Acta Crystallographica Section D
**Biological Crystallography**
ISSN 0907-4449

# The role of CAPS buffer in expanding the crystallization space of the nucleotide-binding domain of the ABC transporter haemolysin B from *Escherichia coli*

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Received 29 January 2004
Accepted 29 March 2004

Nucleotide-binding domains (NBDs), which are roughly 27 kDa in size, are conserved components of the large family of ABC (ATP-binding cassette) transporters, which includes importers and exporters. NBDs, or ABC-ATPases, supply energy for the translocation of a vast range of substrates across biological membranes. Despite their hydrophilic sequence, many NBDs readily associate in some way with membranes but demonstrate extreme instability in solution upon separation from the complete transporter. Conditions that stabilized the purified ABC domain of the *Escherichia coli* haemolysin A (HlyA) transporter were developed. This allowed the screening of unlimited crystallization conditions in the presence of different substrates, the performance of reproducible functional assays and the protection of 50 mg ml$^{-1}$ protein from precipitation on ice for months. As a result, it became possible to obtain crystals of HlyB-NBD in the presence of ADP and ATP that were suitable for X-ray analysis. Although the focus of these investigations was placed on HlyB-NBD, the strategy described here can be directly transferred to other proteins that display instability in solution.

## 1. Abbreviations

ABC, ATP-binding cassette; ADA, *N*-2-acetamidoiminodiacetic acid; ADP, adenosine-5′-diphosphate; AMP, adenosine-5′-monophosphate; AMP-PNP, adenosine-5′-($\beta$,$\gamma$-imido)-triphosphate; ATP, adenosine-5′-triphosphate; ATP$\gamma$S, adenosine-5′-($\gamma$-thio)triphosphate; 2′-Br-ATP, 2′-bromo-ATP; 8-Br-ATP, 8-bromo-ATP; BSA, bovine serum albumin; CHES, 2-cyclohexylaminoethanesulfonic acid; CAPS, 3-(cyclohexylamino)-propanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; DDM, dodecyl-$\beta$-D-maltoside; DTT, dithiothreitol; Hly, haemolysin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; LDAO, lauryldimethylamine-*N*-oxide; MEGA-10, decanoyl-*N*-methylglucamide; $\beta$-ME, $\beta$-mercaptoethanol; MWCO, molecular-weight cutoff; NBD, nucleotide-binding domain; OG, octyl-$\beta$-D-glucopyranoside; SEC, size-exclusion chromatography; TMD, transmembrane domain; TNP, 2′(3′)-*O*-trinitrophenyl; Tris–HCl, tris(hydroxymethyl)aminomethane; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

## 2. Introduction

Secretion of the 107 kDa protein toxin HlyA in *Escherichia coli* is mediated by a dedicated transporter (Holland *et al.*, 2003). This Sec-independent type I protein-translocation

machinery is composed of the outer-membrane protein TolC, the membrane-fusion protein HlyD and HlyB, a member of the ABC-transporter family.

ABC transporters (Higgins, 1992) are found in all living organisms. They play an important role in a variety of physiological processes and have substantial medical relevance. In bacteria, the members of the family are associated with virulence (Blight *et al.*, 1994), antibiotic resistance (Mendez & Salas, 2001) and nutrient uptake (Ames *et al.*, 2001); in humans, they are linked to many disorders, such as cystic fibrosis (Csanady & Gadsby, 1999), drug resistance in cancer cells (Gottesman & Pastan, 1993), suppression of the immune response (Schmitt & Tampe, 2000) and inherited retinal diseases (Sun & Nathans, 2001). ABC transporters are composed of at least four modules: two soluble nucleotide-binding domains/subunits (NBDs), or ABC-ATPases, and two hydrophobic domains/subunits (Holland & Blight, 1999). These modules can be arranged in various ways in different transporters to allow coupling of the ATPase activity of the NBDs to the translocation of the substrate (allocrite) across the membrane. HlyB, one of the constituents of the haemolysin A secretion machinery in *E. coli*, is a so-called 'half-size' ABC transporter. Composed of 707 amino-acid residues, HlyB consists of a fusion of an N-terminal transmembrane region and a C-terminal ATP-binding domain that presumably forms a dimer to accomplish transport activity. Transmembrane domains are specific to each type of ABC transporter and share little sequence similarity. In contrast, the nucleotide-binding domains of different functionally unrelated transporters demonstrate significant sequence conservation in several key regions, such as the Walker A, the Walker B and Signature motifs, a glutamine residue in the Q-loop, a histidine residue in the His-loop and an aspartate residue in the D-loop (Schmitt & Tampé, 2002).

Structural data reported recently for two full-length ABC transporters have significantly contributed to our understanding of the general architecture and possible subunit interactions in the ABC family of transporters (Chang, 2003; Chang & Roth, 2001; Locher *et al.*, 2002). However, the structures of the exporter MsbA, a lipid A flippase, and BtuCD, the vitamin $B_{12}$ importer, obtained in the absence of nucleotides and/or allocrites leaves many open questions regarding the mechanism of ATP hydrolysis and its coupling to transmembrane transport of the allocrite. Isolated nucleotide-binding domains of ABC proteins have proved to be effective in determining the detailed crystal structure of the ABC-ATPases (Chen *et al.*, 2003; Diederichs *et al.*, 2000; Gaudet & Wiley, 2001; Hung *et al.*, 1998; Karpowich *et al.*, 2001; Schmitt *et al.*, 2003; Smith *et al.*, 2002; Verdon, Albers, Dijkstra *et al.*, 2003; Verdon, Albers, van Oosterwijk *et al.*, 2003; Yuan *et al.*, 2001). Recently published structures of certain NBDs with bound ATP from ABC proteins involved in uptake processes have demonstrated the nature of the dimerization of two ATPase subunits (Chen *et al.*, 2003; Smith *et al.*, 2002) and revealed corresponding conformational changes upon ATP binding. In addition, some NBDs have been crystallized with ADP in the binding pocket (Gaudet & Wiley, 2001; Karpowich *et al.*, 2001; Verdon, Albers, Dijkstra *et al.*, 2003; Yuan *et al.*, 2001). However, further information on the various transitional functional states of ABC is required in order to visualize the complete hydrolytic cycle of the ATPase.

