Efficient expression, purification and crystallisation of two hyperthermostable enzymes of histidine biosynthesis

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Abstract Enzymes from hyperthermophiles can be efficiently purified after expression in mesophilic hosts and are well-suited for crystallisation attempts. Two enzymes of histidine biosynthesis from *Thermotoga maritima*, *N*′-((5′-phosphoribosyl)-formimino)-5-aminoimidazol-4-carboxamid ribonucleotide isomerase and the cyclase moiety of imidazoleglycerol phosphate synthase, were overexpressed in *Escherichia coli*, both in their native and seleno-methionine-labelled forms, purified by heat precipitation of host proteins and crystallised. *N*′-((5′-phosphoribosyl)-formimino)-5-aminoimidazol-4-carboxamid ribonucleotide isomerase crystallised in four different forms, all suitable for X-ray structure solution, and the cyclase moiety of imidazoleglycerol phosphate synthase yielded one crystal form that diffracted to atomic resolution. The obtained crystals will enable the determination of the first three-dimensional structures of enzymes from the histidine biosynthetic pathway.

Key words: Histidine biosynthesis; Hyperthermophile; Crystallisation; *Thermotoga maritima*

1. Introduction

The histidine biosynthesis is an ancient and key metabolic pathway that comprises 11 enzymatic reactions that are encoded by eight genes in the enterobacteria [1]. The operon organisation of the *his* genes and the regulation of their expression as well as the catalytic and regulatory properties of the corresponding enzymes have been studied intensively [1,2]. However, in contrast to related amino acid biosynthetic pathways such as tryptophan biosynthesis [3], three-dimensional structures are not yet available for any of the enzymes of the histidine biosynthesis.

Structure determination using X-ray diffraction has rapidly progressed in recent years, mostly by the advances in a variety of phasing methods and the increased availability of synchrotron facilities. Thus, in many projects, the sample preparation by overexpression, purification and crystallisation of the proteins has become the rate-limiting step in the structural analysis. When working with bacterial proteins, the corresponding variants from hyperthermophiles offer considerable advantages in sample preparation compared to their mesophilic orthologues, due to their high intrinsic stability and high solubility [4]. Furthermore, due to the usually weak catalytic activity of hyperthermostable enzymes at low temperatures, enzyme-substrate complexes might have a sufficiently high lifetime for X-ray analysis.

Hence, we have initiated the structural analysis of several histidine biosynthetic enzymes from the hyperthermophilic bacterium *Thermotoga maritima*, where the *his* gene cluster t*his*DCBdHAFI-E was recently sequenced and characterised [5]. Here, we describe the heterologous expression in *Escherichia coli*, purification and crystallisation of *N*′-((5′-phosphoribosyl)-formimino)-5-aminoimidazol-4-carboxamid ribonucleotide isomerase (the product of the t*his*A gene, tHisA) and of the cyclase subunit of imidazoleglycerol phosphate synthase (the product of the t*his*F gene, tHisF). tHisA (241 residues) and tHisF (253 residues) have 25% sequence identity. Both HisA and HisF have been predicted to have evolved from a common ancestor by a series of gene duplication and diversification events [6] and to comprise the $(\beta\alpha)_8$-barrel fold [5,7–9]. The structure determination of tHisA and tHisF by multiple wavelength anomalous diffraction (MAD) analysis of native and seleno-methionine (SeMet)-labelled will allow for testing these predictions.

2. Materials and methods

2.1. Expression and purification of tHisA

The *hisA* gene of *T. maritima* (t*his*A) was amplified by PCR, using the plasmid pUN121/I [5] as a template. The oligonucleotide pDS-t*his*A(upper) 5′-AAGGTGATC**GCATGC**TCGTTG-3′ with a *Sph*I site (in bold) was used as the 5′-primer and the oligonucleotide pDS-t*his*A(lower) 5′-GAGACACGCGATT**AAGCTT**TTAGC-3′ with a *Hin*dIII site (in bold) as the 3′-primer. Using the two new restriction sites, the amplified DNA fragment was cloned into the expression plasmid pDS/RBSII [10] to yield the plasmid pDS/RBSII/t*his*A. Expression was conducted in W3110 *trp*EA2 cells [11] containing the repressor plasmid pDMI,1 [12]. An overnight culture of freshly transformed cells in 500 ml LB medium supplemented with 0.15 mg/ml ampicillin and 0.025 mg/ml kanamycin was used to inoculate a fermenter (Bioengineering, Wald, Switzerland) containing 38 l of the same medium. The culture was incubated at 37°C with a constant

