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Screening orthologs as an important variable in crystallization: preliminary X-ray diffraction studies of the tRNA-modifying enzyme S-adenosylmethionine:tRNA ribosyl transferase/isomerase

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The genes encoding the tRNA-modifying enzyme S-adenosylmethionine:tRNA ribosyl transferase/isomerase (QueA) from 12 eubacterial sources were overexpressed in *Escherichia coli* and the resulting products were purified to homogeneity and subjected to crystallization trials. Using the hanging-drop vapour-diffusion method, crystals suitable for X-ray diffraction experiments were only obtained for the *queA* gene product from *Bacillus subtilis*. The crystals belong to the space group *P*422, with unit-cell parameters $a = b = 100.7$, $c = 150.9$ Å. Using highly focused synchrotron radiation from the EMBL/ESRF beamline ID13 (Grenoble, France), diffraction to at least 3.2 Å could be achieved. A selenomethionyl derivative of the protein was prepared and crystallized for future multiwavelength anomalous diffraction (MAD) experiments.

1. Introduction

The modified tRNA base queuine [Q-base; 7-(4,5-*cis*-dihydroxy-1-cyclopenten-3-yl-aminomethyl)-7-deazaguanine] occurs at the wobble position of the anticodon (position 34) of tRNAs specific for Asp, Asn, His and Tyr and is essential for the pathogenicity of the dysentery-causing bacterium *Shigella*. Mutant *Shigella* strains lacking a Q biosynthesis gene have been shown to exhibit a significantly reduced pathogenicity as a result of inefficient *virF* mRNA translation (Durand *et al.*, 1994). Since the VirF protein directly or indirectly acts as a positive regulator on almost all pathogenicity genes encoded by the large plasmid of *Shigella* (Dorman & Porter, 1998), its reduced concentration in Q biosynthesis mutants leads to a significantly reduced production of most virulence factors. Therefore, enzymes involved in Q biosynthesis seem to be suitable targets for the *de novo* design of specific inhibitors which may act as drugs against shigellosis.

Since its *de novo* synthesis is restricted to eubacteria, Q is a nutrient factor for eukaryotes. The bacterial Q biosynthesis pathway (Fig. 1) starts outside the tRNA with guanosine triphosphate (GTP), which is modified to the Q precursor 7-aminomethyl-7-deazaguanine ($preQ_1$) in a process which is still poorly understood (Slany & Kersten, 1994). The enzyme tRNA-guanine transglycosylase (Tgt) then catalyzes the introduction of $preQ_1$ into the tRNA by a base-exchange mechanism replacing guanine 34 at the wobble position of the cognate tRNAs (Okada & Nishimura, 1979; Okada *et al.*, 1979). Subsequently, the ribosyl moiety of S-adenosylmethionine (AdoMet) is transferred to the tRNA-bound $preQ_1$ and isomerized to the intermediate epoxy-queuine (oQ). This step is catalyzed by the AdoMet-tRNA:ribosyltransferase/isomerase (QueA; Slany *et al.*, 1993). In a final reaction, the epoxide is reduced to a double bond by an unknown cofactor-B_{12}-dependent enzyme, yielding Q (Frey *et al.*, 1988).

While the structure of the Tgt enzyme has been determined (Romier, Reuter *et al.*, 1996*a*) and presently serves as a target for the *de novo* design of a drug against shigellosis, no structural data are available for the remaining enzymes involved in Q biosynthesis. Moreover, apart from the *tgt* gene, *queA* is the only Q-biosynthesis gene which has been cloned and sequenced until now (Reuter *et al.*, 1991). Its gene product is a monomeric protein with a molecular weight of approximately 40 kDa and represents the only enzyme known to transfer the ribose moiety from AdoMet to another substrate (Slany *et al.*, 1993). To obtain crystals from QueA, we used a strategy which resulted from the experience we gained during the crystallization of the Tgt enzyme. In the course of extensive attempts to crystallize Tgt from *E. coli*, we only obtained very thin needles which were unsuitable for X-ray diffraction experiments. Therefore, we had considered using the Tgt from another organism. Since no other complete *tgt* gene was available at this time (1993), we first had to clone and sequence the partially known *tgt* gene from *Zymononas*