Despite the above successes, NBDs of other ABC transporters have proved to be extremely difficult to handle. Although large quantities (at least a few milligrams per litre of culture) of soluble NBDs can be overexpressed and purified, in many cases a problem emerges immediately after purification. Thus, many purified ABC domains tend to precipitate in solution, especially at the concentrations required for structural studies (Kerr *et al.*, 2003; Nikaido *et al.*, 1997; Smith *et al.*, 2002). This instability makes crystallization as well as functional assays very challenging. A possible explanation for the instability of NBD in solution might be the disruption of native protein–protein interactions upon separation of the components from a fully assembled transmembrane complex. Employment of certain additives seems to improve the solubility of nucleotide-binding components after purification; in the case of the ABC constituent of the histidine permease (HisP), glycerol and ATP were added to the purified protein to prevent precipitation (Nikaido *et al.*, 1997). The ABC-ATPase subunit of the open reading frame MJ0796 from *Methanococcus jannaschii* formed an amorphous precipitate in the absence of $Mg^{2+}$-ADP (Yuan *et al.*, 2001). However, it was found that it was possible to resolubilize precipitates of a mutant form of MJ0796 (E171Q) with 1 *M* arginine–HCl (Smith *et al.*, 2002). To increase the solubility of MalK, the ABC component of the *Salmonella typhimurium* maltose transporter, the detergents octyl-$\beta$-D-glucopyranoside (OG) and dodecyl-$\beta$-D-maltoside (DDM) were successfully employed (Schmees *et al.*, 1999). There are, however, disadvantages to these procedures. The presence of ATP or ADP in the protein solution could interfere with functional assays and hamper crystallization when looking for structures containing other nucleotides or the nucleotide-free state. Detergents and a high concentration of arginine could also cause complications in the crystallization screening and biochemical analysis of proteins. The inherently unstable nature of the isolated NBD-HlyB necessitated that only freshly purified protein could be used for crystallization and its concentration had to be kept below a certain level. A similar effect has also been observed for other purified ABC domains (Janas *et al.*, 2003; Kerr *et al.*, 2003). Thus, the problem of protein solubility can set serious limits for the functional and structural analysis of isolated ABC-ATPases.

In the current paper, we describe a successful search for conditions supporting highly concentrated (50 mg ml$^{-1}$ and higher) homogenous and substrate-free HlyB-NBD in solution. This procedure produced protein suitable for long-term storage on ice and reproducible functional assays and provided a starting point for crystallization of HlyB-NBD in various states of the hydrolytic cycle. A simple screening test was utilized to search for the optimal conditions that stabilize the purified NBD in solution. This procedure should also be readily adaptable for other precipitation-prone proteins, providing an important prerequisite for structural studies.

## 3. Materials and methods

### 3.1. HlyB-NBD production and purification

HlyB-NBD (residues 467–707) was produced and purified as described previously (Benabdelhak *et al.*, 2004; Schmitt *et al.*, 2003) with some adjustments in order to improve the protein yield and stability. Since every individual step in the large-scale overexpression and purification can be essential for the quantity and quality of the final product (Kerr *et al.*, 2003; Wang *et al.*, 2003), we consider it important to describe a detailed protocol for the isolation of the HlyB-NBD for structural studies.

DH5$\alpha$ cells, freshly transformed with pPSG122, were grown overnight at 310 K in the presence of ampicillin (100 mg $l^{-1}$). The next morning, new LB media with ampicillin was inoculated 1:100 with an overnight culture and grown at 310 K to an $OD_{600}$ of 0.8. The culture was cooled to 293 K. Overexpression of protein was induced at an $OD_{600}$ of 1.0 by the addition of 0.002% L-arabinose. Cells were grown at 293 K for 3 h and then collected by centrifugation at 277 K for 25 min at 4500*g*. The following purification steps were performed at 277 K. Cells were resuspended in buffer *A* (25 m*M* sodium phosphate pH 8.0, 20% glycerol, 100 m*M* KCl, 10 m*M* imidazole) and lysed by three passes through a French pressure cell at 110 MPa. The lysed cell suspension was centrifuged at 125 000*g* for 1.5 h to pellet unbroken cells, cell debris, protein inclusion bodies and membranes. The clarified supernatant was applied onto a $Zn^{2+}$-charged chelating HiTrap column (Amersham Pharmacia, Freiburg, Germany) that had been equilibrated with buffer *A*. The column was further washed with approximately 20 column volumes of the same buffer. The protein was eluted with a linear gradient of imidazole (10–300 m*M*) in buffer *A* (30 column volumes). Fractions containing HlyB-NBD were combined and concentrated employing an ultrafiltration unit (10 000 MWCO; Ultra-15 centrifugal filter unit, Amicon, Eschborn, Germany). A Hi-Load 16/60 Superdex 200 column (Amersham Pharmacia, Freiburg, Germany) was equilibrated with buffer *B* (10 m*M* CAPS–NaOH pH 10.4, 20% glycerol). After metal-chelating purification, the protein was concentrated by ultrafiltration and loaded onto the gel-filtration column. A major peak was eluted with buffer *B* at about 80 ml, which corresponded to the monomeric size of the protein. Fractions containing homogenous HlyB-NBD were combined and concentrated as above. In some cases, visible protein precipitation started at this point. Adjustment of the CAPS concentration and the pH to 100 m*M* and 10.4, respectively, was necessary to stabilize the protein in solution. A completely clarified sample was further concentrated to increase the protein concentration to 20–50 mg $ml^{-1}$. This HlyB-NBD solution was directly used for crystallization trials or exchanged into buffer with lower CAPS content immediately prior to crystallization (see §4). The typical yield was about 10 mg of purified and homogeneous HlyB-NBD from 1 l of bacterial cell culture.

The quality of protein was routinely estimated by SDS–PAGE (Laemmli, 1970). In order to visualize S–S dimer formation, if any, and for the subsequent separation of the monomeric and dimeric forms of HlyB-NBD, aliquots of fractions after gel-filtration were not heated in SDS–PAGE sample buffer and neither $\beta$-mercaptoethanol ($\beta$-ME) nor dithiothreitol (DTT) was added in this step (for SDS–PAGE see also §3.3). Protein concentration was determined by ultraviolet absorbance at 280 nm using a theoretical molar extinction coefficient of 15 400 $M^{-1}$ $cm^{-1}$ and by the BCA protein assay (Wiechelman *et al.*, 1988). The same procedure was utilized for purification of the HlyB-NBD H662A mutant modified by site-directed mutagenesis, which was overexpressed from pPSG 122 (Benabdelhak *et al.*, 2004).

