆₃₀) essentially as described in [19,20], using K₂HPO₄ as a standard. Alternatively, ATPase activity was measured employing a continuous ATP-regenerating assay, where ATP hydrolysis is coupled to the consumption of NADH [21]. Assays were performed at 22 °C in a temperature-controlled 96-well plate in a spectrophotometer (Polar Start Galaxy, BMG). A typical reaction mixture (600 μ l) contained 10 mM Tris/HCl or sodium phosphate, pH 8, 5 mM MgCl₂, 10 % glycerol, and HlyB ABC protein (8 μ M), with various amounts of ATP and the indicated salt concentrations.

Reactions were started by addition of ATP and monitored at 340 nm. Data points were fitted to either eqn (1):

$$v = \frac{v_{\max} \cdot [S]}{K_m + [S]} \quad (1)$$

which assumes Michaelis–Menten kinetics, or eqn (2):

$$v = \frac{v_{\max} \cdot [S]^h}{K_{0.5}^h + [S]^h} \quad (2)$$

which assumes co-operativity. Here, *v* represents the velocity and *v*_{max} the maximal velocity of the reaction, [S] denotes substrate concentration, *K*_m and *K*_{0.5} are [S] where 50 % of the enzyme is substrate-saturated, and *h* is the Hill coefficient. All reactions were performed in duplicate, and the results presented are means $\pm$ S.E.M.

Photolabelling with 8-azido-[γ -³²P]ATP or non-labelled 8-azido-ATP

8-Azido-[γ -³²P]ATP was purchased from ICN Biochemicals. Labelling was conducted on ice with 20 mM Tris/HCl, pH8, 5 mM MgCl₂, 4 μ M purified HlyB ABC, 50 μ M 8-azido-[γ -³²P]ATP at 0.1 μ Ci/ μ l and a final KCl or NaCl concentration varying between 0 and 300 mM. The reaction mixture was incubated at 4 °C for 5 min to minimize hydrolysis of the azido-ATP. Irradiation was performed using a UV lamp (254 nm) placed directly over the opened tube (Eppendorf, 2 ml), 5 cm above the sample for two 1-min intervals, with a 1-min cooling in between. Labelled bands after SDS/PAGE were scanned and quantified using ImageQuant version 1.11 software.

Oligonucleotide-directed site-specific mutagenesis

For the preparation of mutations in the NBD encoded by *hlyB*, all codon changes were introduced using the specific mutagenic 40-mer oligonucleotide and following the protocol supplied with the Amersham Biosciences U.S.E. Mutagenesis Kit (catalogue number 27-1699-01). The oligonucleotide was synthesized and phosphorylated by Eurogentec. DNA was sequenced using the Terminator Ready Reaction kit (PerkinElmer), following the manufacturer's protocol with the automatic DNA sequencer ABI 373 or ABI 310.

SDS/PAGE and Western blotting

The basic procedure was that of Laemmli [22], using either a 15 % or a 13 % (w/v) acrylamide resolving gel, with a 5 % stacking gel. Gels were stained using Coomassie Brilliant Blue (0.05 %, w/v) in a solution containing 7 % (v/v) ethanoic (acetic) acid and 25 % (v/v) propan-2-ol. Western blotting was as described previously [23].

Steady-state fluorescence experiments and measurements

Intrinsic tryptophan fluorescence emission spectra were recorded on a FLUOLOG-3 spectrometer (Instruments S.A., HORIBA, Paris, France) at room temperature (22 $\pm$ 1 °C) by excitation at 295 nm. Emission scans were performed from 300 to 450 nm. After background correction using buffer (10 mM Tris/HCl, pH 8.0, and varying concentrations of salt), emission maxima and intensities were determined using the software provided by the manufacturer. Data points are the average value of at least two independent experiments.

For quenching experiments, 10 mM Tris/HCl, pH 8.0, buffers with various salt concentrations (10–300 mM NaCl) were supplemented with NaI from 1 μ M to 10 mM to yield final salt concentrations from 10 to 300 mM. Fluorescence spectra were acquired as described above and normalized to the corresponding spectra in the absence of NaI. All spectra are the average of three independent experiments and were analysed according to the Stern–Vollmer equation (eqn 3):

$$\frac{I_0}{I} = 1 + k_Q \tau_0 c_Q \quad (3)$$

where *I*₀ denotes the fluorescence intensity in the absence and *I* in the presence of quencher, *k*_Q is the quenching rate, τ_0 is the

lifetime of excited state in the absence of quencher, and c_Q is the concentration of quencher.