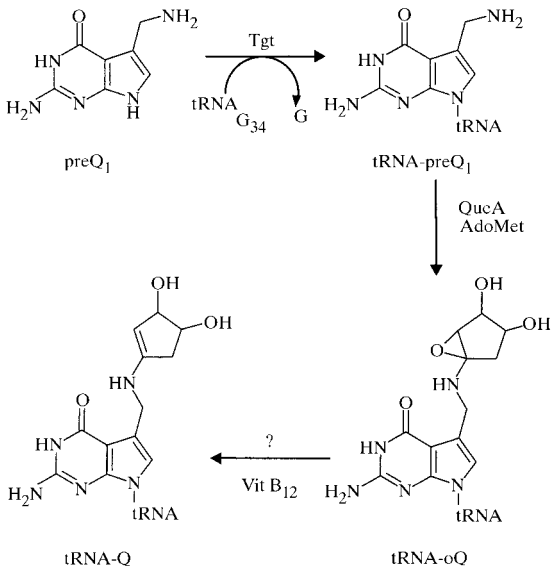

Figure 1
Biosynthesis pathway of the hypermodified tRNA base queuine (Q).

mobilis (Reuter & Ficner, 1995). The *Z. mobilis* Tgt turned out to crystallize easily, allowing the structure determination of its wild type and several mutated forms (Grädler *et al.*, 1999; Romier, Ficner *et al.*, 1996; Romier, Reuter *et al.*, 1996*a*,*b*).

Encouraged by this experience, we overexpressed the *queA* genes from 12 organisms and purified the respective proteins. Here, we report the results of the crystallization screens performed with these QueA proteins.

2. Materials and methods

2.1. Cloning of *queA* genes

The coding regions of the *queA* genes from various organisms were amplified by PCR using the respective chromosomal DNA as a template. Cosmid cs0165 (Kaneko *et al.*, 1996) and λ phage F1339 DNA (Andersson *et al.*, 1998) served as templates for the PCR amplification of the *Synechocystis* sp. and the *Rickettsia prowazekii queA* genes, respectively. The chromosomal DNAs were purchased from the Deutsche Stammsammlung von Mikroorganismen und Zellkulturen (DSZM, Braunschweig, Germany), apart from those acknowledged as gifts. In case of the *E. coli*, *B. subtilis*, *Clostridium acetobutylicum*, *Aquifex aeolicus*, *Haemophilus influenzae*, *Helicobacter pylori*, *Synechocystis* sp. and *R. prowazekii queA* genes, the resulting PCR products were introduced into the multiple-cloning site (MCS) of overexpression vector pGEX-6P-1 (Amersham Pharmacia, Sweden) using a *Bam*HI restriction site (introduced by the 5′ primer) directly preceding the natural start codon and an *Eco*RI restriction site (introduced by the 3′ primer) directly following the natural stop codon. This resulted in GST (coding for *Schistosoma japonicum* glutathione S-transferase) *queA* fusion genes (Smith & Johnson, 1988) with the amino-terminal GST moieties of the respective gene products separated from the carboxy-terminal QueA moieties by the highly specific recognition sequence for PreScission protease (Amersham Pharmacia, Sweden).

These constructs implied that each overproduced QueA protein was preceded by five amino acids at its amino-terminus after removal of the GST moiety by cleavage with PreScission protease. In the course of this study, we modified the pGEX-6P-1 vector by extending its MCS through the introduction of an *Nde*I restriction site between the sequence coding for the PreScission protease recognition and the *Bam*HI restriction site. This enabled us to create constructs where the *queA* genes were each preceeded by only three codons following the PreScission protease cleavage site. The modified pGEX-6P-1 vector was used for overexpression of the *queA* genes from *Staphylococcus aureus*, *Deinococcus radiodurans*, *Borrelia burgdorferi* and *Thermotoga maritima*. Thus, the 5′ primers used for the PCR amplification of the *queA* genes from these organisms each introduced an *Nde*I restriction site containing the *queA* start codon and the 3′ primers introduced a *Bam*HI restriction site directly following the *queA* stop codon.

The inserts of all plasmids generated in this study were resequenced using an ABI 370 automated sequencer (Perkin–Elmer, USA) to exclude unwanted mutations which may have occurred during PCR amplification.