### 3.2. Drop assay for protein stability

HlyB-NBD was purified by $Zn^{2+}$-chelating chromatography and concentrated to 1–2 mg $ml^{-1}$ in the elution buffer (20 m*M* Tris–HCl pH 8.0, 100 m*M* KCl, 150 m*M* imidazole; Kránitz *et al.*, 2002). 1–2 µl of freshly purified protein with trace amounts of precipitation were mixed with 1–2 µl of a test solution on a glass microscope slide at room temperature. The immediate effect of the added component was inspected under a light microscope and monitored for several minutes. Changes in the turbidity of the protein solution were qualitatively assessed.

### 3.3. Stability screening over extended time

To check the long-term stability of HlyB-NBD, slight modifications of the droplet assay were performed in microtitre plates and by microdialysis. Promising solutions selected by the droplet assay were mixed in a 1:1 ratio with the protein (50–100 µl at 1 mg $ml^{-1}$) in the wells of microtitre plates and incubated at various temperatures for a few days. Changes in the turbidity of the tested samples were monitored by absorbance at 600 nm.

Alternatively, protein stability was tested by microdialysis. 50–100 µl of 1 mg $ml^{-1}$ HlyB-NBD was dialyzed against the chosen buffer at 277 K overnight. The degree of precipitation in the samples was qualitatively assessed and compared with the control (the initial protein dialyzed against 20 m*M* Tris–HCl pH 8.0, 100 m*M* KCl, 150 m*M* imidazole).

The final protein solutions were analysed by SDS–PAGE (Laemmli, 1970). The samples were diluted with SDS sample buffer and loaded onto a 12.5% SDS polyacrylamide gel. The same samples were compared after heating at 373 K for 5 min with 200 m*M* $\beta$-ME and without previous treatment. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250 (Andrews, 1981).

### 3.4. ATPase activity

ATPase assays were performed as described previously (Zaitseva *et al.*, 1996) with slight modifications. ATP hydrolysis was measured at room temperature in 100 m*M* HEPES pH 7.0 (or 100 m*M* CAPS pH 10.4), 20% glycerol, 1 m*M* ATP, 10 m*M* $MgCl_2$ and the protein concentration indicated. The reaction was initiated by the addition of $MgCl_2$. 50 µl aliquots were taken at appropriate time points and the reaction was stopped with 350 µl 40 m*M* $H_2SO_4$. The concentration of released inorganic phosphate was determined by measuring the

**Table 1**
Buffers used in the droplet assay.

| No. | Buffer composition | pH value |
|---|---|---|
| 1 | 200 m*M* sodium acetate | 4.65 |
| 2 | 200 m*M* sodium citrate | 5.1 |
| 3 | 200 m*M* sodium malonate | 5.6 |
| 4 | 200 m*M* ADA | 6.5 |
| 5 | 200 m*M* ADA | 7.0 |
| 6 | 200 m*M* HEPES | 7.2 |
| 7 | 50 m*M* sodium phosphate, 100 m*M* KCl | 8.0 |
| 8 | 200 m*M* Tris–HCl | 8.0 |
| 9 | 10 m*M* Tris–HCl, 100 m*M* KCl, 150 m*M* imidazole | 8.0 |
| 10 | 100 m*M* Tris–HCl, 2 *M* ammonium sulfate | 8.0 |
| 11 | 200 m*M* Tricine–HCl | 8.1 |
| 12 | 200 m*M* CHES | 9.3 |
| 13 | 200 m*M* CAPS | 10.4 |

absorbance at 620 nm (Baykov *et al.*, 1988) in microtitre plates 10–20 min after the addition of dye. Calibration of free phosphate was performed with $Na_2HPO_4$ in the working assay solution. All data were corrected for autohydrolysis of ATP, which was insignificant even after 2 h at 1 m*M* ATP under identical assay conditions in the absence of protein.

### 3.5. Analytical gel-filtration

A 20 mg ml$^{-1}$ stock HlyB-NBD solution in 100 m*M* CAPS pH 10.4, 20% glycerol was diluted to 1.5 mg ml$^{-1}$ (54 µ*M*) in the same buffer. After 1 h incubation on ice, a 50 µl sample was subjected to gel-filtration chromatography in order to assess the oligomeric state of the protein. The sample was injected onto a Superdex 75 PC 3.2/30 gel-filtration column (Amersham Pharmacia, Freiburg, Germany) that had been equilibrated with the storage buffer at 281 K and operated at 75 µl min$^{-1}$. Protein elution from the column was monitored by absorbance at 280 nm. 75 µl fractions were collected and the protein content was estimated by SDS–PAGE. Elution volumes were compared with those of the molecular-weight standards BSA (66 kDa), carbonic anhydrase (29 kDa) and hen egg-white lysozyme (14.6 kDa).

### 3.6. Crystallization

Initial screening was performed with the Crystal Screen 1 and 2 solutions (Hampton Research, Laguna Niguel, USA) at 277 K. HlyB-NBD was crystallized by hanging-drop vapour diffusion at 277 K. Drops were set up with a 1:1 precipitant: protein ratio. 5–50 mg ml$^{-1}$ protein solution in storage buffer (100 m*M* CAPS–Na buffer pH 10.4, 20% glycerol) was used for crystallization. If necessary, the protein in the storage buffer was diluted with 20–30% glycerol and further concentrated on an ultrafiltration device (10 000 MWCO; Ultra-15 centrifugal filter unit, Amicon, Eschborn, Germany).

### 3.7. Data collection and processing

Initial experiments were performed in-house using a Rigaku X-ray generator with Cu $K\alpha$ radiation and an R-AXIS IV image plate at 100 K. Data sets were collected either at beamline BW-6 (DESY, Hamburg, Germany) or at the Advanced Swiss Light Source (Paul Scherer Institute, Villigen, Switzerland) equipped with a MAR CCD. All data sets were processed with *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1997) or processed and scaled with *d*TREK* (Pflugrath, 1999).