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Abbreviations: HisA, *N*′-((5′-phosphoribosyl)-formimino)-5-aminoimidazol-4-carboxamid ribonucleotide isomerase; HisF, the cyclase moiety of imidazoleglycerol phosphate synthase

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air supply (5 l/min) and continuous stirring (200 rpm). Overexpression of t*his*A was induced at an optical density at 600 nm (OD_{600}) of 0.56 by adding IPTG to a final concentration of 1 mM. After another 5 h of incubation, the cells reached an OD_{600} of 4.4 and were harvested in a flow-through centrifuge (CEPA Z41G, Lahr, Germany) at 20 000 rpm, washed with 100 mM potassium phosphate buffer at pH 7.5 and centrifuged again (Sorvall GS3, 12 000 rpm, 20 min, 4°C). About 182 g cells (wet weight) was obtained, corresponding to 4.8 g/l culture.

The cells were resuspended at 4°C in 100 mM potassium phosphate buffer at pH 7.5, containing 5 mM EDTA, 1 mM DTT, 0.3 mM phenylmethylsulfonylfluoride, 5 μg/ml of both DNAse and RNAse (1.5 ml buffer/g wet mass), and disrupted by sonification (Branson Sonifier 250, 2×3 min, 50% pulse, output control 6, 0°C). The resulting homogenate was centrifuged (Sorvall GSA, 12 000 rpm, 60 min, 4°C). According to SDS-PAGE, about 90% of tHisA was found in the soluble and 10% in the insoluble fraction. The supernatant was heated to 75°C for 15 min, the suspension was centrifuged (Sorvall GSA, 12 000 rpm, 60 min, 4°C) and the pellet discarded. The supernatant was dialysed at 4°C against 50 mM Tris-HCl buffer at pH 7.5, containing 2 mM EDTA and 0.5 mM DTT, and loaded on a DEAE-Sephacel fast-flow column (5×29.6 cm, Pharmacia) that was equilibrated with the same buffer. The column was washed with two volumes of equilibration buffer and then eluted with 3 l of a linear gradient from 0 to 500 mM NaCl. Fractions judged by SDS-PAGE to contain tHisA were pooled, dialysed against 50 mM Tris-HCl buffer at pH 7.5, 1 mM EDTA, 0.5 mM DTT, and loaded on a Red Sepharose CL-6B column (2.5×16.5 cm, Pharmacia) that was equilibrated with the same buffer. The column was washed with five volumes of equilibration buffer and eluted with 800 ml of a linear gradient of 0 to 500 mM NaCl. The main UV-absorbing peak contained tHisA with a purity above 98%, as judged by SDS-PAGE and reversed phase HPLC. The purification yielded approximately 1.15 mg tHisA/g wet cells, corresponding to 0.2 l cell culture. Fractions containing pure tHisA were pooled, dialysed against 10 mM potassium phosphate at pH 7.5, containing 1 mM EDTA and 0.5 mM DTT, concentrated to 20–29 mg/ml by ultrafiltration (PM 10 membranes, Amicon) and shock-frozen in liquid nitrogen.

For crystallisation attempts, purified tHisA at a concentration of 10 mg/ml was dialysed against 10 mM HEPES/NaOH buffer at pH 7.4, containing 1 mM DTT and 1 mM EDTA.

