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HdcB, a novel enzyme catalysing maturation of pyruvoyl-dependent histidine decarboxylase

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Summary

Pyruvoyl-dependent histidine decarboxylases are produced as proenzymes that mature by cleavage followed by formation of the pyruvoyl prosthetic group. The histidine decarboxylation pathway of *Streptococcus thermophilus* CHCC1524 that consists of the pyruvoyl-dependent histidine decarboxylase HdcA and the histidine/histamine exchanger HdcP was functionally expressed in *Lactococcus lactis*. The operon encoding the pathway contains in addition to the *hdcA* and *hdcP* genes a third gene *hdcB*. Expression of different combinations of the genes in *L. lactis* and *Escherichia coli* followed by analysis of the protein products demonstrated the involvement of HdcB in the cleavage of the HdcA proenzyme. The HdcA proenzyme and HdcB protein were purified to homogeneity and cleavage and activation of the histidine decarboxylase activity was demonstrated *in vitro*. Substoichiometric amounts of HdcB were required to cleave HdcA showing that HdcB functions as an enzyme. In agreement, expression levels of HdcB in the cells were low relative to those of HdcA. The turnover number of HdcB *in vitro* was extremely low (0.05 min⁻¹) which was due to a very slow association/dissociation of the enzyme/substrate complex. In fact, HdcB was shown to co-purify both with the HdcA S82A mutant that mimics the proenzyme and with the mature HdcA complex.

Introduction

Histidine decarboxylation by food-borne bacteria causes histamine spoilage of traditionally fermented foods, such

as cheese and wine (Silla Santos, 1996; Lonvaud-Funel, 2001). The histidine decarboxylation pathway involves the uptake of histidine from the environment, intracellular histidine decarboxylation and excretion of the product, histamine. The pathway consists of two enzymes, the transporter HdcP that mediates both uptake of histidine and excretion of histamine [precursor/product exchange (Molenaar *et al.*, 1993; Lucas *et al.*, 2005)] and the histidine decarboxylase HdcA that removes the α-carboxylate group of histidine (Van Poelje and Snell, 1990a). The pathway generates proton motive force (pmf) by a secondary mechanism (Lolkema *et al.*, 1996). Membrane potential is generated in the transport step in which monovalent histidine is exchanged for divalent histamine thereby transporting net positive charge outside the cell. Inside the cell, the decarboxylation reaction consumes a proton which results in alkalinization of the cytoplasm. Together, these two coupled processes generate an electrical potential (inside negative) and a transmembrane pH gradient (inside alkaline), i.e. proton motive force. Physiological relevances for the bacteria include generation of metabolic energy, acid stress resistance and alkalinization of the medium to counteract acidification by glycolysis.

Histidine decarboxylation is catalysed by two types of unrelated decarboxylases that use different prosthetic groups in their catalytic centre. Histidine decarboxylases found in Gram-negative bacteria and mammals use pyridoxal 5′-phosphate (Kamath *et al.*, 1991; Wu *et al.*, 2008), while histidine decarboxylases found in Gram-positive bacteria use a pyruvoyl moiety (Van Poelje and Snell, 1990a). The pyruvoyl-dependent enzyme of *Lactobacillus saerimneri* 30A (previously *Lactobacillus* 30A) was crystallized and the structure solved at atomic resolution (Parks *et al.*, 1985). The enzyme forms a dumbbell-shaped hexamer consisting of two trimers with threefold symmetry. The pyruvoyl group is formed by cleavage of the 34 kDa proenzyme, termed the π chain, between two conserved serine residues, Ser-81 and Ser-82, yielding an N-terminal, 9 kDa β subunit and a C-terminal, 25 kDa α subunit (Vaaler *et al.*, 1982; Recsei *et al.*, 1983; Huynh *et al.*, 1984). Cleavage proceeds through non-hydrolytic serinolysis with formation of an ester linkage intermediate, followed by β-elimination, which results in Ser-81 as the C-terminal residue of the β subunit and a dehydroalanine residue at the N-terminus of the α subunit. Subsequently,
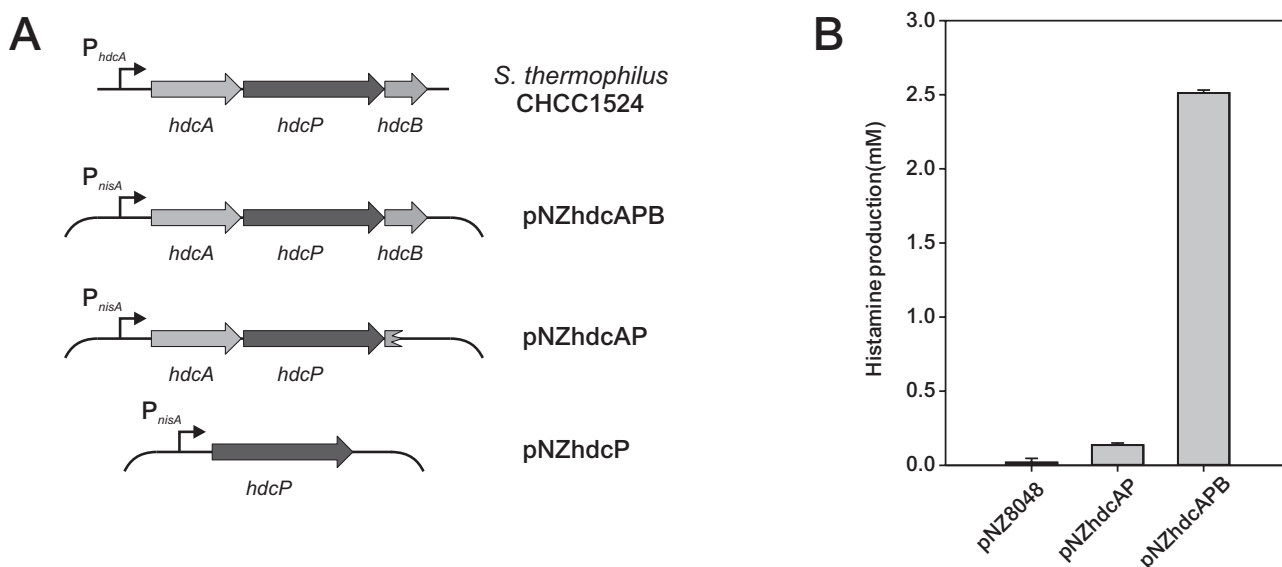
Fig. 1. Functional expression of the histidine decarboxylase pathway of *S. thermophilus* CHCC1524 in *L. lactis* NZ9000.
A. Genetic organization of the *hdcAPB* cluster and derived constructs.
B. Histidine decarboxylase activity by resting cells of *L. lactis*. Histamine produced by cells harbouring plasmids pNZ8048 (control), pNZ-hdcAP and pNZ-hdcAPB as indicated. Cells were resuspended to an OD_{600} of 5.0 in 50 mM KPi pH 6, 10 mM glucose and 25 mM histidine and incubated at 37°C for 1 h. The cell suspension was centrifuged and the histamine concentration in the supernatant determined.

hydrolysis of the dehydroalanine group generates the pyruvoyl group while releasing NH_3 (Recsei *et al.*, 1983; Van Poelje and Snell, 1990a). Since the conversion of the proenzyme (π_6) to the mature enzyme ($\alpha_6\beta_6$) was accompanied by only a 1.9 Å increase in the distance between residues 81 and 82, but no other structural changes, the activation site of the proenzyme was suggested to resemble the active site of the mature enzyme (Van Poelje and Snell, 1990). *In vitro*, maturation of histidine decarboxylase of *L. saerimneri* 30A was a slow, spontaneous process and it was suggested that *in vivo* other factors or conditions might be present to speed up the process.