Orthovanadate preparation

Stock solutions of 40 mM orthovanadate were carefully prepared as described in [24]. To determine potential vanadate inhibition of HlyB NBD ATPase activity in the presence of orthovanadate (5–50 μ M), the Malachite Green assay was used as described above in the presence of the indicated concentrations of orthovanadate at a fixed ATP concentration of 2 mM. The buffer was 10 mM Tris/HCl, pH 8.0, and 10 mM KCl. Data points were analysed according to eqn (4):

$$\text{ATPase activity} = \frac{1}{1 + \left(\frac{[\text{vanadate}]}{\text{IC}_{50}} \right)^n} \quad (4)$$

where n accounts for variations in the slope of the inhibition curve.

Gel filtration

Gel-filtration analysis was performed using a Superdex HR75 10/30 column (Amersham Biosciences). The gel-filtration column was equilibrated with buffer containing 10 mM Tris/HCl, pH 8.0, combined with 0, 10 and 100 mM KCl. In some experiments, unlabelled ATP or 8-azido-ATP (both 0.5 mM) were mixed with the protein (32–500 μ M). The protein concentration varied between 0.9 and 15 mg/ml (32–500 μ M). Elution profiles were monitored by recording the A_{280} using a flow rate of 1 ml/min. The column was calibrated using the following: alcohol dehydrogenase, BSA, carbonic anhydrase, ribonuclease A and uridine, corresponding to a molecular masses of 150, 66, 29, 13.7 and 0.244 kDa respectively. All measurements were performed at 25°C.

Ultracentrifugation analysis

The HlyB ABC ATPase domain was purified in Tris/HCl buffer, pH 8.0, containing 100 mM KCl and 10% glycerol. In order to remove the glycerol and obtain the appropriate KCl concentration, aliquots of the HlyB NBD were desalted using an Econo-Pac 10DG desalting column and exchanged for the appropriate buffer, following the protocol supplied with the Bio-Rad kit (catalogue number 732-2010). Samples were concentrated (0.1–0.2 mg/ml, 4–8 μ M), using the Millipore concentrators (catalogue number UFV2-BGC10). To remove precipitated protein, samples were centrifuged at 3300 g for 30 min. All samples were run in a Beckman analytical ultracentrifuge with or without 1 mM ATP. Sedimentation velocity runs were started immediately after completion of sample preparation in the required buffer. Samples and appropriate buffers (400 μ l each) were loaded into their respective channels in double-sector ultracentrifuge cells and run at speeds of 45 000 rev./min at 20°C in an XL-I analytical ultracentrifuge (Beckman, Palo Alto, CA, U.S.A.) using both interference and absorption (scanning wavelength 280 nm) optical systems. Data was analysed using DCDT + software (version 1.13).

RESULTS

Overexpression and purification of the ABC domain of HlyB

For overexpression of the HlyB ABC ATPase domain, a 725 bp DNA fragment encoding *hlyB* from nucleotide 1399 to 2124 at a site beyond the 3'-end of *hlyB* [23] was cloned into pBAD18, giving plasmid pPSG122. After transformation into *E. coli* strain DH5 α , the conditions for expression of the 28 kDa His-tagged

Figure 1 Overproduction and purification of the His-tagged ABC ATPase domain of HlyB at 25°C

(A) SDS/PAGE of total cell protein stained with Coomassie Brilliant Blue is shown. *E. coli* strain DH5 α was transformed with plasmid pPSG122 expressing the ABC (His₆) domain. Lane 1, molecular-mass standards (in kDa, as indicated to the left of the gel); lane 2, total bacterial proteins before arabinose induction (loaded sample equivalent to an A_{600} of 0.25); lane 3, after induction (loaded sample equivalent to an A_{600} of 0.25); lane 4, cytoplasmic (soluble) fraction; lane 5, membrane plus insoluble aggregated fraction. Lanes 4 and 5 contain equivalent cell loadings. The arrow indicates the ABC ATPase domain of HlyB (28 kDa). (B) Peak fractions eluted from the Sepharose-Ni²⁺, separated and stained with Coomassie Brilliant Blue. Lane 1, molecular-mass standards (in kDa, as indicated to the left of the gel); lanes 2–5, imidazole eluate (180–250 mM); lane 6, overloaded sample from lane 5. The arrow indicates the position of His-tagged HlyB NBD.

ABC domain were optimized with respect to arabinose concentration, culture density at induction of synthesis, time of harvesting and, in particular, the growth temperature. In this way, conditions were established in which 50–60% of the accumulating ABC domain remained in soluble form. The employment of low temperature (25°C) was especially important, combined with high-level expression following induction by addition of 4 mM arabinose at an A_{600} of 2 (see Figure 1A).