2.2. Expression and purification of tHisF

The *his*F gene of *T. maritima* (t*his*F) was amplified by PCR, using the plasmid pUN121/I [5] as a template. The oligonucleotide pET-HisF-U 5′-TGATGAAGA**CATATG**CTCGCTAAAAG-3′ with a *Nde*I site (in bold) was used as the 5′-primer and the oligonucleotide pET-HisF-L 5′-CC**GGATCC**AGCGTCATCACAA-3′ with a *Bam*HI site (in bold) as the 3′-primer. Using the two new restriction sites, the amplified DNA fragment was cloned into the expression plasmid pET11c to yield the plasmid pET11c-t*his*F. Expression was conducted in *E. coli* BL21(DE 3) cells, which contain the gene for the phage T7 polymerase on their chromosome [13]. An overnight culture of freshly transformed cells containing 1 l LB medium supplemented with 0.1 mg/ml ampicillin was used to inoculate the 38 l fermenter as described for tHisA. For reasons unexplained so far, the presence of IPTG inhibits expression of t*his*F. Therefore, cells were grown overnight at 37°C without addition of IPTG, resulting in an OD_{600} value of 5. Cells were harvested in a flow-through centrifuge (CEPA GLE, Lahr, Germany) at 40000 rpm, washed with 100 mM potassium phosphate buffer at pH 7.8, containing 2 mM EDTA and 1 mM DTT, and centrifuged again (Sorvall GS3, 5000 rpm, 7 min, 4°C). About 195 g cells (wet weight) was obtained, corresponding to 5.3 g/l culture.

The cells were resuspended (0.5 ml buffer for 1 g wet mass) in 100 mM potassium phosphate buffer at pH 7.8, containing 5 mM EDTA, 1 mM DTT, 1.2 μg/ml DNAse, 0.4 μg/ml RNAse. About 100 g wet cells was disrupted by sonification (Branson Sonifier 250, 2×2 min, 50% pulse, output control 5–6, 0°C). The resulting homogenate was centrifuged (Sorvall GSA, 12 000 rpm, 45 min, 4°C). According to SDS-PAGE, about equal amounts of tHisF were found in the insoluble and soluble fractions. The supernatant was heated to 75°C for 15 min and centrifuged (Sorvall GSA, 12 000 rpm, 60 min, 4°C) and the pellet discarded. The supernatant was dialysed against 10 mM potassium phosphate, pH 7.8, containing 2 mM EDTA and 1 mM DTT, and loaded on a DEAE-Sepharose fast-flow column (5×29.6 cm, Pharmacia) that was equilibrated with the same buffer, at 4°C. The column was washed with two volumes of equilibration buffer and bound proteins were eluted with 3 l of a linear gradient of 10–500 mM potassium phosphate at pH 7.8, containing 1 mM DTT. tHisF eluted between 220 mM and 255 mM potassium phosphate, as judged from SDS-PAGE and conductivity measurements. Fractions containing tHisF were pooled, dialysed against 10 mM potassium phosphate at pH 7.5 containing 1 mM DTT, loaded on a hydroxylapatite column (3.6×20 cm) and eluted as described above for the DEAE-Sepharose fast-flow column. tHisF eluted between 15 and 85 mM potassium phosphate. The main UV-absorbing peak contained tHisF with a purity above 95%, as judged by SDS-PAGE and reversed phase HPLC. The purification yielded approximately 2.3 mg tHisF/g wet cells, corresponding to 0.2 l cell culture. Fractions containing pure tHisF were pooled, dialysed against 50 mM potassium phosphate at pH 7.0, containing 2 mM EDTA and 1 mM DTT, concentrated to 31 mg/ml by ultrafiltration (PM 10 membranes, Amicon) and shock-frozen in liquid nitrogen.

For crystallisation attempts, tHisF at a concentration of 16 mg/ml was dialysed against 10 mM Bis-Tris buffer at pH 7.2, containing 1 mM DTT and 1 mM EDTA.

2.3. Expression and purification of SeMet derivatives of tHisA and tHisF

Since the methionine-deficient strain B834(DE3)/pLys requires the use of pET vectors for the heterologous expression of SeMet-labelled proteins, the t*hisA* gene was subcloned into pET11c. To this end, t*hisA* was amplified by PCR using the plasmid pUN121/III as a template [5]. The oligonucleotide 5′-AAGGTGAT**CATATG**CTCGTT-GTCC-3′ with a *Nde*I site (in bold) was used as the 5′-primer and the oligonucleotide 5′-GAGACACG**GGATCC**TTCTTTTAGC-3′ introducing a *Bam*HI site (in bold) was used as the 3′-primer. The amplified DNA fragment was cloned into pET11c using the two new restriction sites to yield the plasmid pET11c-t*his*A.