Histidine decarboxylation is a rather rare trait among Gram-positives that is typically strain specific. Histidine decarboxylases have been identified in strains of the lactobacillales *L. saerimneri*, *Lactobacillus hilgardii*, *Lactobacillus buchneri*, *Oenococcus oeni*, *Tetragenococcus muriaticus* and *Tetragenococcus halophilus* (Vanderslice *et al.*, 1986; Coton *et al.*, 1998; Konagaya *et al.*, 2002; Lucas *et al.*, 2005; Martin *et al.*, 2005; Satomi *et al.*, 2008), the bacillales *Clostridium perfringens* and *Staphylococcus capitis* (Van Poelje and Snell, 1990b; De las Rivas *et al.*, 2008) and in the actinomycete *Micrococcus* sp. n. (Prozorovski and Jornvall, 1974). More recently, a histamine-producing strain of the lactic acid bacterium *Streptococcus thermophilus* was identified and the histidine decarboxylation gene cluster was characterized (Calles *et al.*, 2010). The cluster consisted of *hdcA* and *hdcP*, encoding the decarboxylase and transporter, respectively, and a third gene *hdcB* of unknown function.

The HdcA of *S. thermophilus* CHCC1524 shares 67% amino acid sequence identity with that of *L. saerimneri* 30A and the two serine residues at the presumed cleavage site were found at the same positions 81 and 82. The HdcB protein was found before in the *hdc* gene clusters of *Lactobacilli*, *O. oeni*, *T. muriaticus* and *T. halophilus* (Vanderslice *et al.*, 1986; Copeland *et al.*, 1989; Coton *et al.*, 1998; Konagaya *et al.*, 2002; Lucas *et al.*, 2005; Martin *et al.*, 2005; Satomi *et al.*, 2008) but not in those of *C. perfringens* and *S. capitis*.

In this study, the *hdcAPB* cluster of *S. thermophilus* CHCC1524 was functionally expressed in the heterologous host *Lactococcus lactis* and shown to encode a histidine decarboxylation pathway. It is shown that HdcB catalyses maturation of the decarboxylase which results in the formation of the pyruvoyl prosthetic group.

Results

Functional expression of the histidine decarboxylation pathway of S. thermophilus *CHCC1524 requires HdcB*

The histidine decarboxylation gene cluster of *S. thermophilus* strain CHCC1524 consists of the genes *hdcA* and *hdcP*, encoding the putative histidine decarboxylase and histidine/histamine exchanger, respectively, and *hdcB* encoding a protein of unknown function (Fig. 1A) (Calles *et al.*, 2010). The two genes encoding the histidine decarboxylation pathway, *hdcA* and *hdcP*, as well as the complete *hdcAPB* cluster including *hdcB* were cloned in their
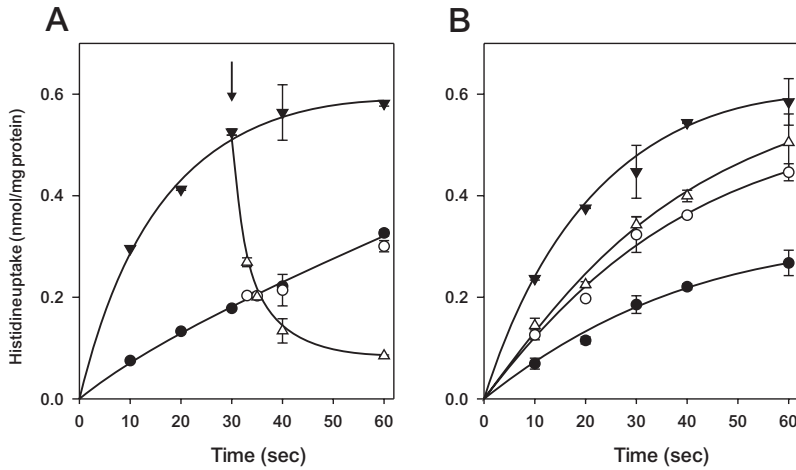
Fig. 2. Histidine uptake by resting cells of *L. lactis* NZ9000 harbouring pNZ-hdcP (▼), pNZ-hdcAPB (B, △), pNZ-hdcAP (B, ○) and pNZ8048 (control, ●). ^{14}C-labelled histidine was added to 1.5 μM final concentration. Unlabelled histamine at a concentration of 100 μM was added after 30 s to cells harbouring plasmids pNZ-hdcP (A, △) and pNZ8048 (A, ○).

native arrangement in pNZ8048 for nisin-inducible expression from the P$_{nisA}$ promoter in *L. lactis* NZ9000 (NICE system) (Kuipers *et al.*, 1998). Nisin-induced cells of *L. lactis* harbouring plasmids pNZ-hdcAPB, pNZ-hdcAP or the empty vector pNZ8048 were incubated for 1 h at 30°C in the presence of 25 mM histidine and 10 mM glucose and the supernatants were assayed for the presence of histamine. Cells expressing the three proteins HdcA, HdcP and HdcB produced 2.5 mM histamine, while cells expressing only the two metabolic enzymes HdcA and HdcP produced an order of magnitude less histamine (Fig. 1B). No significant amount of histamine was detected in the supernatant of the control cells or in the recombinant cells when no histidine was added to the buffer (not shown). The results demonstrate the functional expression of the complete histidine decarboxylation pathway of *S. thermophilus* in the heterologous host *L. lactis*, as well as an important role for the *hdcB* gene in the pathway. In a second construct containing the complete *hdcAPB* cluster, a 600 bp promoter region upstream of *hdcA* was included. *L. lactis* cells harbouring this vector, pNZ-PhdcAPB, showed histidine decarboxylation activity when the cells where grown in the absence of the inducer nisin, indicating that the *S. thermophilus* promoter was active in *L. lactis*. In line with messenger levels determined before (Calles *et al.*, 2010) the activity of the pathway increased two- to threefold when the cells were allowed to grow to stationary phase as compared with exponential growing cells (data not shown).