## 4. Results and discussion

### 4.1. Dependence of protein stability on pH

Previously, purification and crystallization of the HlyB-NBD protein had been performed in either Tris or phosphate buffer (Kránitz *et al.*, 2002). However, protein in concentrations above 1 mg ml$^{-1}$ tended to precipitate in 10 m*M* Tris–HCl pH 8.0, 100 m*M* KCl (Tris buffer) in a few hours, forming tiny white flakes. In an attempt to optimize the purification scheme, Tris buffer was replaced by 10 m*M* sodium phosphate pH 8.0, 100 m*M* KCl (phosphate buffer) for HlyB-NBD isolation (Benabdelhak *et al.*, 2003; Schmitt *et al.*, 2003). The use of phosphate buffer allowed the concentration of soluble HlyB-NBD to 10 mg ml$^{-1}$. Even though the protein did not precipitate immediately in phosphate buffer, only freshly purified protein was used for crystallization trials because this gave more reliable results. Upon 24–48 h of storage at 277 K, HlyB-NBD eventually precipitated from the phosphate-containing solution at protein concentrations of about 10 mg ml$^{-1}$, with the level of precipitation directly related to the protein concentration.

To improve the solubility of the isolated HlyB-NBD in the absence of free inorganic phosphate, the pH-dependence of protein stability was evaluated. A simple screening test was employed to assess the immediate effect of various pH values and buffers on protein stability.

A slightly turbid solution of about 1 mg ml$^{-1}$ HlyB-NBD in Tris buffer was subjected to the droplet assay (see §3) against the buffers listed in Table 1 with pH range 4.65–10.4. The buffer with the lowest pH, 200 m*M* acetate pH 4.65, seemed to induce precipitation compared with the control (10 m*M* Tris–HCl pH 8.0, 100 m*M* KCl, 150 m*M* imidazole). Two buffers appeared to reverse protein aggregation: phosphate buffer (50 m*M* sodium phosphate pH 8.0, 100 m*M* KCl) and the buffer with the highest tested pH, 200 m*M* CAPS pH 10.4. Addition of either buffer visibly clarified a turbid protein solution. All the other buffers seemed to have the same effect as Tris buffer containing 150 m*M* imidazole, causing no significant changes in the amount of precipitated protein. The phosphate buffer was not evaluated further, as this buffer had been used in the structure determination of the nucleotide-free state of the HlyB-NBD (Schmitt *et al.*, 2003). Despite intensive efforts, no condition could be established under which crystals appeared in the presence of nucleotides using the phosphate-buffer system (data not shown). Therefore, all subsequent experiments were continued with the CAPS buffer as the preferred alternative to the phosphate-buffer system.

### 4.2. Protein stability in different buffers

To verify the effect of CAPS on protein stability, freshly purified HlyB-NBD in phosphate buffer supplemented with

150 m*M* imidazole was subjected to a size-exclusion chromatography step in the presence of CAPS pH 10.4. In 25 m*M* CAPS pH 10.4, 100 m*M* KCl, it was feasible to attain 25 mg ml$^{-1}$ protein and to retain solubility. However, the next day the concentrated protein solution had already formed a precipitate. For further screening of conditions ensuring HlyB-NBD stability, a number of supplements were tested at various concentrations by the droplet assay, this time starting from a turbid solution of 20–25 mg ml$^{-1}$ protein in 25 m*M* CAPS buffer pH 10.4, 100 m*M* KCl. These supplements included $\beta$-ME, DTT, glycerol, ATP, ADP and EDTA, various salts such as potassium chloride, sodium chloride, magnesium chloride, lithium sulfate, ammonium sulfate, sodium acetate and sodium citrate, and detergents such as deoxycholic acid, taurocholic acid, CHAPS, OG, LDAO, MEGA-10 and DDM. The addition of glycerol or ATP resulted in partial clarification of the turbid protein solutions in 25 m*M* CAPS pH 10.4, 100 m*M* KCl. However, the most significant improvement in protein solubility was detected upon the introduction of higher CAPS concentrations as an individual component. Various combinations of the above ingredients produced an even greater effect on the solubility of HlyB-NBD. For example, simultaneous addition of ATP and glycerol dissolved most of the precipitated protein, albeit in a not very reproducible manner. The strongest effect of the supplements on HlyB-NBD solubility was observed in the presence of CAPS and glycerol. Cloudy protein suspensions visibly clarified upon the addition of these components, resulting in an absolutely transparent appearance to the solution within a few minutes. Salts of divalent metals seemed to induce precipitation. All other tested components had no noticeable effect.

The stability of the HlyB-NBD was also tested by microtitre-plate assay and dialysis (see §3) against buffers 6, 8, 9, 11, 12 and 13 (see Table 1) with and without 20% glycerol for extended times at 277 K. Microdialysis was performed against tenfold-diluted tested buffers (6, 8, 11, 12, 13) and compared with Tris buffer containing 150 m*M* imidazole. Again, a CAPS buffer with glycerol exhibited the best protein solubility. CAPS alone was not that effective and glycerol proved to be the stabilizing ingredient in all cases, partially protecting the protein from precipitation. After the dialysis or microtitre-plate assay, samples were analyzed by SDS–PAGE. No degradation products were observed in the tested protein solutions by SDS–PAGE.

We have previously observed that a proportion of the HlyB-NBD apparently runs as a dimer during SDS–PAGE when sample boiling and addition of a reducing agent are omitted (Benabdelhak, 2002). In this study, therefore, duplicate samples of the NBD were examined to compare total protein and the dimer:monomer ratio. Thus, before loading for SDS–PAGE analysis, the protein solution under test was either boiled following addition of $\beta$-ME and SDS sample buffer or directly mixed with the latter without prior boiling and omitting $\beta$-ME. SDS–PAGE analysis demonstrated that a significant amount of protein from the turbid samples migrated at the relative size of the HlyB-NBD dimer. The molecular weight of these 56 kDa bands was reduced to that of monomeric protein in the SDS–PAGE gel in the presence of $\beta$-ME. Apparently, the formation of intermolecular disulfide bridges through the single cysteine (Cys652) of HlyB-NBD monomers serves as an indication of protein denaturation in solution. A direct correlation between the amount of S–S dimers detected by SDS–PAGE and the level of protein precipitation was observed in the assayed samples. The lowest ratio of dimer to monomer was observed in CAPS–glycerol samples.

Based on the results of all the screening procedures, 10 m*M* CAPS pH 10.4, 20% glycerol was finally chosen for the last step of HlyB-NBD purification, size-exclusion chromatography (see §3), as the most efficient buffer for stabilization. For long-term storage of concentrated protein solutions 100 m*M* CAPS pH 10.4, 20% glycerol was used. Thus, no precipitation or aggregation was observed in a 20–50 mg ml$^{-1}$ protein solution in this buffer stored for 2–4 months at 277 K. Furthermore, when maintained in CAPS–glycerol the ABC domain of HlyB was found to be suitable for crystallization for at least a month after purification.

