Purification and characterization of L-histidine aminotransferase from nikkomycin-producing *Streptomyces tendae* Tü901

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Cell extracts of *Streptomyces tendae* grown in nikkomycin production media contained an enzyme (HisAT) that transaminated L-histidine as the sole amino substrate with pyruvate as the amino group acceptor. HisAT was purified about 190-fold from the crude extract of *S. tendae*. The enzyme was determined by gel filtration and SDS-PAGE to be a homodimer with a subunit molecular mass of approximately 45 kDa. The aminotransferase had maximum activity at pH 7.0 and 37 °C. The enzyme was highly specific for L-histidine; pyruvate, 2-oxobutyrates, 2-oxovalerate and 2-oxocaproate were used as keto acceptors to about the same extent. The reaction mechanism was ping-pong. The K_m values for L-histidine and pyruvate, determined from Lineweaver–Burk plots, were 25 mM and 10 mM, respectively. Neither cell extracts of non-producing *S. tendae* mutants nor extracts of *Streptomyces lividans*, a species that does not synthesize nikkomycins, showed transaminating activity with a narrow substrate specificity for L-histidine as the amino donor. This strongly suggests that the formation of HisAT is essential for nikkomycin production.

Introduction

Aminotransferases play a major role in the metabolism of most amino acids. Many aminotransferases have broad and overlapping substrate specificities and function in more than one biochemical pathway. In addition, they catalyse freely reversible reactions providing a high degree of versatility (Jensen & Calhoun, 1981). While aminotransferases that utilize aromatic amino acids in the presence of a 2-oxo acid acceptor are widely distributed in micro-organisms (Gelfand & Steinberg, 1977; Paris & Magasanik, 1981a; Kradolfer *et al.*, 1982; Whitaker *et al.*, 1982; Lee & Desmazeaud, 1985; Lewis Kittell *et al.*, 1989), there are only a few reports of histidine aminotransferases (HisAT). Some aromatic aminotransferases (AAT) are also able to utilize histidine as amino substrate. A multispecific AAT using histidine as amino donor has been detected in extracts of *Escherichia coli* B grown under conditions of nitrogen limitation (Wickramasinghe, 1969a, b). Its postulated physiological role was to scavenge nitrogen from histidine and other amino acids during nitrogen deprivation. In *Klebsiella aerogenes* (*K. pneumoniae*) amino-

transferase I plays an essential role in the utilization of tryptophan and phenylalanine as sources of nitrogen. It can also transaminate histidine, although its affinity for this amino acid is very low (Paris & Magasanik, 1981b). Furthermore, an AAT acting on aromatic amino acids and histidine has been purified from extracts of *Rhizobium leguminosarum* (Perez-Galdona *et al.*, 1992). In contrast, the L-histidine-2-oxoglutarate aminotransferase from *Pseudomonas testosteroni*, which is essential for the utilization of imidazolyl-L-lactate as sole carbon source, exhibits great specificity for histidine as amino donor (Hacking & Hassall, 1975).

In crude protein extracts of nikkomycin-producing *S. tendae* Tü901/hut-11 separated on polyacrylamide gels, we detected a protein exhibiting transaminase activity with L-histidine in the presence of pyridoxal phosphate and pyruvate (Roos *et al.*, 1992). This protein did not use aromatic amino acids as amino substrates. During growth of *S. tendae* in nikkomycin production medium, HisAT activity was at its maximum in mycelium harvested at the onset of the nikkomycin production phase. As L-histidine and imidazole pyruvate are precursors of the 4-formyl-4-imidazolone base of nikkomycins (Schmidt & Pape, 1985; Kamps, 1989), it seemed that the HisAT might be involved in the nikkomycin biosynthetic pathway. In the present paper we relate the formation of HisAT protein to nikkomycin production,

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Abbreviations: AAT, aromatic aminotransferase; HisAT, histidine aminotransferase.