Expression of *hdcA* and *hdcP* in *L. lactis* measured by semi-quantitative RT-PCR was not significantly changed after deletion of *hdcB* from the construct (not shown) suggesting a role of HdcB at a post-translational level, either affecting the decarboxylase (HdcA) or affecting the histidine/histamine exchanger (HdcP). The *hdcP* gene was cloned in vector pNZ8048 yielding plasmid pNZ-hdcP. Resting cells of *L. lactis* harbouring pNZ-hdcP were assayed for histidine uptake using ^{14}C-labelled histidine.

At a concentration of 1.5 μM a significantly higher initial rate of histidine uptake was observed than in control cells containing the empty vector demonstrating functional expression of HdcP (Fig. 2A). Addition of excess unlabelled histamine after the cells were allowed to take up ^{14}C-histidine to a steady-state level resulted in rapid release of radioactivity from the cells. Adding the same concentration of histamine to the control cells had no effect on the accumulation level. It follows that HdcP of *S. thermophilus* effectively catalyses histidine/histamine exchange. The data resemble the results obtained with HdcP of *L. hilgardii* 0006 (Lucas *et al.*, 2005) that shares 45% amino acid sequence identity with the *S. thermophilus* HdcP. Histidine uptake by cells containing HdcP together with HdcA or HdcA plus HdcB expressed from plasmids pNZ-hdcAP and pNZ-hdcAPB, respectively, was significantly higher than by the control cells, showing functional expression from these constructs. The initial rate of uptake was lower than in cells expressing *hdcP* alone, but, more importantly, not significantly different in the presence or absence of HdcB indicating that the activity of HdcP is not affected by the HdcB protein (Fig. 2B).

HdcB catalyses cleavage of the HdcA proenzyme. Activation of the HdcA proenzyme (π chain) involves cleavage between two serine residues resulting in the α and β subunits of 25 and 9 kDa respectively. The gene encoding green fluorescent protein (GFP) was inserted at the 5′ end of the *hdcA* gene in construct pNZ-PhdcAPB. The construct encodes, in addition to the HdcP and HdcB proteins, a hybrid protein consisting of HdcA with GFP fused at the N-terminus which allows for visualization of the protein by in-gel fluorescence imaging following SDS-PAGE. Two additional constructs were derived from the plasmid by deleting, subsequently (major parts of) the *hdcB* and *hdcP* genes (Fig. 3A). Crude cell extracts of *L. lactis* cells harbouring the constructs revealed fluorescent bands running at apparent molecular masses of ~27
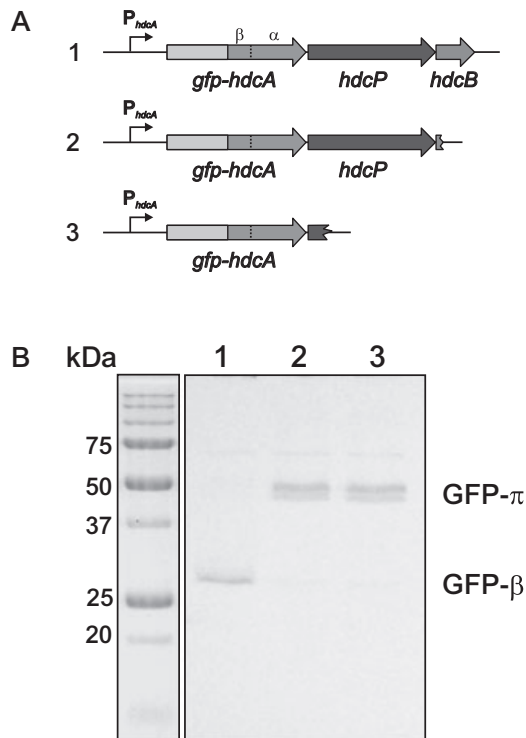
Fig. 3. In-gel fluorescence of GFP–HdcA translational fusions.
A. Schematic diagram of the GFP fusion constructs. In the first gene, the GFP (light grey), β subunit and α subunit encoded parts were indicated.
B. Fluorescence image of a standard 12% SDS-PAGE gel loaded with 15 μl of cell extracts of *L. lactis* harbouring constructs 1–3 indicated in (A) as indicated (right, lanes 1–3). The left shows the Coomassie Brilliant Blue-stained part of the gel containing the marker proteins with molecular masses in kDa as indicated. Bands corresponding to GFP-π chain and GFP-β subunit were indicated. Cell extracts were prepared by resuspending cells grown to stationary phase in 1/50 of the culture volume. Lysozyme was added to 0.5 μg ml^{-1} followed by incubation at 37°C for 30 min and solubilization in a 5× SDS-PAGE resuspension buffer adjusted to pH 8. The gel was exposed to a LAS-4000 luminescent image analyser (Fujifilm), with excitation at 460 nm and using a 510 nm cut-off filter.

and ~50 kDa (Fig. 3B). The high molecular mass was observed when HdcA was expressed alone or in the presence of HdcP, the lower mass when also HdcB was present, strongly suggesting that HdcB was responsible for cleavage of the π chain. In the absence of HdcB, a small fraction of less than 5% of the total GFP signal was found with the same mobility as the smaller product, suggesting a low level of spontaneous cleavage. The mobility of the GFP-π and GFP-β hybrids was higher than expected based on the calculated masses (61 kDa and 35.7 kDa respectively).

The *hdcA* and *hdcB* genes were cloned separately in a pBAD24 (Guzman *et al.*, 1995) derived vector with an N-terminal His-tag and enterokinase cleavage site coding sequence, for arabinose-induced expression in *Escherichia coli* (pBADH-hdcA and pBADH-hdcB respectively). In

addition, an artificial operon was constructed by cloning *hdcA* in tandem with the *hdcB* gene in the same vector (pBADH-hdcAB). His-tagged HdcA synthesized by *E. coli* DH5α cells harbouring plasmid pBADH-hdcA was purified by Ni^{2+}-NTA affinity chromatography (Fig. 4, lane 1). The purified protein appeared on SDS-PAGE with an apparent molecular mass of 37 kDa, corresponding to the uncleaved proenzyme. Coexpression of HdcB (without His-tag) resulted in the appearance of two products with apparent masses of 24 and 12 kDa corresponding to the α and β subunits (lane 2). A small fraction of HdcA was not cleaved. Co-purification of the α subunit with the His-tagged β subunit indicates strong association of the two in a complex. At the positions of the α and β subunits in the gel, minor bands were observed when HdcA was synthesized in the absence of HdcB (lane 1) indicating a low level of spontaneous cleavage, similarly as observed above in *L. lactis*.

Cleavage of HdcA by HdcB was also observed after synthesis in separate cell cultures. Combining equal amounts of cells containing HdcA (plasmid pBADH-hdcA) and cells containing HdcB (pBADH-hdcB) followed by Ni^{2+}-NTA affinity chromatography of the crude cell extract prepared from the mixture resulted in mainly the α and β subunits of HdcA (Fig. 4A, lane 3). Remarkably, His-tagged HdcB was hardly visible on the SDS-PAGE gel suggesting low levels of expression relative to HdcA and showing that only catalytic amounts of HdcB were required to cleave the HdcA protein pool.