2.2. Expression and purification

E. coli BL21(DE3)(plysS) cells (Novagen, UK) transformed with the appropriate overexpression plasmid were grown aerobically in 1 l LB medium (Miller, 1972) with 10 mg l^{-1} chloramphenicol and 100 mg l^{-1} ampicillin at 303 K using 2 l flasks. When the culture had reached an optical density at wavelength 600 nm (OD_{600}) of 0.8, the expression of the fusion gene was induced by addition of isopropyl-1-thio-β-D-galactoside (IPTG) to a final concentration of 0.5 m*M*. After an additional incubation period of 4 h, the cells were harvested by centrifugation and resuspended in 15 ml lysis buffer [50 m*M* Tris–HCl pH 8.5, 5 m*M* dithiothreitol (DTT) plus one tablet of Complete (EDTA-free) protease-inhibitor cocktail (Roche Mannheim, Germany) plus 150 µg DNAase I (Roche Mannheim, Germany)] and immediately frozen at 255 K. After thawing and incubation at 293 K for 25 min, lysis was complete. Cellular debris was sedimented by centrifugation of the lysate at 10 000*g*. The supernatant was incubated at 293 K for 1 h with 2.5 ml glutathione Sepharose 4B suspension (Amersham Pharmacia, Sweden). After a short centrifugation at 4000*g*, the supernatant was discarded. Subsequently, the pelleted glutathione Sepharose 4B was resuspended with washing buffer (50 m*M* Tris pH 8.5, 100 m*M* NaCl, 2 m*M* DTT), centrifuged and the supernatant discarded again. This step was repeated twice. Subsequently, the glutathione Sepharose 4B was resuspended in 1 ml scission buffer (50 m*M* Tris pH 8.5, 100 m*M* NaCl, 2 m*M* DTT, 50 U ml^{-1} PreScission protease) per millilitre of glutathione Sepharose 4B and incubated overnight at 293 K. The scission buffer containing the QueA protein cleaved off from the GST moiety was then separated from the GST-bound affinity chromatography material by filtration. The protein solution, which typically contained >80% pure QueA protein as judged by SDS–gel electrophoresis, was then concentrated using a Centricon 30 concentrator (Amicon, USA) and loaded onto a HiLoad 26/60 Superdex 75 prep-grade gel-filtration column (Amersham Pharmacia, Sweden). The column was pre-equilibrated and eluted with gel-filtration buffer (50 m*M* Tris pH 8.5, 100 m*M* NaCl, 2 m*M* DTT, 1 m*M* EDTA) and fractions containing the QueA protein were pooled, concentrated and rebuffered to a final protein concentration of 15 g l^{-1} in 10 m*M* Tris–HCl pH 8.5. The resulting QueA concentrate contained in no case more than 5% protein impurities as judged by SDS–gel electrophoresis. The gel filtration was carried out on an Äkta Explorer system (Amersham Pharmacia, Sweden) at room temperature. The yield of purified QueA protein was roughly equal for all organisms (except *R. prowazekii*, *Haem. influenzae* and *Helico. pylori*, which yielded no soluble fusion protein) and amounted to ∼10 mg QueA per litre of *E. coli* culture.

The identity of each purified protein was verified by amino-terminal sequencing.

2.3. Crystallization

All crystallization experiments were carried out both at 277 and 293 K in VDX plates (Hampton Research, USA) using the hanging-drop vapour-diffusion technique. For initial screens, the 98 solutions of the Crystal Screen and Crystal Screen II kit (Hampton-Research, USA) were used; 1 µl protein solution (15 g l^{-1} QueA protein in 10 m*M* Tris–HCl pH 8.5 plus 5 m*M* AdoMet) was mixed with 1 µl reservoir solution and equilibrated against 0.5 ml of reservoir solution. For further screens, the crystallization droplets were sealed against 1 ml reservoir solution to which 0.02%(*w*/*v*) sodium azide and 2 m*M* DTT were always added.