The soluble His-tagged HlyB ABC domain was purified to homogeneity in a single IMAC (immobilized metal-affinity chromatography) step as shown in Figure 1(B). The ATPase domain was stable in phosphate or Tris buffer, pH 8, in the absence of ATP at concentrations of up to 25 mg/ml, for at least 2 days at 4°C or indefinitely at –80°C without loss of ATPase activity.

ATPase activity of the isolated HlyB ABC

Enzyme kinetic parameters under a variety of conditions are summarized in Table 1. Notably, k_{cat} decreased, while the $K_{0.5}$ value increased, with increasing salt concentrations. As a consequence, there was a fall in catalytic efficiency of the ATPase, and no steady-state ATPase activity was detectable for NaCl or KCl concentrations above 300 mM. In the presence of 10 mM salt, we obtained a $K_{0.5}$ of $650 \pm 20 \mu$ M, with a catalytic centre activity of approx. 0.33 ATP/s. The values obtained for HlyB compare with a range of reported K_m and k_{cat} values (0.05–0.9 ATP/s) for different purified preparations of HisP and MalK [8,25]. Surprisingly, HlyB ATPase activity (Figure 2) did not obey Michaelis–Menten kinetics, while a nearly perfect fit was obtained using the Hill equation (eqn 2). This is in striking contrast with the ATPase activity of HisP, MalK, Mdl1p NBD or OpuAA, which does not display ATP-dependent positive co-operativity [8,9,11,25,26]. In contrast with the k_{cat} and $K_{0.5}$ for HlyB, which were sensitive to the salt concentration employed, the Hill coefficient remained constant ($h = 1.7 \pm 0.1$) over the whole range of NaCl concentration (10–200 mM). We also investigated the unusual co-operative behaviour of the HlyB NBD as a function of pH and cation requirements, with the results in this case typical of other ABC ATPases

Table 1 Kinetic parameters for the ATPase activity of HlyB NBD calculated from data at 25 °C and fitted to the Hill equation

Results are means ± S.D. for two independent experiments. (a) Effect of NaCl at pH 8.0. (b) Effect of pH at 3 mM MgCl₂ and 10 mM KCl. (c) Effect of bivalent cations at pH 8.0 and 10 mM KCl.

(a)				
NaCl concentration (mM) ...	10	50	100	200
Hill coefficient	1.7 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	1.7 ± 0.1
K _{0.5} (mM)	0.65 ± 0.02	1.09 ± 0.06	1.50 ± 0.06	2.33 ± 0.15
k _{cat} (s ⁻¹)	0.33	0.31	0.20	0.11
k _{cat} /K _{0.5} (s ⁻¹ · mM ⁻¹)	0.51	0.28	0.13	0.04
(b)		(c)		
pH	Activity	Cation	Activity	
8.5	85	None	0	
8.0	100	Mg ²⁺	100	
7.5	90	Mn ²⁺	125	
7.0	75	Co ²⁺	47	
		Ca ²⁺	3	
		Zn ²⁺	1

Figure 2 ATPase activity of the purified ABC domain of HlyB

ATPase activity as a function of different salt concentrations, using the enzyme-coupled assay at a protein concentration of 8 μM. Data within 10 % deviation of ATPase activity, at 200 mM NaCl, were analysed by the Hill equation (eqn 2, solid line) and the Michaelis–Menten equation (eqn 1, broken line).

(Tables 1b and 1c). Nevertheless, under all of these conditions, positive co-operativity was still observed (results not shown).

Analysis of *in vitro* activity of purified HlyB ABC carrying different mutations

Using site-directed mutagenesis, several mutations were created affecting different conserved motifs in the ABC domain, and the activity of the corresponding ABC polypeptides was determined (Table 2). The ATPase activity of mutant polypeptides Lys⁵⁰⁸ → Met and Asp⁶³⁰ → Met, affecting the Walker A and B motifs respectively, was 0.6 % and 1.3 % with respect to the wild-type. The highly conserved His⁶⁶² (His²¹¹ in HisP) in the so-called switch region of the ABC domain [27] was replaced by alanine and was completely inactive. In contrast, replacement of the single cysteine (Cys⁶⁵²) by alanine, had no effect. Using γ-³²P-labelled 8-azido-ATP, we confirmed that all these mutant forms still bound this derivative of ATP as efficiently as the wild-type protein (results not shown).