Fig. 1. Examination of crude cell extracts of *S. lividans* TK23, *S. tendae* Tü901/8c and NP47 for HisAT activity on non-denaturing polyacrylamide gradient gels. Lanes 1–5: extracts (135 µg protein) from mycelium cultivated in SP medium for 17, 23, 41, 47 and 69 h, respectively. Gels (a)–(c) were stained with L-histidine and pyruvate; gel (d) was stained with tyrosine and pyruvate. HisAT activity is marked by an arrow.

and describe the purification and properties of HisAT from crude extracts of *S. tendae*.

Methods

Organisms and conditions of cultivation. *S. tendae* Tü901/8c was obtained from the *Streptomyces* Culture Collection of the Institute of Microbiology I, University of Tübingen, Germany. *S. tendae* Tü901/hut-11 is a histidine-ammonia-lyase-negative mutant (Roos *et al.*, 1992) and Tü901/NP9, NP47 and NP55 are nikkomycin non-producing mutants (Bormann *et al.*, 1989). *S. lividans* TK23 (Hopwood *et al.*, 1985) was obtained from D. A. Hopwood, John Innes Institute, Norwich, UK.

For nikkomycin production, mycelia were grown in SM medium consisting of 3% (w/v) mannitol, 1% (w/v) starch, 2% (w/v) soy bean meal, and 1% (w/v) yeast extract, pH 6.0, and in SP medium (3% mannitol, 1% soluble starch, 0.5% soy peptone and 0.8% yeast extract, pH 6.0). *S. lividans* was grown in CRM medium (Bormann *et al.*, 1990) and YEME (Hopwood *et al.*, 1985).

Strains were usually cultivated in 500 ml baffled Erlenmeyer flasks containing 100 ml medium on a rotatory shaker (120 r.p.m.) at 27 °C. However, for purification of HisAT, *S. tendae* Tü901/hut-11 was grown in 2 litre baffled Erlenmeyer flasks containing 500 ml SP medium; cultures were inoculated with 5 ml of a preculture grown in the same medium for 2 d. At intervals during the large-scale culture, samples were taken for determination of HisAT activity. When the mycelium exhibited highest HisAT activity (Roos *et al.*, 1992), it was harvested by centrifugation at 4500 g for 5 min at 4 °C, washed twice with 100 mM-Tris/HCl, pH 8.0, and stored frozen at –20 °C.

Enzyme assays. The assay for aromatic aminotransferases based on the method of Spolter & Baldrige (1963) is described by Roos *et al.* (1992) but the concentrations of reactants are incorrect. The reaction mixture used in the present investigation to determine HisAT activity contained 0.114 M-sodium tetraborate, pH 8.0, unless otherwise stated, 50 mM-2-oxo acid, 100 mM-L-histidine, 30 µM-pyridoxal-5'-phosphate and enzyme. Absorbance changes were monitored at 310 nm. One unit of HisAT activity was defined as the amount of enzyme required to form 1 µmol imidazolylpyruvate min⁻¹ using the molar absorption coefficient $9.5 \times 10^6 \text{ cm}^{-2} \text{ mol}^{-1}$ according to Lin *et al.* (1958).

The assay for transaminating activity with histidinol phosphate was as described by Ames & Horecker (1956).

Protein was estimated by the method of Bradford (1976) with bovine serum albumin as standard.

Assay for aminotransferase activity on polyacrylamide gels. Electrophoresis using native polyacrylamide gradient gels has been described by Roos *et al.* (1992). The staining method for aminotransferase activity described by Gelfand *et al.* (1977) and Paris & Magasanik (1981a) was modified. Gels were stained at 37 °C in the dark for 30–60 min. The staining mixture contained 50 mM-amino acid, 50 mM-2-oxo acid, 0.19 mM-pyridoxal phosphate, 0.48 mM-thiazolyl blue, 0.098 mM-phenazine methosulphate, 0.3 mM-NAD, 0.6 U alanine dehydrogenase (Sigma) or 6 U glutamate dehydrogenase (Sigma), and 100 mM-Tris/HCl, pH 7.5, in a total volume of 50 ml. The dark-stained bands indicating aminotransferase activity were not detected in controls stained without 2-oxo acid.

Molecular masses were estimated using the high-molecular-mass protein standard from Pharmacia containing thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and bovine serum albumin (67 kDa).