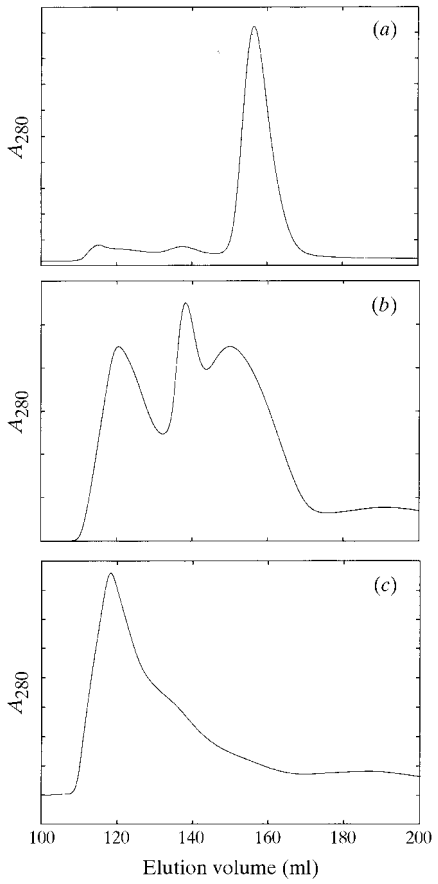

Figure 2
The elution profiles of gel-filtration runs performed with the QueA proteins from three different organisms. (*a*) The elution volume of *E. coli* QueA corresponds to a molecular weight of ∼40 kDa, consistent with a monomeric state of this protein. An identical migration behaviour was seen for *C. acetobutylicum*, *D. radiodurans*, *Haem. influenzae*, *Staph. aureus* and *B. subtilis* QueA. (*b*) QueA from *Synechocystis* sp. is obviously able to form stable aggregates, since three peaks are observed in the elution profile corresponding to a monomeric state and states of higher order. (*c*) The elution profile of *A. aeolicus* QueA displays one peak with a long trail, whose elution volume corresponds to more than 80 kDa. This migration behaviour indicates the tendency of *A. aeolicus* QueA to reversibly aggregate. A similar profile was obtained with *T. maritima* QueA.

2.4. X-ray diffraction experiments and data processing

Crystals were first tested in-house for their power to diffract X-ray beams using a Rigaku RU-300 rotating-anode generator equipped with an R-AXIS IV image-plate system at 50 kV and 100 mA and focusing mirrors (MSC, USA) under cryo-conditions (100 K). The nitrogen cryo-stream was provided by an X-stream cryosystem from MSC (USA).

A complete native data set of a *B. subtilis* QueA crystal was collected at beamline ID13 (ESRF, Grenoble, France). 0.5° frames were collected at a wavelength of 0.906 Å using a MAR CCD camera at a distance of 200 mm from the frozen crystal. A solution of 30% PEG 4000, 100 m*M* Tris–HCl pH 8.0, 200 m*M* $(NH_4)_2SO_4$ and 5% glycerol served as a cryobuffer.

Data processing was performed with the program *MOSFLM* using the *DPS* autoindexing algorithm (Powell, 1999; Steller *et al.*, 1997) and data were scaled using the program *SCALA* (Collaborative Computational Project, Number 4, 1994).

2.5. Preparation of a selenomethionyl derivative of *B. subtilis* QueA

For a future multiple-wavelength anomalous diffraction (MAD) experiment, a selenomethionyl derivative (Hendrickson *et al.*, 1990) of *B. subtilis* QueA was prepared. For this purpose cells of the *met*$^-$ *E. coli* strain B834(DE3)(plysS) (Novagen, UK) transformed with the *B. subtilis* queA containing pGEX-6P-1 derivative were grown at 303 K in 1 l minimal medium with glucose (10 g l^{-1}) as carbon source. The medium contained 100 mg l^{-1} ampicillin and 10 mg l^{-1} chloramphenicol. It was supplemented with biotin, thiamine (2 mg l^{-1} each) and L-methionine (500 mg l^{-1}). After the culture had reached an OD_{600} of 0.6, cells were spun down by centrifugation and resuspended in 1 l of the above medium devoid of methionine. After 1 h, 50 mg of DL-selenomethionine were added. After an additional 30 min, induction of selenomethioninyl QueA production was achieved through addition of IPTG to a final concentration of 0.5 m*M*. Expression was allowed to continue for 8 h, resulting in a final $OD_{600} \simeq 2.5$, when cells were harvested. Purification and crystallization of the selenomethioninyl QueA was identical to that of the native protein, except that oxidation of selenomethionine was prevented by adding 5 m*M* DTT to all buffers.