E. coli B834(DE3)/pLys cells were transformed with pET11c-t*his*F or pET11c-t*his*A and streaked out on LB plates supplemented with 150 μg/ml ampicillin and 35 μg/ml chloramphenicol. For confirming methionine auxotrophy, two colonies from each LB plate harbouring cells with pET11c-t*his*F or pET11c-t*his*A were resuspended in Vogel-Bonner minimal medium [14], supplemented per l with 1 mg thiamine, 2 g glucose, 150 mg ampicillin, 35 mg chloramphenicol, a heavy metal mix containing 10 mg $MoNa_2O_4$, 2 mg $CoCl_2$, 2 mg $CuSO_4{\cdot}5H_2O$, 10 mg $MnCl_2{\cdot}4H_2O$, 10 mg $ZnCl_2$, 5 mg $FeSO_4{\cdot}7H_2O$, 50 mg $CaCl_2{\cdot}2H_2O$, 10 mg H_3BO_3, an amino acid mix containing 50 mg of each tryptophan, tyrosine, valine, isoleucine, leucine, serine, cysteine, aspartate, glutamate, lysine, arginine, histidine, alanine, threonine and optionally 50 mg methionine or D/L-SeMet, and streaked out on minimal medium plates with or without methionine. Clones with a confirmed methionine auxotrophy were used to inoculate 50 ml of minimal medium containing methionine. These pre-cultures were shaken at 200 rpm and 37°C for 20 h. Two 10 ml portions were used to inoculate two 1 l cultures each containing the same medium and shaking was continued at 100 rpm and 37°C. After the OD_{600} had reached a value of 0.6, the cultures were centrifuged (Sorvall RC-3B, 4500 rpm, 30 min, 4°C) and the cells were resuspended in the same volume of pre-warmed minimal medium lacking methionine. The cultures were then incubated for another 8 h under the same conditions for consumption of residual methionine. During this incubation, the OD_{600} increased only slightly to a value of 0.62. 50 mg D/L-SeMet was added and the culture was shaken for another 2 h, during which the OD_{600} increased to a value of 0.8. Expression of SeMet containing tHisA ([SeMet]tHisA) or tHisF ([SeMet]tHisF) was induced by adding IPTG to a final concentration of 1 mM, followed by incubation for another 12 h. Cells were centrifuged (Sorvall RC-3B, 4500 rpm, 30 min, 4°C), washed with 100 mM potassium phosphate buffer at pH 7.8, containing 2 mM EDTA and 5 mM DTT, and centrifuged again (Sorvall RC-5Cplus, SS34 rotor, 8000 rpm, 20 min, 4°C). About 3.8 g of [SeMet]tHisA and 6.4 g of [SeMet]tHisF wet cells were obtained from 2 l cultures each.

All buffers contained 5 mM DTT during purification of both [SeMet]tHisA and [SeMet]tHisF in order to prevent oxidation of SeMet [15]. The purification of [SeMet]tHisA followed the established procedure for wild-type tHisA as described above. The only change in the purification protocol was the use of a smaller DEAE-Sephacel column (3.5×25.4 cm), with a total gradient volume of 1.5 l. Peak fractions of pure [SeMet]tHisA eluting from the Red Sepharose column were pooled and dialysed against 10 mM potassium phosphate at pH 7.5, containing 1 mM EDTA and 5 mM DTT. The purification

yielded 13.3 mg of [SeMet]tHisA per g of wet cells. [SeMet]tHisA was concentrated to 17 mg/ml and shock-frozen in liquid nitrogen.

About 80% of [SeMet]tHisF was found in the soluble fraction of the cell homogenates, in contrast to only about 50% in the case of native tHisF. [SeMet]tHisF was purified essentially as described for native tHisF, with the following exceptions. A smaller DEAE-Sepharose fast-flow column (2.5×29 cm) was used, with a total gradient volume of 0.9 l. Furthermore, the last purification step with hydroxylapatite was omitted. Peak fractions of pure [SeMet]tHisF from the DEAE-Sepharose column were pooled and dialysed against 10 mM potassium phosphate at pH 7.5, containing 2 mM EDTA and 5 mM DTT. The purification yielded 11.7 mg of [SeMet]tHisF per g wet cells. [SeMet]tHisF was concentrated to 24.6 mg/ml (PM10 membranes, Amicon) and shock-frozen in liquid nitrogen.