Table 2 Properties of mutants of the HlyB NBD

ND, not determined.			
Codon change	Amino acid change	Solubility	ATPase activity
AAA → ATG	Lys ⁵⁰⁸ → Met WalkerA	Soluble	Inactive, binds ATP
GAT → ATG	Asp ⁶³⁰ → Met WalkerB	Soluble	Inactive, binds ATP
TGT → GCT	Cys ⁶⁵² → Ala	Soluble	Active
CAT → GCG	His ⁶⁶² → Ala	Soluble	Inactive, binds ATP
GTG → GCG	Val ⁵⁴⁸ → Ala	Insoluble	ND
CCT → CTT	Pro ⁶²⁴ → Leu	Insoluble	ND
CCT → GCG	Pro ⁶²⁴ → Ser	Insoluble	ND
CCT → GCG	Pro ⁶²⁴ → Cys	Insoluble	ND
CCT → GCG	Pro ⁶²⁴ → Arg	Insoluble	ND

Figure 3 Effect of salt concentration on ATPase activity and the fixation of 8-azido-γ-³²P]ATP by the HlyB ABC ATPase domain

(A) ATPase activity of the purified ABC ATPase (1 μM) at different salt concentrations. The rate of hydrolysis was measured by following the release of P_i for 20 min at 25 °C. (B) Autoradiogram after SDS/PAGE showing the effect of salt on binding 8-azido-γ-³²P]ATP.

Two additional mutations, Val⁵⁴⁸ → Ala and Pro⁶²⁴ → Leu, corresponding to HisP mutations Val⁹⁸ → Ala and Pro¹⁷² → Leu respectively, that affect the regulation of the ATPase activity of HisP [5] were also constructed. However, both the HisP-like constitutive mutants, Val⁵⁴⁸ → Ala and Pro⁶²⁴ → Leu, were completely insoluble, as were other mutations of Pro⁶²⁴ to serine, cysteine or arginine (see Table 2).

The *in vitro* activity and binding of 8-azido-ATP to the HlyB ATPase domain are modulated by the salt concentration

The activity of the NBD was seen to be decreased at high (physiological) salt concentrations (Table 1a). This effect was examined in more detail, and we detected only 3–5 % residual activity at 200–300 mM salt. Importantly, the decrease of activity at high salt is completely reversible (see Figure 3A; rightmost column), consistent with a change in conformation and/or the oligomerization state of the ABC domain, which turns off activity at physiological salt concentrations.

Figure 4 Reciprocal relationship between Trp⁵⁴⁰ fluorescence and ATPase activity of the HlyB ABC ATPase domain with increasing salt concentration

Maximal fluorescence intensity (▲) and ATPase activity, normalized according to Figure 3 (■), were plotted against salt concentration. The lines were fitted to a standard mono-exponential model for dissociation with offset and association respectively.

We then investigated the binding of the ATP analogue 8-azido-ATP as a function of salt concentration. Figure 3(B) shows that increasing the salt concentration decreased the binding of radio-labelled 8-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, as detected by SDS/PAGE analysis and autoradiography. When the labelled bands were scanned, the results indicated an approx. 7-fold decrease in azido-ATP binding at 200 mM compared with 10 mM KCl (results not shown).

Changes in intrinsic tryptophan fluorescence of the ABC domain as a function of salt concentration

Decreased binding of ATP at high salt concentrations could result from changes in the conformation and/or the oligomerization states of the purified HlyB ATPase domain. The ABC domain contains a single tryptophan residue at position 540. Therefore intrinsic tryptophan fluorescence, as an indication of conformational changes, resulting from the presence of salt, was measured. As shown in Figure 4, intrinsic fluorescence indeed increased reciprocally with the decrease in ATPase activity as the salt concentration increased.

HlyB NBD is orthovanadate-sensitive

The activity of the NBDs of intact ABC transporters is reported as normally vanadate-sensitive due to the formation of a vanadate-trapped ADP intermediate [28]. Curiously, this is usually only observed with the ABC domain in the presence of the membrane components of the transporter; for example, with HisP or MalK [8,25]. As shown in Figure 5, orthovanadate inhibited the isolated HlyB ATPase activity over the range 5–50 μM , with an IC_{50} of $16.3 \pm 1.6 \mu\text{M}$, behaving as a competitive inhibitor.

Attempts to identify dimers of the HlyB ABC domain

Many studies indicate that ABC proteins function as dimers, with increasing evidence that the membrane domains are 'constitutively' dimerized, while the ABC domains may only interact transiently during the catalytic cycle linked to the transport process [29–31]. The question of the dimerization of the HlyB ABC domain, including conditions of varying salt concentrations, was therefore investigated in a number of ways.