3. Results and discussion

The queA genes from 12 organisms (*A. aeolicus*, *B. subtilis*, *Borr. burgdorferi*, *C. acetobutylicum*, *D. radiodurans*, *E. coli*, *Haem. influenzae*, *Helico. pylori*, *R. prowazekii*, *Staph. aureus*, *Synechocystis* sp. and *T. maritima*) were cloned as GST (coding for *Schist. japonicum* glutathione S-transferase) fusion genes (Smith & Johnson, 1988) and overexpressed in *E. coli*. All fusion genes resulted in the production of high amounts of soluble fusion protein, apart from those containing the *queA* genes from *Borr. burgdorferi*, *Helico. pylori* and *R. prowazekii*. While the GST–*R. prowazekii queA* fusion gene was not expressed in detectable amounts, the other two constructs lead to insoluble fusion proteins after overproduction. In case of the *Borr. burgdorferi queA* gene, the fusion protein was truncated. This turned out to be because of a one-nucleotide deletion resulting in a frame shift in the *queA* gene of the *Borr. burgdorferi* type strain used (DSM4680) chromosomal DNA. This deletion is also present in the sequence of the complete *Borr. burgdorferi* genome (Fraser *et al.*, 1997). The QueA proteins from these three organisms were not considered for further studies. The GST moieties of the remaining fusion proteins allowed their affinity purification by means of glutathione Sepharose. After the fusion proteins had been bound to the glutathione Sepharose material, the QueA moieties were proteolytically cleaved off and eluted. Finally, the QueA proteins were separated from the GST-bound affinity chromatography material by filtration and subjected to gel filtration as described in §2.2 to effectively remove residual impurities. Owing to the expression strategy used, each QueA protein contained an additional three or five amino acids at its amino-terminus compared with its natural counterpart (see §2.1). This was not thought to negatively influence crystallization behaviour, particularly since the amino-termini of the QueA proteins vary somewhat dependent on the organism they are derived from. The QueA proteins from *C. acetobutylicum*, *D. radiodurans*, *E. coli*, *Haem. influenzae*, *Staph. aureus* and *B. subtilis* showed a migration behaviour on the gel-filtration column corresponding to a monomeric state of these protein molecules (Fig. 2*a*) as judged by gel-

Table 1
X-ray data-collection statistics.

No. of crystals	1
Resolution (Å)	30–3.2
Wavelength (Å)	0.906
Space group	*P*422
Unit-cell parameters (Å)	$a = b = 100.7$, $c = 150.9$
Temperature of data collection (K)	100
No. of observed reflections	72948
No. of unique reflections	13356
Completeness of all data (%)	99.7
R_{sym} for all data (%)	8.2
Completeness of outer shell (3.37–3.20 Å) (%)	99.6
R_{sym} in outer shell (%)	26.3

filtration runs of standard proteins (data not shown). In contrast, the elution profile of the QueA protein from *Synechocystis* sp. displayed three major peaks (Fig. 2*b*), indicating that this protein is capable of forming stable aggregates of higher order in addition to the monomeric state. The QueA proteins of *A. aeolicus* and *T. maritima* both eluted at a volume beyond the separation limits of the column used, corresponding to a molecular weight of more than 80 kDa. In both cases, the respective peak on the elution profile had a long trail (Fig. 2*c*). This indicated the tendency of these proteins to reversibly aggregate, as was previously observed for the Tgt enzyme of *E. coli* (Reuter & Ficner, 1995).

All nine purified QueA proteins were subjected to a sparse-matrix crystallization screen (Cudney *et al.*, 1994; Jancarik & Kim, 1991) using commercially available kits (see §2.3). Subsequently, additional screens were performed to find crystallization conditions or to improve conditions when crystals were obtained during the initial screening. For all QueA proteins, screens were carried out using $(NH_4)_2SO_4$, Li_2SO_4, NaCl, sodium/potassium phosphate, sodium formate, sodium citrate and PEGs of varying molecular weight as precipitants. Various pH values and precipitant concentrations as well as the influence of several additives were systematically tested in each case. The results of this extensive effort were crystals of five different habits obtained from the QueA proteins from *T. maritima*, *Haem. influenzae*, *E. coli* and *B. subtilis*.

Figure 3
Crystals of *B. subtilis* QueA. The crystals have approximate dimensions of 0.4 × 0.4 mm and a thickness of up to 0.02 mm.

After a period of more than three weeks, the *T. maritima* QueA protein yielded thin plates at 293 K with 30% PEG 4000 as a precipitant and 200 m*M* NaCl as an additive in the pH range 6.0–8.5. The crystals were embedded in a protein skin and had dimensions of approximately 0.1 × 0.1 × 0.01 mm. Despite intensive efforts, it was not possible to increase the size of these crystals.

After four to five weeks, the same protein yielded crystals of a cubic shape with an edge length of 0.1 mm within the pH range 7.5–9.0 using 5–10% PEG 8000 as a precipitant. However, these crystals were unable to diffract X-rays generated by a conventional X-ray source. In this case, it was also impossible to increase the size or quality of these crystals.