Together, the experiments demonstrate that HdcB catalyses cleavage of HdcA. Cleavage occurred during coexpression in the heterologous hosts *L. lactis* and *E. coli* and also when the two proteins were synthesized separately. In both expression systems a low level of HdcB-independent cleavage was observed.

In vitro *maturation of HdcA catalysed by HdcB*

Over 95% pure HdcA of *S. thermophilus* was easily obtained in sufficient quantities in a single step by Ni^{2+}-NTA chromatography following expression of the His-tagged protein in *E. coli* (see Fig. 4A, lane 1). An adapted procedure in which imidazole was replaced by histidine as the eluent was developed to obtain cleavable HdcA after purification. The high concentrations of imidazole used in standard protocols irreversibly inhibited the cleavage of HdcA (not shown). Replacement by histidine which requires much lower concentrations for elution (see *Experimental procedures*) solved the problem. In contrast to HdcA, His-tagged HdcB was expressed to much lower levels in *E. coli* resulting in impure protein preparations following Ni^{2+}-NTA affinity chromatography (Fig. 4B, left lane). Strategies using different tags to improve the yield were not successful. The elution fractions obtained from
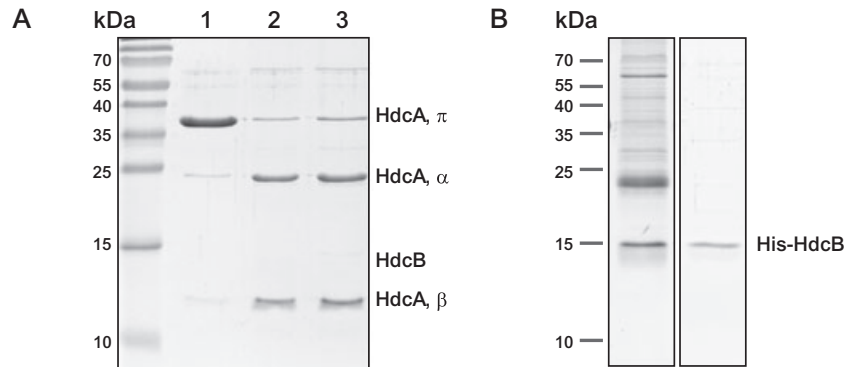
Fig. 4. Expression and purification of HdcA and HdcB in *E. coli*.
A. HdcA. Ni²⁺-NTA affinity chromatography purified HdcA from cells expressing His-tagged HdcA alone (lane 1), and coexpressed with HdcB (lane 2), and from cells expressing HdcA mixed with cells expressing HdcB (lane 3). Cells were broken by sonication. The mixture of cells expressing HdcA and HdcB was incubated for 1 h at 37°C after sonication.
B. HdcB. Partial purified HdcB obtained by Ni²⁺-NTA affinity chromatography from cells expressing His-tagged HdcB (left lane) and purified HdcB obtained by subsequent anion exchange chromatography (right lane; see *Experimental procedures* for details). Samples were loaded onto a standard 15% SDS-PAGE gel. At the left, molecular masses of marker proteins were indicated.

the Ni²⁺-NTA column were further purified by anion exchange chromatography using a HiTrap Q HP column. The HdcB protein did not bind to the resin under the conditions employed, whereas the major impurities did. A pure HdcB fraction was used for further *in vitro* experiments (Fig. 4B, right lane).

Purified HdcA proenzyme (π chain) was incubated with purified HdcB in an approximately 3:1 molecular ratio or with buffer as a control in 50 mM KPi pH 7, 40 mM NaCl. Samples taken at different times were analysed for cleavage of the π chain by SDS-PAGE (Fig. 5A) and for pyruvoyl group formation by measuring histidine decarboxylation activity (Fig. 5B). In the presence of HdcB, more than 50% of the HdcA proenzyme was processed into α and β subunit after 30 min, and approximately 90% after 90 min. A small fraction of HdcA remained uncleaved even after prolonged incubation. In the absence of HdcB, slow autocatalytic processing of HdcA was observed resulting in less than 5% cleavage after 90 min. The histidine decarboxylation activity correlated with the pattern of proenzyme cleavage, showing that HdcB catalyses *in vitro* the complete maturation of the HdcA enzyme. Low levels of histidine decarboxylation observed in the control samples correlate with the low levels of autocatalytic cleavage.

HdcB is associated with HdcA

The cleavage of HdcA proenzyme occurs between two serine residues followed by conversion of the N-terminal serine of the α subunit into the pyruvoyl group (Recsei *et al.*, 1983). The corresponding serine residue in the HdcA proenzyme of *S. thermophilus* was mutated to alanine (S82A). Purification of the mutant from *E. coli* cells revealed the uncleaved proenzyme (Fig. 6, lane 2)

without the low level of α and β subunits observed with wild-type HdcA (lane 1). Similarly, mutation of the corresponding serine in HdcA of *L. saerimneri* 30A into a cysteine or threonine strongly reduced spontaneous cleavage rates down to 3.3% and 1.4%, respectively (Vanderslice *et al.*, 1988), whereas mutation to alanine in *C. perfringens* HdcA prevented spontaneous cleavage completely (Van Poelje and Snell, 1990b). Expression of the S82A mutant of *S. thermophilus* together with (untagged) HdcB did not show any processed enzyme as well, while more than 90% was cleaved in the control experiment with wild-type HdcA (lane 4 and 3 respectively). Thus, the Ser-82 to Ala mutation prevents both spontaneous and HdcB catalysed cleavage of the π chain.

More interestingly, an additional protein running at an apparent molecular mass of 14 kDa was observed in the purified HdcA-S82A preparation when synthesized together with HdcB and not in the absence of HdcB (compare Fig. 6, lanes 4 and 2). The apparent molecular mass of the band corresponded to the molecular mass of untagged HdcB prepared from purified His-tagged HdcB treated by enterokinase (not shown). The band was confirmed to be HdcB by mass spectrometry analysis. Closer inspection revealed the same band in the purified wild-type HdcA preparation when produced in the presence of HdcB and not in its absence (lanes 3 and 1) but the intensity was lower. Apparently, HdcB which was synthesized without His-tag in these experiments, co-purified with the uncleaved S82A mutant and to a lesser extent with wild-type cleaved HdcA. The amounts of co-purified HdcB were substoichiometric relative to HdcA. The constructs used to produce the HdcA varieties together with HdcB were modified to introduce a His-tag at the N-terminus of HdcB which allowed for isolation of total
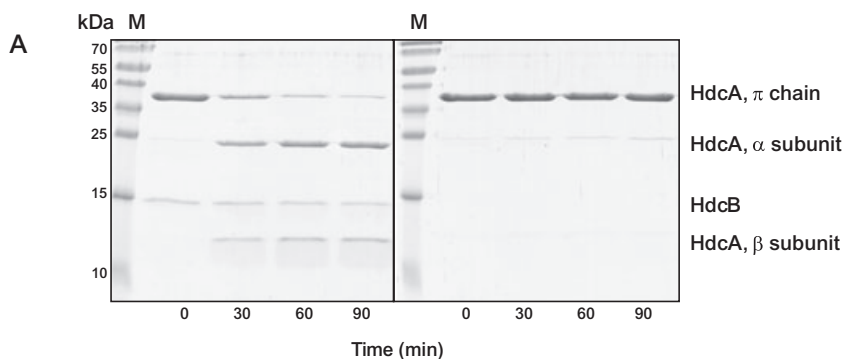
A

Fig. 5. *In vitro* cleavage and activation of HdcA catalysed by HdcB.