(i) Gel-filtration analysis of the HlyB ABC domain

Purified HlyB ABC ($1.7 \text{ mg/ml} = 60 \mu\text{M}$) was analysed by gel filtration over a range of salt concentrations (0, 10 and 100 mM).

Figure 5 Vanadate inhibition of the ATPase activity of the HlyB NBD

Experiments were performed in 10 mM Tris/HCl, pH 8.0, and 10 mM KCl with 2 mM ATP, and data were analysed according to eqn (4).

Figure 6 Size-exclusion chromatography of the HlyB NBD

Purified ABC ATPase was eluted from a Superdex HR 75 column with 10 mM Tris/HCl, pH 8.0, and 100 mM KCl, in the absence (profile 1) or presence (profile 2) of non-labelled 8-azido-ATP. For convenience, the two profiles were superimposed after two separate experiments injecting 1.5 mg and 340 μg of protein respectively; however, identical results were obtained with equal 1.5 mg injections. The monomer (28 kDa) and presumed dimer (56 kDa) of His-tagged HlyB ABC are indicated. MM, molecular mass.

As shown in Figure 6, the HlyB ATPase always fractionated as a single peak, as expected for the 28 kDa monomer, with no indication of dimerization. These experiments were also performed in the presence of ATP (1 mM) and with a range of protein concentrations (30–500 μM), but no dimers were eluted at the expected position of 56 kDa (results not shown). In contrast, when the ATPase domain was first pre-incubated with 8-azido-ATP, followed by UV irradiation, a small proportion (approx. 20%)

Table 3 Analytical ultracentrifugation studies of the HlyB NBD in the presence or absence of ATP at various KCl concentrations

MM, molecular mass; ND, not determined.

KCl concentration (nM)	(-) ATP		(+) ATP	
	$s_{20,w}$ (S)	MM (kDa)	$s_{20,w}$ (S)	MM (kDa)
100	ND	ND	2.46 ± 0.1	27.5 ± 1.2
10	2.42 ± 0.1	27.7 ± 1.2	2.46 ± 0.1	30.8 ± 2.3
0	2.88 ± 0.2	$35.7 \pm 2.0^*$	ND	ND

was eluted from the Superdex column in the position of a dimer. Since 8-azido-ATP may undergo slow hydrolysis by ABC proteins, this suggested that a transient dimer might be trapped under these conditions.

(ii) Ultracentrifugation analysis of the HlyB ABC domain

The properties of the purified HlyB ABC domain were also examined by sedimentation velocity analysis, and the results are summarized in Table 3. In the presence of a range of salt concentrations (5–100 mM) and with or without 1 mM ATP, the polypeptide sedimented as a single monodisperse band, with a sedimentation behaviour calculated to be close to that of a 28 kDa monomer. In the absence of added salt, the sedimentation coefficient increased, indicating a change in conformation or molecular mass. However, fitting the data to either a single- or a two-species model was difficult, suggesting a rapid equilibrium between two species; for example, a monomer–dimer interconversion.

DISCUSSION

Difficulties in overexpressing significant quantities of ABC ATPase domains in soluble form have been a major factor limiting the production of structural information concerning some important ABC ATPases [32]. Previous studies with Pgp (Mdr1) [33], CFTR (cystic fibrosis transmembrane conductance regulator) [34–36], and J. R. Riordan, personal communication) or TAP [37] have all shown that the overexpressed ATPase largely forms inclusion bodies. Attempts to overexpress either NBD1 or NBD2 of human Mdr1 in soluble form in our laboratory have been similarly unsuccessful (H. Benabdelhak and M. A. Blight, unpublished work). Several recent successful structural studies of ABC (NBD) domains have been obtained with proteins derived from thermophiles, and this may avoid this insolubility problem [10,38,39]. Unfortunately, however, this approach is offset by the lack of any knowledge so far of the mechanism of action of such thermophile proteins *in vivo*. The careful optimization of conditions for overexpression of the ABC ATPase of HlyB described in the present study, with, in particular, employment of a low incubation temperature (25 °C), allowed the production of large amounts of soluble polypeptide that remained soluble and stable at concentrations up to 15–20 mg/ml. Indeed, this has permitted the recent determination of a high-resolution structure for this NBD [12].