Similarly, crystals obtained from *Haem. influenzae* QueA, which grew at 277 K with 15% PEG 4000 as a precipitant buffered with 100 m*M* 3-(cyclohexylamino)-1-propanesulfonic acid (CHES; pH 9.0) in the presence of 100 m*M* calcium acetate, showed no X-ray diffraction. The dimensions of the tested crystals amounted to approximately 0.15 × 0.1 × 0.1 mm.

Small crystals of *E. coli* QueA first grew during a period of about two weeks at 277 K in the presence of 28% PEG 4000 and 200 m*M* Li_2SO_4 buffered with 100 m*M* Tris–HCl pH 8.5. The size of these crystals could be increased significantly when crystals were grown in a drop consisting of 2 µl protein solution (whose concentration was increased to 50 g l^{-1}) and 1.33 µl reservoir solution with 17% PEG 4000, 100 m*M* Tris–HCl pH 8.5, 200 m*M* $MgCl_2$ and 1.23 µ*M* β-octyl-D-glucoside (0.05% of its critical micellar concentration). After nearly two months, the crystals reached dimensions of approximately 0.5 × 0.1 × 0.1 mm. It was not possible to accelerate crystal growth by seeding techniques. Using conventional X-ray equipment, these crystals only diffracted to about 12 Å. Despite extensive efforts, further improvement of these crystals was not achieved.

Within 2 d, small plate-like crystals of *B. subtilis* QueA were obtained with 30% PEG 4000, 100 m*M* Tris–HCl pH 8.0 and 200 m*M* $(NH_4)_2SO_4$ at 277 K as well as at 293 K. Upon reduction of the PEG 4000 concentration to 22% and increasing the initial drop size to 16 µl (8 µl protein solution plus 8 µl reservoir solution), the very thin crystal plates reached edge lengths of more than 0.4 × 0.4 mm but a thickness of less than 0.02 mm (Fig. 3). Using a conventional rotating-anode X-ray source, these crystals diffracted to ∼8 Å. Using synchrotron radiation on the MAD beamlines 5.2R at the ELETTRA synchrotron (Trieste, Italy) and BW7A at DESY (Hamburg, Germany) the crystals diffracted to about 5 Å, which is still not sufficient for a structure determination. Finally, a test experiment at the high-intensity microfocus beamline ID13 at EMBL/ESRF (Grenoble, France), which is endowed with a 30 µm collimator and designated for the examination of protein microcrystals (Perrakis *et al.*, 1999), enabled us to collect a complete native data set at 3.2 Å resolution (see §2.3). The crystals belong to a tetragonal crystal system. The space group was determined to be *P*422, with unit-cell prameters $a = b = 100.7$, $c = 150.9$ Å. Table 1 lists the data-collection statistics of the processed data set. Assuming two molecules in the asymmetric unit, the Matthews coefficient (Matthews, 1968) was 2.39 $Å^3$ Da^{-1}, corresponding to a solvent content of 48%. Owing to the high intensity and excellent brilliance of the beam used for data collection, the data are of sufficient quality that a structure determination by means of multiple isomorphous replacement or MAD seems feasible, provided that equally good data can be collected from a selenomethionyl or heavy-atom derivative. Since QueA shows no homology to any protein of known structure, it is not possible to obtain phase information through the molecular-replacement method. We have prepared and crystallized a selenomethionyl derivative of *B. subtilis* QueA for a future MAD experiment (see §2.4). The success of selenomethionine incorporation was verified by electrospray mass spectrometry. A difference in molecular weight of 379 Da between the natural and selenomethionyl protein was measured, corresponding to the replacement of all eight methionines present in *B. subtilis* QueA by selenomethionines. The crystals obtained from selenomethionyl QueA were morphologically identical to those from the native protein and diffracted X-ray beams generated by a rotating-anode generator to the same resolution as native crystals. In addition, native *B. subtilis* QueA was co-crystallized with several heavy-atom additives in order to investigate their tolerance to the respective substances.

This paper emphasizes the advantage of the recent success in different genome-sequencing projects for protein crystallization, since it renders the use of orthologous proteins from several organisms an attractive and easily accessible variable if a

protein shows low tendency to crystallize. In addition, the results presented point out the importance of high-intensity microfocus beamlines such as ID13, which are able to yield reasonable data sets from crystals lacking the size or quality necessary for a structure determination at conventional synchrotron-radiation sources.

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