A. Purified HdcA was incubated with (left panel) or without (right panel) HdcB at 42°C and analysed for cleavage at the indicated time points by SDS-PAGE. Lanes marked M contain the marker proteins and the molecular masses in kDa were indicated.

B. Histamine produced by samples taken at the indicated times. Amounts corresponding to 0.2 μg of HdcA were taken from the samples and added to 200 μl of 0.2 M ammonium acetate pH 4.8, containing 50 mM L-histidine, followed by 1 h incubation at 42°C. The reaction was stopped by incubation for 5 min at 90°C.

B
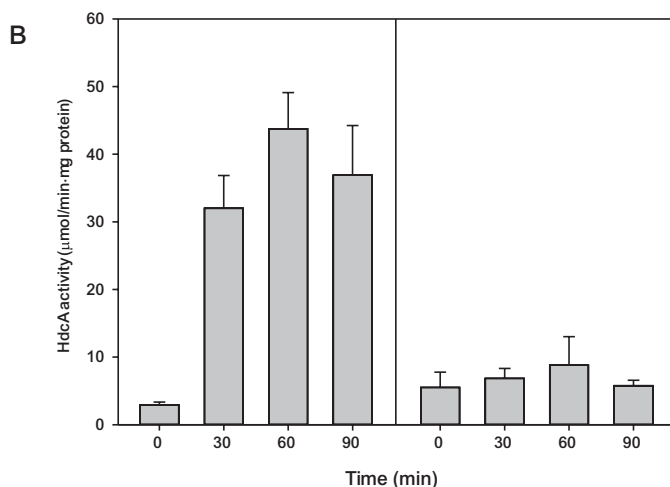
(bound plus free) HdcB produced by the cells. Following Ni^{2+}-NTA affinity chromatography, two additional bands appeared which both were identified by mass spectroscopy as HdcB. The two bands corresponded to His-tagged HdcB and HdcB without His-tag (Fig. 6, lane 5 and 6). Apparently, a fraction of the HdcB molecules lost the His-tag by proteolytic cleavage. The yield of HdcB was the same when the purification was performed in the presence of 0.1% SDS excluding a reduced yield caused by a buried His-tag or proteolytic activity in the crude cell extract. It follows that the (total) amount of HdcB purified when synthesized as a His-tagged version was similar to the amount co-purified with the HdcA mutant when synthesized without His-tag. It suggests that no pool of free HdcB is present in the cells, i.e. all HdcB is associated with HdcA, either processed or not, and that HdcB is produced at a significantly lower protein level than HdcA in these expression systems.
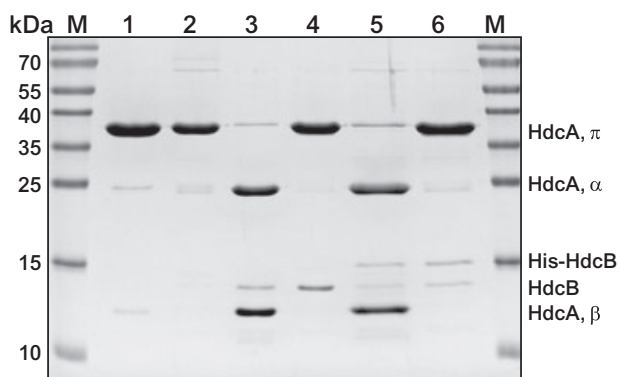

Fig. 6. Co-purification of HdcB with wild-type HdcA and the HdcA S82A mutant. SDS-PAGE analysis of His-tagged HdcA (lanes 1, 3, 5) and His-tagged HdcA S82A (lanes 2, 4, 6) purified from *E. coli* expressing HdcA alone (lanes 1, 2), together with HdcB (lanes 3, 4) and together with His-tagged HdcB (lanes 5, 6). Lane marked M contains the marker proteins with the corresponding molecular masses in kDa indicated.

Maturation does not affect the oligomeric state of HdcA

The oligomeric state of matured HdcA of *S. thermophilus* produced in *E. coli* in the presence of HdcB and purified by Ni^{2+}-NTA affinity chromatography (Fig. 4, lane 2) was determined by size exclusion chromatography (Fig. 7). In a buffer at pH 7, the protein eluted as a symmetric peak at an elution volume corresponding to a molecular mass of 107 kDa based on a calibration curve obtained with standard proteins of known masses. The calculated mass of a
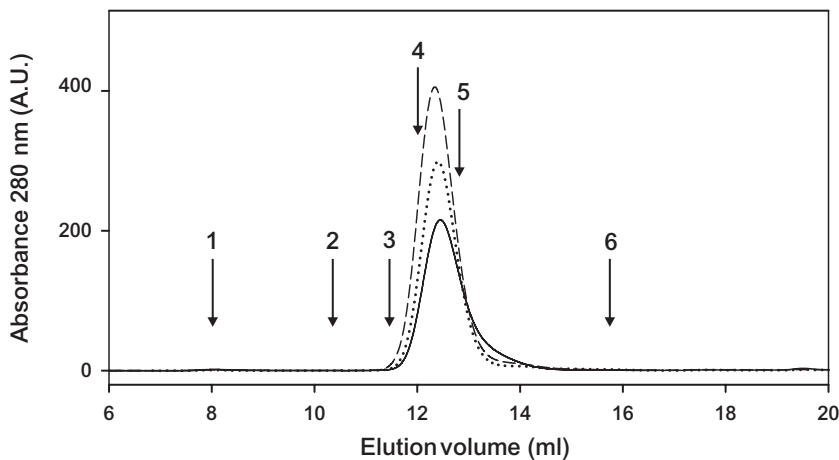
Fig. 7. Size exclusion chromatography of HdcA proenzyme (continuous line), matured HdcA (dotted line) and HdcA S82A (dashed line). The latter two contain small amounts of HdcB, co-purified during isolation (see Fig. 6). Elution positions of standard proteins and Blue Dextran (1) for calibration were indicated by arrows: 2, ferritin (443 kDa); 3, β-amylase (200 kDa); 4, alcohol dehydrogenase (150 kDa); 5, bovine serum albumine (66 kDa); 6, cytochrome c (12.4 kDa).

trimeric $(\alpha\beta)_3$ complex of the His-tagged enzyme is 107.7 kDa. The crystal structure of the *L. saerimneri* 30A HdcA revealed a dimer of loosely associated trimers (Van Poelje and Snell, 1990a). In solution, an equilibrium between trimers and hexamers was observed, shifting towards hexamers at lower pH (Hackert *et al.*, 1981). Nevertheless, the oligomeric state of matured HdcA of *S. thermophilus* was determined to be trimeric as well in a buffer of pH 5 (not shown).