2.4. Reversed phase HPLC

Reversed phase HPLC was performed on a Hewlett Packard HPLC workstation (TI-SERIES 1050). The protein samples were acidified with 0.1% (v/v) TFA and aggregated material was removed by centrifugation. The supernatants (total volume of 150 μl containing 35 μg tHisA, 42 μg [SeMet]tHisA, 100 μg tHisF or 160 μg [SeMet]tHisF each) were loaded on a Vydac C18 218TP54 column, eluted with a gradient of 1.6–64% acetonitrile in 0.08% (v/v) TFA at flow rates of 1 ml/min and monitored at 278 nm.

2.5. Electrospray mass spectrometry

Electrospray mass spectrometry was performed with tHisF and [SeMet]tHisF. Mass determinations were carried out on a TSQ7000 triple quadrupole mass spectrometer (Finnigan, San José, CA, USA). All measurements were carried out in the positive ion mode, scanning between 200 and 2000 Da. The resolution of the instrument was set to 1 Da. The protein was introduced into a micro ion source [16] by injection onto a 100 μm in diameter capillary column packed with POROS R2 (20 μm particle size, 3–5 cm in length). Desorption was performed with a 10 min gradient consisting of 0–80% acetonitrile in 0.02% acetic acid at flow rates of 100–200 nl/min. The spray voltage was set to 1100 V.

2.6. Crystallisation and preliminary X-ray characterisation

Crystallisation trials were set up as vapour diffusion experiments using the hanging drop method on siliconised cover slips over 1 ml reservoir limbro multi-well tissue culture plates (ICN Biomedicals, Eschwege, Germany). Crystallisation drops consisted of 2.0 μl concentrated protein as described above and 2.0 μl reservoir solution and trials were maintained at 20°C. Crystallisation conditions were screened by the protocol of Jancarik and Kim [17] using the multifactorial crystal screens (Hampton Research, Riverside, USA). Initial successful crystallisation conditions were further optimised by varying the concentration of protein, precipitant and buffer system as well as the pH. Some crystals were further improved by the macroseeding method [18]. Seeds were obtained by either crushing larger twinned crystals or by using smaller ones. The concentration of the precipitant in macroseeding experiments was lowered by 50%.

The diffraction properties of crystals were investigated by collecting test X-ray images. Crystals were mounted into thin-walled capillaries with diameters between 0.4 and 1.0 mm (Mueller, Berlin, Germany). Alternatively, crystals were mounted into nylon cryo loops (Hampton Research, Riverside, USA) and shock-frozen in a nitrogen stream at 100 K (Oxford Cryosystem, UK). Test images were recorded on one of the protein crystallography synchrotron beam lines at the EMBL Hamburg Outstation.

3. Results

3.1. Heterologous expression and purification of tHisA, tHisF and their SeMet derivatives

Different expression plasmids, pDS56/RBS II and pET11c, were used for the production of tHisA and tHisF in *E. coli*. In the pDS system, proteins are expressed under the control of a lac promoter/repressor system, in which the expression can be induced by IPTG [10]. In the pET system, proteins are expressed from a phage T7 promoter, the phage T7 polymerase being encoded on the chromosome of the host cells [13].

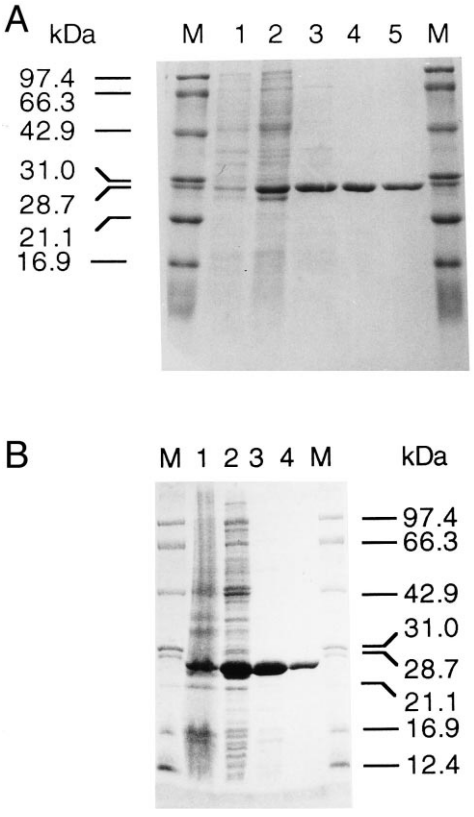

Fig. 1. Purification of [SeMet]tHisA (A) and [SeMet]tHisF (B) from transformed *E. coli* cells monitored by electrophoresis on 12.5% polyacrylamide gels in the presence of SDS. Lanes M, standard proteins with molecular weights given in kDa; lanes 1, insoluble fractions of the cell extracts; lanes 2: soluble fractions of the cell extracts; lanes 3: supernatants after heat steps (15 min, 75°C); lanes 4: eluates from DEAE-Sephacel FF (A) or DEAE-Sepharose FF (B) column; lane 5: eluate from Red Sepharose FF column.