### 4.3. Disulfide dimers as a monitor for protein denaturation over time

Freshly purified protein in 100 m*M* CAPS pH 10.4, 20% glycerol usually demonstrated more than 99% monomeric protein content when analyzed by SDS–PAGE omitting the treatment with a reducing agent. However, the percentage of the disulfide HlyB-NBD dimers increased progressively during storage of protein in the buffer on ice, even though no visible precipitation was observed (Fig. 1). After 18 d the amount of dimer reached 5–20% of the total protein and 20–50% of protein was converted to the S–S dimeric species in two months, as estimated by either SDS–PAGE or SEC (data not shown). The HlyB-NBD eventually precipitated upon 3–6 months storage at 277 K, forming white fluffy flakes. The addition of reducing agents such as $\beta$-ME and DTT protected

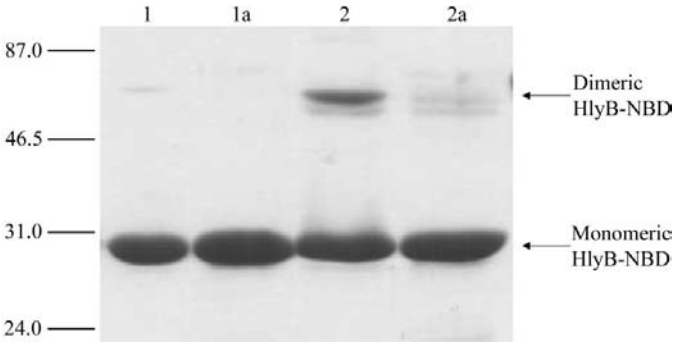


**Figure 1**
SDS–PAGE analysis of the purified HlyB-NBD stored at 277 K in 100 m*M* CAPS pH 10.4, 20% glycerol. Approximately 2.5 µg protein was subjected to electrophoresis as described in §3. Lane 1, freshly purified protein without boiling and treatment with $\beta$-ME. Lane 1a, as lane 1 but boiled and treated with $\beta$-ME prior to analysis. Lane 2, two-and-a-half-month-old protein without boiling and treatment with $\beta$-ME. Lane 2a, as lane 2 but boiled and treated with $\beta$-ME prior to analysis. The molecular weights (in kDa) of the marker proteins are indicated to the left. Arrows indicate the monomeric and dimeric forms of HlyB-NBD.

the protein from disulfide bridging of monomers, but did not appear to extend the storage life of the isolated HlyB-NBD.

### 4.4. The ATPase activity of the NBD is reversibly inactivated in the CAPS buffer

In order to evaluate the oligomeric state of the HlyB-NBD in storage buffer, one-week-old protein was subjected to gel filtration on a Superdex 75 column in 100 m*M* CAPS pH 10.4 and 20% glycerol. The protein appeared to run according to its monomeric size (Fig. 2). The injected sample was eluted as a relatively sharp peak coinciding with a 29 kDa protein, carbonic anhydrase, which was run in the same buffer for column calibration. The slight asymmetry of the peak detectable in Fig. 2 indicates the presence of a higher molecular weight species in the sample. This is likely to be attributable to disulfide protein dimers, since small amounts of dimers can be detected in the one-week-old sample by SDS–PAGE analysis.

Unexpectedly, HlyB-NBD was found to be completely inactivated when ATPase activity was measured in CAPS storage buffer (Fig. 3). However, activity was fully restored by protein dilution into a conventional assay buffer. Fig. 3 shows the time-dependent ATPase activity of HlyB-NBD in 100 m*M* Na HEPES pH 7.0, 20% glycerol following an approximately 1000-fold dilution from the storage buffer 100 m*M* CAPS pH 10.4, 20% glycerol. Thus, exposure of HlyB-NBD to high pH (for example CAPS pH 10.4) inactivated the protein only temporarily, shifting the ABC domain to a 'latent mode' and protecting it from denaturation. The significantly improved stability of HlyB-NBD in the storage buffer made it possible to perform highly reproducible ATPase assays up to two weeks after protein purification. After three weeks, the ATPase activity still corresponded to 90% of that of freshly purified protein when tested by dilution in 100 m*M* Na HEPES pH 7.0, 20% glycerol assay buffer (data not shown).

As a possible explanation for the effect of CAPS pH 10.4 on HlyB-NBD stability and activity, we propose that this is a consequence of structural modifications of the protein accompanying the change in pH. For example, in the case of retinol-binding protein, pH-induced structural changes were reported, resulting in the rearrangement of salt bridges, hydrogen bonds, the network of solvent molecules, alteration of charge distribution on the protein surface, changes in flexibility in some regions of the protein and in the volume of the internal substrate-binding cavity (Calderone *et al.*, 2003). Similar structural alterations might take place in the case of HlyB-NBD upon switching to high pH. Elevation of pH above the theoretical isoelectric point (pI = 8.9, as calculated with ExPASy; http://ca.expasy.org/tools/pi_tool.html) of the HlyB-NBD could strengthen close-range electrostatic interactions and introduce additional hydrogen bonds and salt bridges into the protein. On the one hand, the predicted modifications could potentially contribute to the conformational stability of the protein, making the whole molecular structure more rigid. On the other hand, these structural rearrangements could limit protein flexibility, modify van der Waals surfaces and the charge distribution, resulting in a lowered affinity for ATP and/or an inability to cleave ATP. The aforementioned effect of CAPS buffer could also be explained by an interaction between CAPS and the protein at one or more potential low-affinity binding sites. Surprisingly, the chemically highly related CHES with buffering capacity in a somewhat lower pH range ($pK_a$ = 9.3) failed to improve HlyB-NBD solubility. This makes us believe that the high pH, not protein–CAPS interactions, has a major impact on protein stability. However, we cannot yet rule out the significance of the chemical structure and the ability to maintain constant pH in more basic regions, a combination which makes CAPS buffer unique for our studies.