At pH values of both 5 and 7, the proenzyme of HdcA and the S82A mutant were trimeric with elution volumes corresponding to molecular masses at pH 7 of 104 kDa and 112 kDa respectively (Fig. 7). Also, the presence of HdcB did not affect the oligomeric state of mature HdcA or the S82A mutant. The results strongly suggest that HdcA of *S. thermophilus* forms a trimeric $(\alpha\beta)_3$ complex and that a change of the oligomeric state is not part of the maturation process.

Discussion

The *hdcAPB* operon of *S. thermophilus* CHCC1524 encodes the histidine decarboxylase HdcA and the histidine/histamine exchanger HdcP, the enzyme and transporter, respectively, that constitute the histidine decarboxylation pathway, and the protein HdcB. Here it is shown that HdcB catalyses maturation of the pyruvoyl-dependent HdcA. *In vivo* experiments with the heterologously produced proteins in *L. lactis* and *E. coli* and *in vitro* experiments with purified HdcA proenzyme and HdcB demonstrate that the proenzyme is cleaved by HdcB yielding the α and β subunits and, subsequently, the pyruvoyl group is formed resulting in mature enzyme catalysing histidine decarboxylation. Size exclusion chromatography showed that uncleaved HdcA proenzyme, mature HdcA and the uncleavable mutant HdcA S82A, the latter two in presence of co-purified HdcB, form trimers, demonstrating that HdcB plays no role in the oligomeric

state of HdcA. In addition, *in vitro* cleavage of HdcA by HdcB after purification of the proenzyme indicates that HdcB cleaves the π chain after assembly of the trimer. Therefore, biogenesis in the cell, most likely, involves assembly of the complex followed by cleavage and formation of the pyruvoyl group. In agreement, the oligomeric structure of HdcA from *L. saerimneri* 30A, i.e. hexameric, was shown to be the same for the proenzyme and the mature enzyme and structural differences between the two were minor (Van Poelje and Snell, 1990a). Enzyme catalysed maturation of histidine decarboxylases is exceptional among pyruvoyl-dependent enzymes. Other well-studied enzymes that contain the pyruvoyl prosthetic group like *S*-adenosylmethionine decarboxylase (AdoMetDC) (Ekstrom *et al.*, 2001), L-aspartate α-decarboxylase of *E. coli* (Albert *et al.*, 1998) and arginine decarboxylase of *Methanococcus jannaschii* (Tolbert *et al.*, 2003) appear to mature by an autocatalytic cleavage mechanism. Even among histidine decarboxylases of Gram-positive bacteria, HdcB-catalysed activation of HdcA is not the only mechanism (see below).

The genetic organization of histidine decarboxylation loci among histamine-producing Gram-positive bacteria differs both in composition and gene order. Three types may be discriminated. The largest group described to date is found in the lactic acid bacteria *L. hilgardii* 0006, *L. buchneri* B301, *L. reuteri* F275 and *T. halophilus*. The gene cluster contains, in addition to *hdcP*, *hdcA* and *hdcB*, a fourth gene *hisRS* encoding histidyl-tRNA synthetase (Lucas *et al.*, 2005; Martin *et al.*, 2005; Satomi *et al.*, 2008). The role of the latter in the pathway is unknown. The *hdcP* and *hdcA* genes are separated by an approximately 300 bp intergenic region and are transcribed from different promoters, while for *hdcA* and *hdcB* bicistronic transcripts were found (Copeland *et al.*, 1989; Martin *et al.*, 2005). The *hisRS* gene is most likely transcribed from its own promoter. A second type, found in *C. perfringens* strain 13 and *S. capitis*, does not contain homologues of *hdcB* and *hisRS*

and the order of *hdcA* and *hdcP* is reverse, separated by only a small intergenic region (Shimizu *et al.*, 2002; De las Rivas *et al.*, 2008). Presumably both genes are under control of one promoter. Finally, in *S. thermophilus* CHCC1524, the organization of the *hdcA* and *hdcP* genes is as found in *C. perfringens* and *S. capitis* but in addition, the *hdcB* gene is present. HdcB of *S. thermophilus* is a somewhat smaller protein (14 kDa) than HdcBs of the other LAB in the first group (19.5 kDa). Overall sequence identity is relatively low (approximately 25%) but regions in the C-terminal part share much higher identities. A similar function of HdcB of *L. saerimneri* 30A is supported by purification of the uncleaved HdcA π chain from *E. coli* expressing the *hdcA* gene in the absence of the *hdcB* gene. The slow self-catalysed cleavage of the purified proenzyme into α and β subunits does not seem to be physiologically relevant (Copeland *et al.*, 1987). The authors suggested a missing activation factor. The absence of an *hdcB* homologue in the *C. perfringens hdc* cluster suggests that the HdcA proenzyme does not require a cofactor for cleavage. In agreement, following expression of the histidine decarboxylase of *C. perfringens* in *E. coli*, the protein appeared only in its matured form, i.e. as α and β subunits (Van Poelje and Snell, 1990b), suggesting that cleavage of the proenzyme is fully autocatalytic. Remarkably, HdcA of *S. capitis* which also lacks HdcB appeared mainly as uncleaved π chain after cloning in *E. coli* (De las Rivas *et al.*, 2008). Phylogenetically, HdcA of *S. capitis* is closer to HdcA in the clusters of the *lactobacilli* (containing *hdcB*) than to *C. perfringens* HdcA (59–66% and 47% identity respectively). Possibly, the *hdcA* and *hdcP* genes were recently acquired from this group without *hdcB* and the low level of autocatalysed proenzyme activation may have been a sufficient advantage for maintaining the genes.

Substoichiometric amounts of HdcB were sufficient to cleave HdcA both *in vitro* (Fig. 5) and *in vivo* (Fig. 6, lanes 3 and 5) which shows that HdcB functions as an enzyme; one HdcB molecule cleaves more than one HdcA molecule. In this context, the consistently lower levels of expression of HdcB relative to HdcA observed here may be of physiological relevance. Low levels of HdcB protein relative to HdcA were obtained when *hdcA* and *hdcB* were transcribed in *E. coli* from a single, artificial operon *hdcAB* (Figs 4A and 6), but also when *hdcB* was expressed alone (Fig. 4B). Since inefficient isolation or protein instability was not observed, HdcB expression appears to be inefficient at the level of translation, probably by intrinsic properties of the *hdcB* gene. It is likely that the same situation exists in *S. thermophilus* providing the organism with a mechanism to synthesize a catalytic amount of HdcB relative to HdcA.