About 90% of tHisA and 50% of tHisF were found in the soluble fractions of the cell homogenate. Since slow refolding and a high tendency to aggregate often hamper the refolding of thermostable proteins ([19], Sterner, unpublished observations), both tHisA and tHisF were purified from the soluble extracts only. The main purification step for both enzymes was the heating of the extracts to 75°C for 15 min, which removed more than 80% of the *E. coli* host proteins. Subsequent purification steps included anion exchange chromatography, followed by affinity chromatography on Red Sepharose for tHisA and hydroxylapatite chromatography for tHisF. The final yields were 1.15 mg of tHisA and 2.3 mg of tHisF per g wet cells.

The molar extinction coefficients of tHisA and tHisF at 280 nm were determined from the amino acid sequence [20]. For tHisA, a value of $\varepsilon_{280} = 12\,950\ M^{-1}\ cm^{-1}$ was calculated. This value is similar to $\varepsilon_{280} = 11\,060\ M^{-1}\ cm^{-1}$ as determined by second derivative spectroscopy according to Levine and Federici [21]. With a molecular mass of 27 031 Da [5], this corresponds to $A_{280}^{0.1\%} = 0.41\ cm^2\ mg^{-1}$. For tHisF, a value of $\varepsilon_{280} = 11\,500\ M^{-1}\ cm^{-1}$ was calculated. With a molecular mass of 27 719 Da [5], this corresponds to $A_{280}^{0.1\%} = 0.42\ cm^2\ mg^{-1}$.

The SeMet derivatives [SeMet]tHisA and [SeMet]tHisF were expressed using the pET system and *E. coli* B834(DE3)

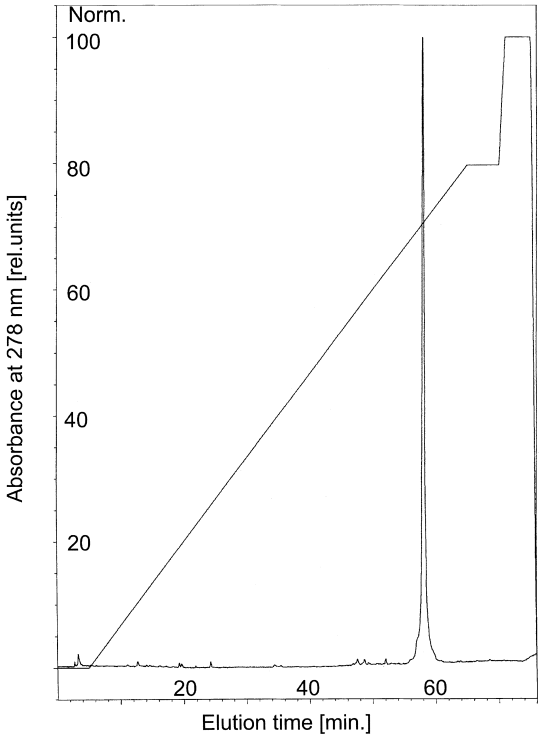

Fig. 2. Reversed phase HPLC testifies to a high purity of [SeMet]-tHisF. Elution of 160 μg protein was performed at 1 ml/min with an acetonitrile gradient (1.6–64%, dotted line) in 0.08% trifluroacetic acid and monitored on-line at 278 nm. [SeMet]tHisF eluted as a symmetrical peak at 58 min and integration of the peak areas yielded a purity of at least 98%.