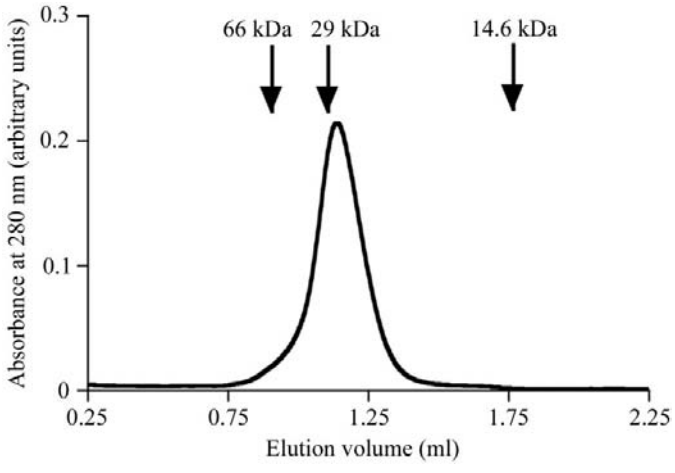


**Figure 2**
Analytical size-exclusion chromatography of one-week-old HlyB-NBD prepared in 100 m*M* CAPS pH 10.4, 20% glycerol on a Superdex 75 PC 3.2/30 column. Protein was eluted according to its monomeric size, 28 kDa. The column was calibrated with standard proteins in the same buffer (for further details see §3).

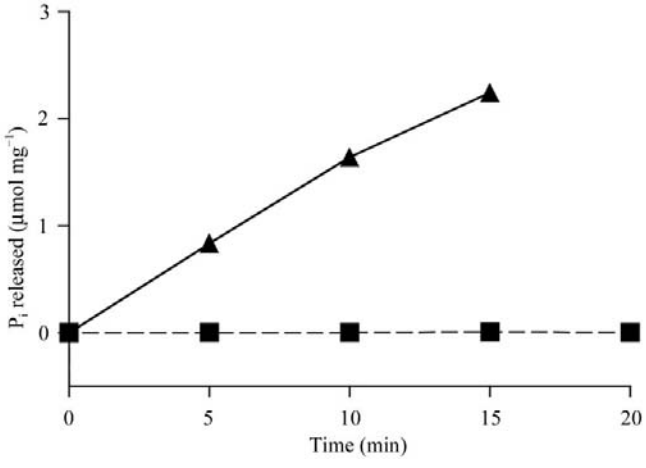


**Figure 3**
Time-dependent ATPase activity of purified HlyB-NBD. Protein from the storage buffer was diluted for ATPase assay in 100 m*M* CAPS pH 10.4 and 20% glycerol (final protein concentration 14.4 μ*M*, squares) or assay buffer, 100 m*M* HEPES pH 7.0, 20% glycerol (final protein concentration 1.1 μ*M*, triangles). Data points are the average of three independent experiments and were corrected for ATP autohydrolysis.

### 4.5. Crystallization of HlyB-NBD and its H662A mutant

The use of the nucleotide-free HlyB-NBD maintained in the 'sleeping' CAPS storage buffer permitted the screening of crystallization conditions with different substrates: ATP, ADP, AMP-PNP, TNP-ATP, TNP-AMP, ATP$\gamma$S, 2′-Br-ATP and 8-Br-ATP. Since 100 m*M* CAPS in the storage buffer can be a potential problem for crystallization, an opportunity to decrease the CAPS concentration would be highly desirable for the starting protein solution for crystallization. If necessary, the HlyB-NBD stabilization buffer was therefore exchanged for 1–10 m*M* CAPS pH 10.4, 20–30% glycerol by simple dilution into 20–30% glycerol solution and then re-concentrated by ultrafiltration. HlyB-NBD was crystallized in hanging drops in the presence of ADP or ATP containing 1 µl protein solution (25 mg ml$^{-1}$ in 70 m*M* CAPS pH 10.4, 30% glycerol, 10 m*M* ATP or 10 m*M* ADP) and 1 µl well solution (100 m*M* Tris pH 8.0, 10% PEG 6000, 5% MPD). Crystal plates fused together usually appeared the next day and continued to grow to full size over 7–10 d (Fig. 4*a*). The largest crystals obtained were 450 × 350 × 30 µm. Crystals were directly flash-frozen in liquid nitrogen and diffracted to 1.6 Å at the Advanced Swiss Light Source (Villigen, Switzerland) (Fig. 4*b* and Table 2). Identically shaped crystals were also obtained from solutions containing ATP/$Mg^{2+}$ and ADP/$Mg^{2+}$. No crystals appeared from solutions containing ATP/EDTA, ATP$\gamma$S or AMP-PNP with and without $Mg^{2+}$.

In an attempt to crystallize HlyB-NBD in the ATP-bound state, cubic shaped crystals were produced by mixing 1 µl protein solution (40 mg ml$^{-1}$ in 8 m*M* CAPS pH 10.4, 30% glycerol, 10 m*M* ATP, 0.8 m*M* EDTA) and 1 µl well solution (100 m*M* citrate pH 5.6, 30% PEG 4000, 0.2 *M* ammonium sulfate). However, the shape of the crystals and growth conditions closely resembled those of the previously reported nucleotide-free state of HlyB-NBD (Schmitt *et al.*, 2003). Alternatively, long rod-shaped crystals were grown from the same protein solution containing 2 *M* sodium malonate pH 6.0–6.2 as precipitant (McPherson, 2001). However, this type of crystal was also easily reproduced in the presence of ADP and AMP-PNP as well as in the absence of nucleotides. In addition, both the cubic and rod-shaped crystals failed to bind TNP-ATP or TNP-ADP, as demonstrated by their ability to absorb the yellow colour of the fluorophore. Therefore, we considered both crystal forms as resembling the nucleotide-free state of the protein and they were not evaluated further.

**Table 2**
Summary of the crystallographic analysis and data-set collection of the HlyB-NBD wild type and H662A mutant with ADP.