The ATPase activity of the isolated HlyB NBD, in contrast with those of purified HisP and MalK, but like that of the NBD of Mdl1p [9], is sensitive to vanadate and displays positive cooperativity with respect to ATP concentration, with a Hill coefficient of 1.7 ± 0.1 at 10–200 mM NaCl. A further indication of the unique features of the HlyB NBD is reflected by the so far novel characteristic of salt sensitivity of an isolated NBD, whereby loss of activity with increasing salt concentration is accompanied by

decreased binding of ATP. Intrinsic fluorescence analysis demonstrated that this was accompanied by conformational changes. Furthermore, fluorescence quenching experiments in the presence of KI (1 μ M–10 mM) and analysis of results by the Stern–Vollmer (eqn 3) method indicated that there was only a single molecular species of tryptophan at all salt concentrations (results not shown). However, the rate of quenching increased 4-fold at lower ionic strength. These data support the conclusion that the ABC domain adopts an ionic-strength-dependent conformation, favouring binding of ATP in low salt *in vitro* due to an increased flexibility of the protein.

In relation to this unique feature of HlyB, it is interesting to note two additional properties of the HlyB NBD reported previously [12]. Thus we have shown that the transport substrate, or allocrite, HlyA, interacts directly and specifically with the NBD of HlyB, apparently involving the C-terminal secretion signal of HlyA [40]. In addition, we have recently reported that the crystal structure of the HlyB NBD, in the absence of nucleotide, includes an unusual structural organization of the catalytic site, resulting in an unexpected interaction of the Walker A lysine and Walker B glutamate. This is incompatible with the binding of ATP [12]. We postulated therefore that this unusual architecture of the empty HlyB NBD in the crystal structure may reflect a molecular switch, capable of reversibly repressing the activity of the ABC domain *in vivo*, for example, as a function of the salt concentration effect seen here. Thus we suggest that the binding of the allocrite HlyA to the NBD would return the Walker A lysine to its functional position, despite the high cellular salt concentration.

In the present study, we have examined the properties of the purified ABC ATPase domain of HlyB separated from its membrane domain, with particular attention to the possible formation of dimers. Under a wide range of salt conditions and in the presence or absence of ATP, we could find no evidence of dimer formation by either analytical ultracentrifugation or gel filtration; in the latter case, with concentrations of the HlyB ABC domain up to 15 mg/ml. In contrast, in the complete absence of salt, ultracentrifugation analysis indicated the existence of a rapid equilibrium between monomers and dimers. Attempts to confirm this by gel filtration in the complete absence of salt was precluded because of the altered characteristics of the matrix under low-salt conditions. However, small amounts of dimers were apparently detected by SDS/PAGE in the presence of 8-azido-ATP. Such dimers were also detected by gel filtration even in the presence of 100 mM NaCl. This may indicate that such dimers are trapped at some point in the catalytic cycle, as in the case of the ‘sandwich’ dimers crystallized by Smith et al. [38].

Importantly, our conclusion that the ABC domain of HlyB can form at least transient, but rapidly dissociating, dimers, was supported further by the demonstration that *in vitro* ATPase activity showed positive co-operativity. This may, for example, indicate that binding of a molecule of ATP to one subunit of a transient dimer then increases the probability of ATP binding to the second subunit, or that two ATP molecules are required to form an active dimer [41].

This study emphasizes that, despite its overall high-level conservation, the HlyB NBD displays some unique properties. This, together with other reports indicating varying degrees of dimer stability by different ABC proteins [7–11], combined with some structural diversity of the ABC ATPases [12], leads to an important conclusion that these molecules are more varied than might have been expected. We propose that this reflects the obvious versatility of the many ABC ATPases in energizing the transport of such a wide variety of substrates, with the specificity of a given transporter conferred by the interaction between the NBD, the cognate membrane domain and the transport substrate.