At first sight, the co-purification of HdcB with HdcA seems to be at variance with the catalytic function of HdcB. The small amount of HdcB present in the cells relative to HdcA appears to be firmly bound to a fraction of HdcA both in the mature form and the S82A mutant that mimics the proenzyme (Fig. 6). A catalytic cycle would involve binding of HdcB to an HdcA subunit of the trimeric complex, cleaving of the π chain followed by dissociation of HdcB after which a new cycle may start. The usual scenario is that the enzyme (HdcB) looses affinity for the product (αβ) and the enzyme/product complex dissociates. Here, the stability of the proenzyme and mature forms in the complex do not seem to make a big difference. Even though the *in vitro* conditions are different, both HdcB–HdcA complexes are strong enough to survive the purification procedure, suggesting very slow dissociation rates and, consequently, low concentrations of free HdcB in the cell. The latter was confirmed by isolating all HdcB, free and bound, from the cells by His-tagging the protein. The amount of HdcB obtained was similar to the amount that co-purified with HdcA, i.e. the bound fraction. The observations may explain the very low turnover rates of HdcB in the *in vitro* assay with the purified proteins. At an HdcB to HdcA molar ratio of 1:3, approximately 50% of HdcA was cleaved in 30 min which corresponds to a turnover number of 0.05 min^{-1}. The low activity would be caused by the slow association/dissociation of the enzyme/substrate complex. In the bacterial cell, the free HdcB concentration apparently is high enough to cope with the HdcA proenzyme synthesis rate in growing cells. Of course, other factors in the internal cellular milieu may play a role in the process.

Experimental procedures

Growth conditions

Streptococcus thermophilus CHCC1524, obtained from the Christian Hansen Culture Collection (Hørsholm, Denmark), was grown at 42°C in M17 media supplemented with 1% lactose. *L. lactis* NZ9000 (Kuipers *et al.*, 1998) and derived strains were grown in M17 medium supplemented with 0.5% glucose (GM17) and 5 μg ml^{-1} chloramphenicol when appropriate. Nisin was added to 5 ng ml^{-1} to induce expression when the culture reached an OD$_{600}$ of 0.6, followed by another hour of growth. *E. coli* strain DH5α was grown in LB medium supplemented with 50 μg ml^{-1} ampicillin when appropriate. At an OD$_{600}$ of 0.6 expression was induced with 0.2% arabinose and the cells were allowed to grow for another 2 h. Cells were harvested by centrifugation at 4°C, followed by washing and resuspension in the indicated buffer.

Plasmid construction

Plasmids used in this study were listed in Table 1. Cloning was performed essentially as described by Sambrook *et al.* (2009). Genetic manipulations to construct the plasmids were performed following standard procedures. Mutations were intro-

Table 1. Plasmids used in this study.

Plasmid	Properties	Reference
pNZ8048	Cm^r, expression vector carrying the nisin-inducible P$_{nisA}$	Kuipers *et al.* (1998)
pNZ-hdcP	pNZ8048 containing *hdcP*	This work
pNZ-hdcAPB	pNZ8048 containing *hdcAPB* cluster	This work
pNZ-PhdcAPB	pNZ8048 containing *hdcAPB* cluster including 600 bp upstream sequence	This work
pNZ-hdcAP	pNZ8048 containing *hdcA* and *hdcP*	This work
pSG1151	Ap^r Cm^r, plasmid for construction of GFP fusions, containing *gfpmut1*	Lewis and Marston (1999)
pNZ-PgfphdcAPB	pNZ-PhdcAPB with *gfpmut1* upstream *hdcA*	This work
pNZ-PgfphdcAP	pNZ-PhdcAP with *gfpmut1* upstream *hdcA*	This work
pNZ-PgfphdcA	pNZ-PgfphdcAP with deletion of *hdcP*	This work
pNZ-Pgfp	pNZ-PgfphdcA with deletion of *hdcA*	This work
pBADCitS	Ap^r, derived from *E. coli* expression vector pBAD24, carrying arabinose-inducible P$_{BAD}$, containing *citS*	Sobczak and Lolkema (2003)
pBADH-hdcA	pBAD24 containing *hdcA* with His-tag coding sequence	This work
pBADH-hdcAB	pBADH-hdcA with *hdcB*	This work
pBADH-hdcAS82A	pBADH-hdcA with S82A mutation in *hdcA*	This work
pBADH-hdcAS82A-hdcB	pBADH-hdcAB with S82A mutation in *hdcA*	This work
pBADH-hdcB	pBAD24 containing *hdcB* with His-tag coding sequence	This work
pBADH-hdcA-H-hdcB	pBADH-hdcAB with addition of His-tag coding sequence to *hdcB*	This work
pBADH-hdcAS82A-H-hdcB	pBADH-hdcAS82A-B with addition of His-tag coding sequence to *hdcB*	This work

duced by PCR. The *hdcAPB* cluster was amplified by PCR from chromosomal DNA of *S. thermophilus* CHCC1524 with forward primer hdcAPB-fw2 (Table S1) containing a PagI restriction site and annealing to the start of *hdcA*, and primer hdcAPB-rv with a PstI restriction site and annealing 70 bp downstream of the stop codon of *hdcB*. The 2.9 kb fragment was ligated into pNZ8048, digested with NcoI and PstI, yielding pNZ-hdcAPB. Plasmid pNZ-PhdcAPB was constructed similarly, using forward primer hdcAPB-fw1, thereby including a 600 bp promoter region in addition to the *hdcAPB* cluster. pNZ-hdcAP was obtained from pNZ-hdcAPB by deletion of a 404 bp NheI–XbaI fragment. To construct pNZ-hdcP, the gene encoding HdcP was amplified using primers hdcP-St-fw and hdcP-St-rv, introducing NcoI and XbaI restriction sites and ligated into vector pNZ8048 digested with the same enzymes.

The *gfpmut1* gene encoding GFP was amplified from pSG1151 (Lewis and Marston, 1999) with primers gfp-hdcA-fw and gfp-hdcA-rv, introducing AatII and ApaI restriction sites. AatII and ApaI restriction sites were introduced in pNZ-PhdcAPB in between the RBS and start codon of *hdcA* and immediately behind the start codon, respectively, using primers hdcA-gfp-rv and hdcA-gfp-fw. Ligation of *gfpmut1* PCR product yielded plasmid pNZ-PgfphdcAPB containing an N-terminal translational fusion of *gfpmut1* with *hdcA*, under control of the *hdcA* promoter. pNZ-PgfphdcAP and pNZ-PgfphdcA were derived from pNZ-PgfphdcAPB by deleting a 404 bp NheI–XbaI fragment and a 1769 bp NsiI–PstI fragment respectively.

pBADH-hdcA was constructed by replacing the *citS* gene of pBADCitS (Sobczak and Lolkema, 2003) with *hdcA*, amplified from *S. thermophilus* CHCC1524 chromosomal DNA using primers hdcA-St-fw2, containing a PagI site, and hdcA-St-rv, containing a SalI site. The plasmid encodes HdcA extended with a His-tag and enterokinase cleavage site at the N-terminus. pBADH-hdcB was constructed similarly, using primers hdcB-St-fw and hdcB-St-rv. pBADH-hdcAB was obtained by first replacing *citS* in pBADCitS with *hdcA* amplified with primers hdcA-St-fw and hdcA-St-rv2, using PagI and SalI restriction sites. Then *hdcB* plus the upstream RBS was

amplified with primers hdcB-fw3 and hdcB-St-rv2 and cloned downstream *hdcA*, using SalI and PstI restriction sites.