Resolution limits are based on $I/\sigma(I)$ statistics. Values in parentheses refer to the highest resolution shell (1.65–1.6 Å for the wild type and 1.74–1.7 Å for the H662A protein). $R_{sym}$ is defined as $\sum|I_{hkl} - \langle I_{hkl}\rangle|/\sum I_{hkl}$. Data sets were scaled and processed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

| | Wild type | H662A |
|---|---|---|
| Space group | *C*2 | *C*2 |
| Unit-cell parameters at 100 K | | |
| *a* (Å) | 180.37 | 180.16 |
| *b* (Å) | 34.84 | 34.77 |
| *c* (Å) | 37.82 | 38.1 |
| $\alpha$ (°) | 90 | 90 |
| $\beta$ (°) | 98.41 | 98.58 |
| $\gamma$ (°) | 90 | 90 |
| Wavelength (Å) | 1.005 | 1.005 |
| Resolution (Å) | 20–1.6 (1.65–1.60) | 20–1.7 (1.78–1.70) |
| Mean redundancy | 10.9 | 12.2 |
| Completeness (%) | 90.5 (69.5) | 99.2 (88.5) |
| $I/\sigma(I)$ | 14.7 (2.1) | 26 (3.2) |
| $R_{sym}$ (%) | 8.8 (25.4) | 4.2 (16.3) |

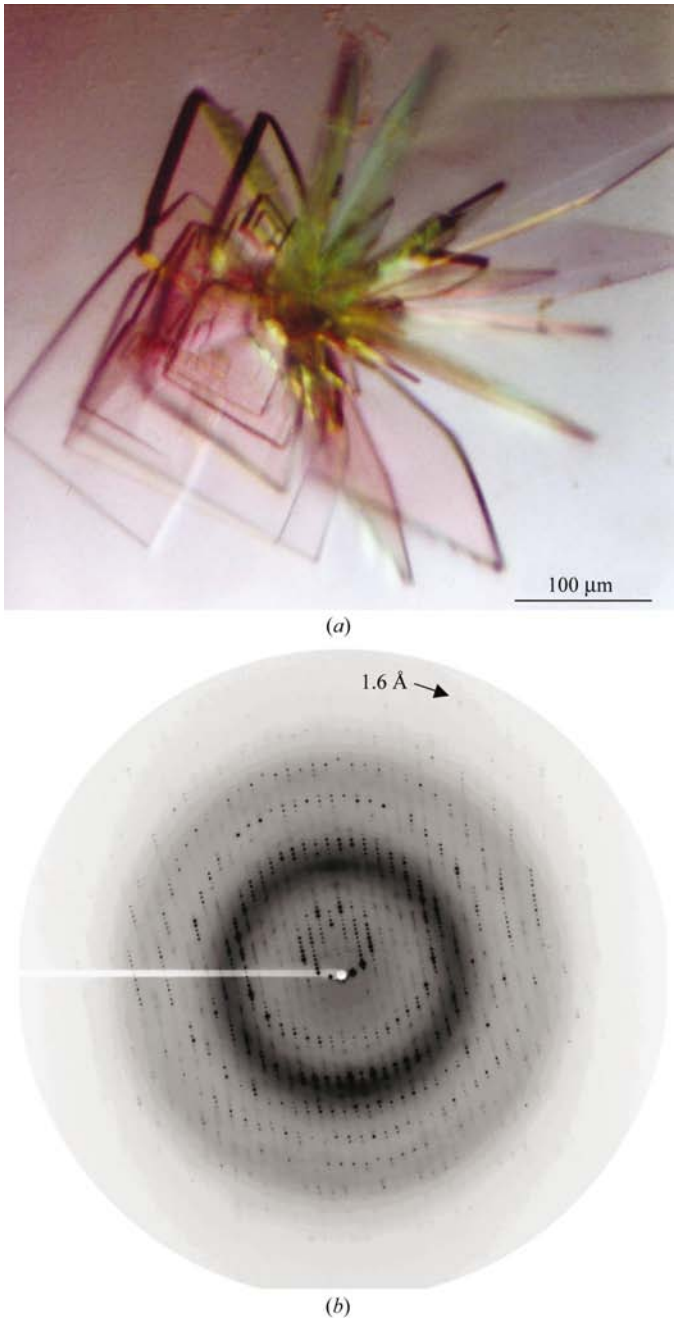


**Figure 4**
(*a*) Crystals of HlyB-NBD in the presence of ADP. For crystallization conditions and further details, see §3. (*b*) Oscillation photograph of HlyB-NBD obtained at the Advanced Swiss Light Source. Protein was crystallized in the presence of ADP/$Mg^{2+}$. Further details are given in Table 2.

Screening of different crystallization conditions revealed one further crystal type formed in the presence of ATP: 30 mg ml$^{-1}$ HlyB-NBD in 60 m*M* CAPS pH 10.4, 35% glycerol, 10 m*M* ATP and 20 m*M* $MgCl_2$ was mixed in a 1:1 ratio with 100 m*M* Tris–HCl pH 8.0, 2 *M* ammonium sulfate and 10% DMSO. The formation of these crystals was only observed upon pre-incubation of the protein with ATP. Neither ADP, AMP-PNP nor ATP$\gamma$S supported crystal growth under the above conditions. Crystals produced under the same conditions with the addition of 50 µ*M* TNP-ATP were coloured opalescent yellow. Inclusion of 50 µ*M* TNP-AMP in the droplet did not lead to accumulation of colour in the protein crystals, suggesting the specific binding of the fluorescent analogue of ATP but not AMP to the protein under these conditions. However, those crystals only diffracted to 4.5–5 Å even at a synchrotron beamline.

Crystallization and determination of the high-resolution structure of the MJ0796 NBD carrying the E171Q mutation presented the very first evidence in relation to the ABC-transporter family for the nature of the dimer formed upon ATP binding (Smith *et al.*, 2002). This result also indicated the potential importance of mutant proteins for the elucidation of the catalytic cycle of NBD. Having had no success in obtaining crystals of wild-type HlyB-NBD with ATP and ATP analogues (AMP-PNP, ATP$\gamma$S) that diffracted to high resolution even using the optimized conditions described here, we considered the employment of HlyB-NBD mutants in order to extend our screening efforts of the various functional states of the ABC domain. Mutation of a conserved histidine (His662) to alanine in HlyB-NBD completely eliminated its ability to hydrolyse ATP, whilst preserving its ATP-binding capability (manuscript in preparation). The same effect on replacement of a conserved histidine (His211) was reported for HisP from *Salmonella typhimurium* (Nikaido & Ames, 1999). Crystals of HlyB-NBD H662A in the presence of ATP were obtained 3–5 d after mixing 1 µl protein solution (10 mg ml$^{-1}$ in 1 m*M* CAPS pH 10.4, 30% glycerol, 2 m*M* ATP) and 1 µl reservoir solution (100 m*M* sodium malonate pH 5.6, 10% PEG MME 5500, 0.25 *M* sodium acetate). Crystals continued to grow for 2–3 weeks, reaching maximum dimensions of 250 × 50 × 50 µm. Diffraction data were collected to 2.5 Å on flash-frozen crystals at beamline BW-6 (Fig. 5 and Table 3). The H662A mutant also produced crystals under similar conditions with 8-Br-ATP instead of ATP, but not in the presence of 2′-Br-ATP. The HlyB-NBD H662A mutant formed crystals in the presence of ADP that were identical in shape to those of the wild-type protein obtained under exactly the same conditions (Fig. 4*a*), except that the H662A mutant specifically required the presence of ADP to produce crystals. As expected, the space groups of both crystal types were identical and the unit-cell parameters were rather similar (Table 2).