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REFERENCES

- Holland, I. B. and Blight, M. A. (1999) ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. *J. Mol. Biol.* **293**, 381–399
- Linton, K. and Higgins, C. F. (1998) The *Escherichia coli* ATP-binding cassette (ABC) proteins. *Mol. Microbiol.* **28**, 5–13
- Young, J. and Holland, I. B. (1999) ABC transporters: bacterial exporters – revisited five years on. *Biochim. Biophys. Acta* **1461**, 177–200
- Holland, I. B., Benabdelhak, H., Young, J., Pimenta, A. L., Schmitt, L. and Blight, M. (2003) Bacterial ABC transporters involved in protein translocation. In *ABC Proteins: From Bacteria to Man* (Holland, I. B., Cole, S. P. C., Kuchler, K. and Higgins, C. F., eds.), pp. 209–241, Academic Press, London
- Kerppola, R. E., Shyamala, V. K., Klebba, P. and Ames, G. F.-L. (1991) The membrane-bound proteins of periplasmic permeases form a complex: identification of the histidine permease HisQMP complex. *J. Biol. Chem.* **266**, 9857–9865
- Davidson, A. L. and Sharma, S. (1997) Mutation of a single MalK subunit severely impairs maltose transport activity in *Escherichia coli*. *J. Bacteriol.* **179**, 5458–5464
- Kennedy, K. A. and Traxler, B. (1999) MalK forms a dimer independent of its assembly into the MalFGK2 ATP-binding cassette transporter of *Escherichia coli*. *J. Biol. Chem.* **274**, 6259–6264
- Nikaido, K. and Ames, G. F. (1999) One intact ATP-binding subunit is sufficient to support ATP hydrolysis and translocation in an ABC transporter, the histidine permease. *J. Biol. Chem.* **274**, 26727–26735
- Janas, E., Hofacker, M., Chen, M., Gompf, S., van der Does, C. and Tampé, R. (2003) The ATP hydrolysis cycle of the nucleotide-binding domain of the mitochondrial ATP-binding cassette transporter Mdl1p. *J. Biol. Chem.* **278**, 26862–26869
- Verdon, G., Albers, S. V., van Oosterwijk, N., Dijkstra, B. W., Driessen, A. J. and Thunnissen, A. M. (2003) Formation of the productive ATP-Mg²⁺-bound dimer of GlcV, an ABC-ATPase from *Sulfolobus solfataricus*. *J. Mol. Biol.* **334**, 255–267
- Horn, C., Bremer, E. and Schmitt, L. (2003) Nucleotide dependent monomer/dimer equilibrium of OpuAA, the nucleotide-binding protein of the osmotically regulated ABC transporter OpuA from *Bacillus subtilis*. *J. Mol. Biol.* **334**, 403–419
- Schmitt, L., Benabdelhak, H., Blight, M. A., Holland, I. B. and Milton, T. (2003) Crystal structure of the nucleotide binding domain of the ABC-transporter haemolysin B: identification of a variable region within ABC helical domains. *J. Mol. Biol.* **330**, 333–342
- Karpowich, N., Martsinkevich, O., Millen, L., Yuan, Y. R., Dai, P. L., MacVey, K., Thomas, P. J. and Hunt, J. F. (2001) Crystal structures of the MJ1267 ATP binding cassette reveal an induced-fit effect at the ATPase active site of an ABC transporter. *Structure* **9**, 571–586
- Yuan, Y. R., Blecker, S., Martsinkevich, O., Millen, L., Thomas, P. J. and Hunt, J. F. (2001) The crystal structure of the MJ0796 ATP-binding cassette: implications for the structural consequences of ATP hydrolysis in the active site of an ABC-transporter. *J. Biol. Chem.* **276**, 32313–32321
- Lewis, H. A., Buchanan, S. G., Burley, S. K., Connors, K., Dickey, M., Dorwart, M., Fowler, R., Gao, X., Guggino, W. B., Hendrickson, W. A. et al. (2004) Structure of nucleotide-binding domain 1 of the cystic fibrosis transmembrane conductance regulator. *EMBO J.* **23**, 282–293
- Guzman, L.-M., Belin, D., Carson, M. J. and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose pBAD promoter. *J. Bacteriol.* **177**, 4121–4130
- Mackman, N., Nicaud, J. M., Gray, L. and Holland, I. B. (1985) Genetical and functional organisation of the *Escherichia coli* haemolysin determinant 2001. *Mol. Gen. Genet.* **201**, 282–288
- Gonzalez-Romo, P., Sanchez-Neito, S. and Gavilanes-Ruiz, M. (1992) A modified colorimetric method for determination of orthophosphate in the presence of high ATP concentrations. *Anal. Biochem.* **200**, 235–238
- Zaitseva, J., Zhang, H., Binnie, R. A. and Hermanson, M. (1996) The proteins encoded by the rbs operon of *Escherichia coli*. II. Use of chimeric protein constructs to isolate and characterize RbsC. *Protein Sci.* **5**, 1100–1107