Plasmid pBADH-hdcAS82A-hdcB was derived from pBADH-hdcAB, by introducing a point mutation using primers hdcA-SA-f and hdcA-SA-r and inverted PCR (Reikofski and Tao, 1992). This mutation leads to a serine to alanine mutation at position 82 in HdcA.

Plasmid pBADH-hdcA-H-hdcB was constructed by amplifying *hdcB* including the His-tag coding sequence from pBADH-hdcB using primers hishdcB-f and hishdcB-r, incorporating SalI and PstI restriction sites, and cloning the PCR product into SalI- and PstI-digested pBADH-hdcAB, with replacement of the *hdcB*. pBADH-hdcAS82A-H-hdcB was obtained by replacing an MluI–NcoI fragment of pBADH-hdcA-H-hdcB with that of pBADH-hdcAS82A. The sequence of all constructs was confirmed by sequencing (ServiceXS, the Netherlands).

Transport assays in whole cells

Lactococcus lactis NZ9000 cells containing the appropriate plasmid were washed and resuspended in ice-cold 50 mM KPi pH 6.0 to an OD$_{600}$ of 2.0. Glucose was added to a concentration of 10 mM. After 5 min of incubation at 30°C with stirring, 1.5 μM L-[U-^{14}C]histidine was added to 100 μl of cell suspension. Uptake was stopped by addition of 2 ml of ice-cold 0.1 M LiCl followed by filtration through a 0.45-μm-pore-size nitrocellulose filter (BA85; Schleicher & Schuell GmbH). The filter was washed once with 2 ml of ice-cold 0.1 M LiCl and submerged in Emulsifier Scintillator Plus scintillation fluid (Packard Bioscience). Retained radioactivity was counted in a Tri-Carb 2000CA liquid scintillation analyser (Packard Instrumentation).

HdcA activity assay

Histidine decarboxylation activity was measured by the production of histamine. Histamine concentrations were measured using an enzymatic assay described before (Landete

et al., 2004) with modifications. Forty microlitres of undiluted samples as well as 10× and 100× dilutions in 50 mM KPi pH 6.8, were added to 185 µl of reaction mixture containing 0.15 U ml⁻¹ diamine oxidase (from porcine kidney, Sigma), 0.3 U ml⁻¹ horseradish peroxidase, 28 µg ml⁻¹ diaminobenzidine in 100 mM KPi pH 6.8. After 1 h of incubation at 37°C, the OD at 405 nm was measured in a microtitre plate reader. Standard curves were derived from histamine solutions of known concentration ranging from 0 to 500 µM in 50 mM KPi pH 6.8.

Purification of HdcA and HdcB

Escherichia coli DH5α containing pBADH-hdcA or pBADH-hdcB were disrupted by sonication or by three passages through a French press operated at 13 kpsi in 50 mM KPi pH 7 buffer. Unbroken cells and debris were removed by centrifugation at 14000 *g*. About 10 ml of HdcA containing cell extract was mixed with 0.5 ml Ni²⁺-NTA sepharose (Qiagen) in the same buffer containing 4 mM histidine and 600 mM NaCl, and incubated for 1 h at 4°C with rotation. The resin was washed four times with 4 ml of 50 mM KPi pH 8.0, 12 mM histidine and 300 mM NaCl and protein was eluted four times with 0.5 ml of 50 mM KPi pH 7.0, 30 mM histidine and 300 mM NaCl. Similarly, HdcB-containing cell extract was incubated with Ni²⁺-NTA sepharose containing 10 mM imidazole and 600 mM NaCl. Beads were washed with 50 mM KPi pH 8.0, 300 mM NaCl and 40 mM imidazole and eluted with 50 mM KPi pH 7.0, 300 mM NaCl and 150 mM imidazole. The elution fractions containing HdcB were pooled, dialysed against 50 mM KPi pH 7.0, 50 mM NaCl, and subjected to anion exchange chromatography using a HiTrap Q HP, 1 ml column (GE Healthcare) and a FPLC system. After loading the column with 2 ml of HdcB-containing sample and washing with 5 ml of 50 mM KPi pH 7.0, 50 mM NaCl, the column was eluted with a continuous NaCl gradient in 50 mM KPi pH 7.0. HdcB was recovered from the flow-through. Protein concentrations were determined using the *DC* Protein Assay (Bio-Rad).

Mass spectrometry

Protein bands of interest were excised from an SDS-PAGE gel, destained in 12 mM ammonium bicarbonate, 50% acetonitrile, and dehydrated by treatment with acetonitrile and drying in an oven. The gel pieces were re-swollen by adding 20 µl of 10 ng µl⁻¹ trypsin (porcine, Promega) and incubation overnight at 37°C. Peptides were extracted from the fluid by shaking for 20 min with 10 µl of 75% acetonitrile, 1.25% formic acid. The extracted peptides were dried in a SpeedVac centrifuge and dissolved in 10 µl of 0.1% formic acid. Aliquots of 0.75 µl of the peptide suspension were spotted on the MALDI target and mixed in a 1:1 ratio with the matrix solution consisting of 5 mg ml⁻¹ α-cyano-4-hydroxycinnamic acid (dissolved in 50% acetonitrile and 0.1% TFA). After drying, the spots were analysed by MALDI-TOF-MS using a Voyager DE-Pro mass spectrometer (Applied Biosystems, Foster City, CA, USA).

Size exclusion chromatography

Size exclusion chromatography was performed using a Superose 12 10/300 GL column (GE Healthcare). After equili-

bration of the column with elution buffer, samples of 200 µl containing approximately 1 mg ml⁻¹ purified HdcA in 50 mM KPi buffer pH 7 or pH 5, 300 mM NaCl, 30 mM histidine were injected onto the column. The column was eluted with 50 mM KPi pH 7 or pH 5, 300 mM NaCl at a flow rate of 0.8 ml min⁻¹. Protein standards for calibration were applied in two mixes containing ferritin (0.3 mg ml⁻¹) and alcohol dehydrogenase (3 mg ml⁻¹) (mix 1), and β-amylase, bovine serum albumine (6 mg ml⁻¹) and cytochrome *c* (3 mg ml⁻¹) (mix 2). Protein content in the eluent was measured continuously by absorbance at 280 nm.

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