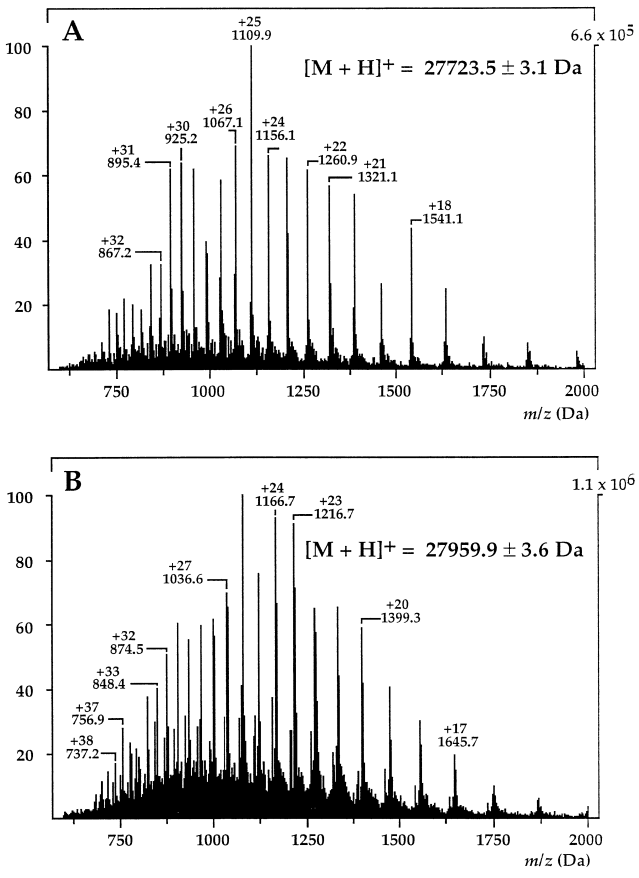

Fig. 3. [SeMet]tHisF is completely labelled with SeMet. Electrospray mass spectra of tHisF (A) and [SeMet]tHisF (B) were obtained on a TSQ7000 triple quadrupole mass spectrometer. The labelled peaks indicate the charge states of measured *m/z* values of the two proteins. $[M+H]^+$ indicates the mass of the two proteins (27 723.5 ± 3.1 Da for tHisF and 27 959 ± 3.6 Da for [SeMet]tHisF) as obtained after deconvolution of the measured *m/z* values. The mass difference of 236 Da shows that all five methionine residues in tHisF are completely replaced by SeMet in [SeMet]tHisF.

cells containing the plasmid pLys, which encodes the phage T7 lysozyme. T7 lysozyme is an inhibitor of phage T7 polymerase, providing a tight repression of transcription in the absence of IPTG. The yields for [SeMet]tHisA and [SeMet]tHisF were 13 mg and 12 mg per g wet cells, which is about 10 and five times higher compared to the native proteins. These large differences are probably due to the small scale expression of [SeMet]tHisA and [SeMet]tHisF being carried out in glass flasks, instead of the large scale expression of the native proteins being carried out in a fermenter. In addition, for [SeMet]tHisA, the use of a pET instead of the pDS plasmid could have resulted in a higher expression level and for [SeMet]tHisF, the omission of the hydroxylapaptite column might have reduced the loss of protein during purification.

3.2. Purity and SeMet content of proteins

Fig. 1 shows that the purity of [SeMet]tHisA and [SeMet]-tHisF followed by SDS-PAGE is at least 90%. Similar results were obtained for native tHisA and tHisF (data not shown). Reversed phase HPLC, performed with [SeMet]tHisF and using an acetonitrile gradient for elution (Fig. 2), gives a purity better than 98%. Similar purities were obtained also for [SeMet]tHisA, tHisA and tHisF (data not shown).

Electrospray mass spectrometry was performed with native tHisF and [SeMet]tHisF to analyse the content of SeMet (Fig. 3). The observed molecular masses were 27 723.5 ± 3.1 Da for tHisF and 27 959 ± 3.6 Da for [SeMet]tHisF. The difference between the two masses (236 Da) corresponds accurately to the mass difference expected for replacing the five methionines of tHisF completely by SeMets.