- Chan, K. W., Delfert, D. and Junger, K. D. (1986) A direct colorimetric assay for Ca²⁺-stimulated ATPase activity. *Anal. Biochem.* **157**, 375–380
- Senior, A. E., al-Shawi, M. K. and Urbatsch, I. L. (1998) ATPase activity of Chinese hamster P-glycoprotein. *Methods Enzymol.* **292**, 514–523
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- Blight, M. A., Menichi, B. and Holland, I. B. (1995) Evidence for post-transcriptional regulation of the synthesis of the *Escherichia coli* HlyB haemolysin translocator and production of polyclonal anti-HlyB antibody. *Mol. Gen. Genet.* **247**, 73–85
- Goodno, C. C. (1982) Myosin active site trapping with vanadate ion. *Methods Enzymol.* **85**, 116–123
- Morbach, S., Tebbe, S. and Schneider, E. (1993) The ATP-binding cassette (ABC) transporter for maltose/maltodextrins of *Salmonella typhimurium*: characterization of the ATPase activity associated with the purified MalK subunit. *J. Biol. Mol.* **268**, 18617–18621
- Liu, P. Q. and Ames, G. F. L. (1997) Characterization of the adenosine triphosphatase activity of the periplasmic histidine permease, a traffic ATPase (ABC transporter). *J. Biol. Chem.* **272**, 21883–21891
- Hunke, S., Mourez, M., Jehanno, M., Dassa, E. and Schneider, E. (2000) ATP modulates subunit-subunit interactions in an ATP-binding cassette transporter (MalFGK2) determined by site-directed chemical cross-linking. *J. Biol. Chem.* **275**, 15526–15534
- Urbatsch, I. L., Sankaran, B., Bhagat, S. and Senior, A. E. (1995) Both P-glycoprotein nucleotide-binding sites are catalytically active. *J. Biol. Chem.* **270**, 26956–26961
- Locher, K. P., Lee, A. T. and Rees, D. C. (2002) The *E. coli* BtuCD structure: a framework for ABC transporter architecture and mechanism. *Science* **296**, 1091–1098
- Chang, G. and Roth, C. B. (2001) Structure of MsbA from *E. coli*: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. *Science* **293**, 1793–1800
- Chang, G. (2003) Structure of MsbA from *Vibrio cholera*: a multidrug resistance ABC transporter homolog in a closed conformation. *J. Mol. Biol.* **330**, 419–430
- Kerr, I. D. (2002) Structure and association of ATP-binding cassette transporter nucleotide binding domains. *Biochim. Biophys. Acta* **1561**, 47–64
- Dayan, G., Baubichon-Cortay, H., Jault, J. M., Cortay, J. C., Deléage, G. and Di Pietro, A. (1996) Recombinant N-terminal nucleotide-binding domain from mouse P-glycoprotein. *J. Biol. Chem.* **271**, 11652–11658
- Ko, Y. H. and Pedersen, P. L. (1995) The first nucleotide binding fold of the cystic fibrosis transmembrane conductance regulator can function as an active ATPase. *J. Biol. Chem.* **270**, 22093–22096
- Qu, B. H., Strickland, E. H. and Thomas, P. J. (1997) Localization and suppression of a kinetic defect in cystic fibrosis transmembrane conductance regulator folding. *J. Biol. Chem.* **272**, 15739–15744
- Duffieux, F., Annereau, J. P., Boucher, J., Miclet, E., Pamard, O., Schneider, M., Stoven, V. and Lallemand, J. Y. (2000) Nucleotide-binding domain 1 of cystic fibrosis transmembrane conductance regulator production of a suitable protein for structural studies. *Eur. J. Biochem.* **267**, 5306–5312
- Müller, K., Ebersperger, C. and Tampé, R. (1994) Nucleotide binding to the hydrophilic C-terminal domain of the transporter associated with antigen processing (TAP). *J. Biol. Chem.* **269**, 14032–14037
- Smith, P. C., Karpowich, N., Millen, L., Moody, J. E., Rosen, J., Thomas, P. J. and Hunt, J. F. (2002) ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimer. *Mol. Cell* **10**, 139–149
- Verdon, G., Albers, S. V., Dijkstra, B. W., Driessen, A. J. and Thunnissen, A. M. (2002) Purification, crystallization and preliminary X-ray diffraction analysis of an archaeal ABC-ATPase. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **58**, 362–365
- Benabdelhak, H., Kiontke, S., Horn, C., Blight, M. A., Holland, I. B. and Schmitt, L. (2003) A specific interaction between the NBD of the ABC-transporter, HlyB and a C terminal fragment of its transport substrate, haemolysin A. *J. Mol. Biol.* **327**, 1169–1179
- Chen, J., Lu, G., Lin, J., Davidson, A. L. and Quiocho, F. A. (2003) A tweezers-like motion of the ATP-binding cassette dimer in an ABC transport cycle. *Mol. Cell* **12**, 651–661