**Table 3**
Summary of the crystallographic analysis and data-set collection of the H662A mutant NBD with ATP.

Resolution limits are based on $I/\sigma(I)$ statistics. Values in parentheses refer to the highest resolution shell (2.56–2.50 Å). $R_{sym}$ is defined as $\sum|I_{hkl} - \langle I_{hkl}\rangle|/\sum I_{hkl}$. Data were processed and scaled with *d*TREK* (Pflugrath, 1999).

| | |
|---|---|
| Space group | $P2_1$ |
| Unit-cell parameters at 100 K | |
| *a* (Å) | 46.95 |
| *b* (Å) | 196.26 |
| *c* (Å) | 63.69 |
| $\alpha$ (°) | 90 |
| $\beta$ (°) | 110.85 |
| $\gamma$ (°) | 90 |
| Wavelength (Å) | 1.005 |
| Resolution (Å) | 19.2–2.5 (2.59–2.50) |
| Mean redundancy | 4.9 (2.8) |
| Completeness (%) | 94.8 (94.7) |
| $I/\sigma(I)$ | 7.8 (3.1) |
| $R_{sym}$ (%) | 6.8 (30.5) |

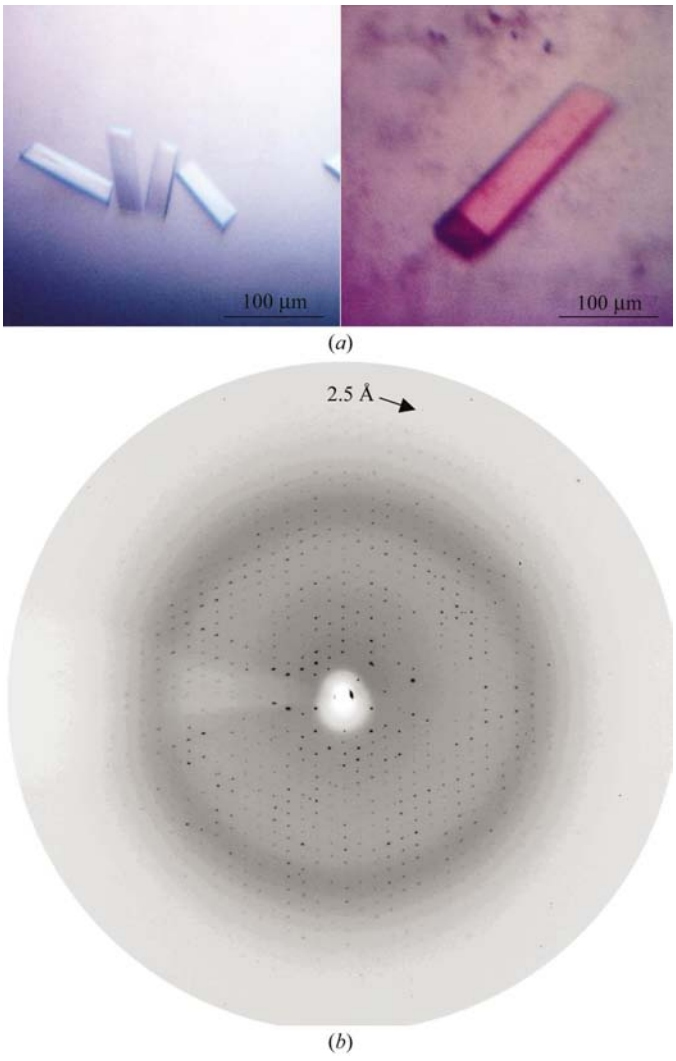


**Figure 5**
(*a*) Crystals of the HlyB-NBD H662A in the presence of ATP. For crystallization conditions and further details, see §3. (*b*) Oscillation photograph of HlyB-NBD obtained at beamline BW-6. Protein was crystallized in the presence of ATP. Further details are given in Table 3.

## 5. Conclusions

Conditions were developed for obtaining highly concentrated (50 mg ml$^{-1}$ and higher) nucleotide-free HlyB-NBD that was

stable at 277 K and remained suitable for crystallization for at least a month. The availability of this protein stock solution allowed the testing of many different substrates and a variety of buffers for crystallization and also provided an opportunity to prevent protein precipitation until the moment of introduction of the protein into a functional assay buffer or crystallization conditions. Significantly improved protein stability allowed us to obtain crystals of HlyB-NBD trapped in various states of the ATP hydrolytic cycle, *e.g.* in the presence of ADP and ATP.

We would like to stress the importance of the buffer chosen for protein purification and storage as the crucial point for successful crystallization. Previous attempts to crystallize the nucleotide-bound states of HlyB-NBD, purified either in Tris or phosphate buffer, never yielded crystals. CAPS buffer on the other hand allowed us to obtain high-quality crystals of the purified NBD of HlyB. Based on the ATPase assays, the CAPS-glycerol buffer system retained the HlyB-NBD in a reversible 'sleeping state' that was unable to hydrolyze ATP. It is likely that the ability of this particular buffer system to switch off the enzymatic activity of the ATPase domain of HlyB is the molecular reason for its extreme effect on protein stability.

A simple screening assay was employed to determine those conditions that provided stability of the purified NBD of HlyB. Although the focus of our study was the NBD of HlyB, we are convinced that a screening procedure similar to that employed here as well as the use of CAPS buffer could be utilized for other proteins to prevent their aggregation or denaturation during storage.

We thank Uli Ermler, Eckhard Hoffmann and Milton T. Stubbs for many fruitful and stimulating discussions and Uli Ermler and Harmut Michel for in-house facilities. We are indebted to Sander Smits for his support during data acquisition and the staff of the Paul Scherer Institute and of the BW6 beamline (DESY, Hamburg), especially Gleb Bourenkov, for their support. We also acknowledge the support of Jim Pflugrath, MSI during data processing using *d*TREK*. LS thanks Robert Tampé for constant support and encouragement. This work was supported by ABCF (Paris), CNRS and the University Paris-Sud (to IBH), and the Deutsche Forschungsgemeinschaft (Emmy Noether Program Grant Schm1279/2-2 to LS).

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