3.3. Crystals of tHisA

Crystals of tHisA were obtained under four different crys-

Table 1
Crystallisaton conditions

Name	Precipitant	Buffer	Additives
tHisA-1	25% (v/v) PEG MME 500	0.1 M MES/NaOH, pH 6.5	10 mM $ZnSO_4$, 10% (v/v) glycerol
tHisA-2	18% (w/v) PEG 8000	0.1 M Na-cacodylate/HCl, pH 6.6	200 mM $Ca(Ac)_2$
tHisA-3	29–35% (w/v) PEG 4000	0.1 M citrate, pH 5.6	200 mM NH_4Ac, 3% (v/v) MPD
tHisA-4	28% (w/v) PEG 4000	0.1 M citrate, pH 5.6	200 mM $NH_4(H_2PO_4)$, 3% (v/v) MPD
tHisF	0.9–1.2 M $NH_4(H_2PO_4)$	0.1 M citrate, pH 5.6	

Table 2
Crystal properties

Crystal form	Morphology	Space group	Cell parameters			Diffraction limit (Å)	V_M (Å^3/Da)	Number of mol per asymmetric unit
			a (Å)	b (Å)	c (Å)			
tHisA-1	Hexagonal plates	$C222_1$	47.3	81.2	121.9	2.5	2.2	1
tHisA-2	Rectangular plates	$P2_12_12_1$	77.5	103.6	135.3	3.5	2.5	4
tHisA-3	Rods	$P2_1$ ($\beta = 98.1°$)	46.4	72.9	62.0	1.8	1.9	2
tHisA-4	Pyramides	$P2_12_12_1$	70.6	78.5	212.9	1.9	2.7	4
tHisF	Squares	C2 ($\beta = 112°$)	79.6	44.4	63.9	1.0	2.0	1

tallisation conditions within the pH range from 5.6 to 6.6 and were always comprising polyethyleneglycols (PEG) of a different molecular weight as precipitants (Table 1). The main differences are the nature of the ionic additives. Since the substrate of tHisA, 5′-ProFAR, contains two phosphate groups, it is expected that in tHisA-1 and tHisA-4, sulfate or phosphate anions are bound to the active site, respectively. All the four crystal forms display distinct habits and crystallographic parameters (Table 2). Similarities in the unit cell dimensions indicate related packing arrangements with permutations of the crystallographic axes. Crystal forms tHisA-1 and tHisA-3 have similar unit cell dimensions of about 47 Å × 80 Å × 62 Å (c/2 in crystal tHisA-1). The crystal packing parameter V_M [22] suggests one and two molecules per asymmetric unit for tHisA-1 and tHisA-3, respectively. Crystal forms tHisA-2 and tHisA-4 both have $P2_12_12_1$ symmetry and related cell dimensions of 78 Å (a in tHisA-2, b in tHisA-4) × 70 Å (c/2 in tHisA-2, a in tHisA-4) × 105 Å (b in tHisA- 2, c/2 in tHisA-4). Since crystal forms 3 and 4 display superior diffraction qualities, they will be further used for structural analysis. These two crystal forms have been obtained and tested with [SeMet]tHisA too.

3.4. Crystals of tHisF

Crystals of tHisF and [SeMet]tHisF were obtained with ammonium phosphate as precipitant (Table 1). Initial crystals grew within days as small multicrystalline clusters. Single crystals, obtained by macroseeding, grew to a maximum size of 400 μm × 250 μm × 250 μm. These tHisF crystals diffract to about 1.0 Å resolution (Table 2). Interestingly, their unit cell parameters are similar to those of tHisA-3 crystals, suggesting similarities in the shape of the molecules and crystal packing arrangement.

4. Discussion

4.1. Use of thermostable proteins for X-ray crystallography

Tryptophan biosynthesis is the first amino acid biosynthetic pathway for which the crystal structures of all enzymes have been determined ([3] and citations within, [23]) or where structure determination is in progress (Mayans, O., Ivens, A., Kirschner, K. and Wilmanns, M., unpublished results). In the tryptophan biosynthesis, progress in structure determination was hampered for many years by difficulties in crystallising enzymes from mesophilic species. The switch to tryptophan enzymes from hyperthermophiles such as *Sulfolobus sulfataricus* and *T. maritima* facilitated the crystallisation and structure determination of unliganded structures as well as of various ligand complexes. Based on this experience with tryptophan enzymes, we chose thermostable variants in order to grow crystals for structure determination of two enzymes of the histidine biosynthesis. The efficiency of purification and crystallisation of tHisA and tHisF from the hyperthermophile *T. maritima* confirms our expectations, underscoring the specific advantages of proteins from hyperthermophiles for X-ray crystallography. Hyperthermophilic proteins display a high solubility and overall rigidity at temperatures where crystallisation is performed [24]. We speculate that these properties contribute to the increased tendency of thermostable proteins to yield excellent crystals with a higher probability than their thermolabile orthologues.

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