Purification of HisAT. All enzyme purification procedures were carried out at 4 °C unless otherwise indicated and all buffers, including those used for equilibration and elution, contained 10% (w/v) glycerol, 10 mM-EDTA and 1 mM-dithiothreitol (DTT).

Preparation of crude extract. Mycelium (180 g wet wt) was thawed in 180 ml 100 mM-Tris/HCl buffer, pH 8.0, containing 40% (w/v) glycerol and 20 mM-EDTA, and ruptured by sonication on ice (Branson sonifier W-250; 20% duty cycle, power level 10, three times for 2.5 min with a 2 min delay). Phenylmethylsulphonyl fluoride (PMSF) was added to a concentration of 2 mM to the crude extract, and cell debris was removed by centrifugation at 36000 g for 30 min. Nucleic acids were precipitated from the supernatant by adding protamine sulphate [5% (w/v) in 100 mM-Tris/HCl, pH 7.0] to a final concentration of 0.2 mg (mg protein)⁻¹ and stirring the solution for 30 min on ice. The precipitate was removed by centrifugation at 20000 g for 15 min.

Ammonium sulphate precipitation. Ground solid ammonium sulphate was added to the extract to 40% saturation with constant stirring over

30 min. Following centrifugation at 20000 *g* for 15 min, ammonium sulphate was added to 60% saturation in the same manner and centrifugation was repeated. The pellets were dissolved in 20 mM-Tris/HCl, pH 8.0. The solution was centrifuged at 100000 *g* for 30 min to remove particles which interfered with column chromatography, then it was desalted on a Sephadex G25 column (5 × 20 cm) equilibrated with 20 mM-Tris/HCl, pH 8.0, at a flow rate of 7.5 ml min⁻¹.

Q-Sepharose chromatography. Protein-containing fractions from the Sephadex column were applied to a Q-Sepharose FF column (2.6 × 10 cm) equilibrated with 20 mM-Tris/HCl, pH 8.0. Proteins were eluted with a linear gradient of 0–0.5 M-NaCl in the same buffer at a flow rate of 2.5 ml min⁻¹. The total elution volume was 400 ml.

Butyl-Sepharose chromatography. Fractions containing HisAT activity from the Q-Sepharose column were pooled and ammonium sulphate was slowly added to a concentration of 1 M. The sample was applied to a butyl-Sepharose column (1.6 × 10 cm) equilibrated with 50 mM-Tris/HCl buffer, pH 7.0, containing 1 M-ammonium sulphate. The column was eluted with 200 ml of a linear gradient of 1–0 M-ammonium sulphate in 50 mM-Tris/HCl, pH 7.0, at a flow rate of 0.2 ml min⁻¹. The active fractions were pooled and exchanged into 20 mM-Tris/HCl buffer, pH 8.0, using Pharmacia PD10 desalting columns.

Mono Q chromatography. The enzyme preparation was loaded on a Mono Q column (1 × 10 cm) that had been equilibrated with 20 mM-Tris/HCl, pH 8.0. The column was then washed with equilibration buffer, and proteins were eluted from the column with a linear gradient of 0–0.5 M-NaCl in 20 mM-Tris/HCl buffer, pH 8.0 (80 ml). The flow rate was maintained at 1 ml min⁻¹.

Gel filtration. Active fractions obtained from the Mono Q column were exchanged into 20 mM-Tris/HCl, pH 8.0, without glycerol. After lyophilization, protein was dissolved in 200 µl 20 mM-Tris/HCl, pH 8.0, and applied to a Superose 12 column (1 × 30 cm) equilibrated with the same buffer. Gel filtration was performed at a flow rate of 0.1 ml min⁻¹. For determination of the molecular mass of HisAT the column was calibrated with reference proteins from Sigma (blue dextran, 2000 kDa; β-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; carbonic anhydrase, 29 kDa).

PAGE. SDS-PAGE was carried out according to Laemmli (1970), using a 12% (w/v) resolving gel and a 4% (w/v) stacking gel. Molecular mass standards, obtained from Gibco BRL, were lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (68 kDa) and phosphorylase b (97.4 kDa). Protein in gels was detected with Coomassie Blue G250.

Results

Formation of HisAT in correlation to nikkomycin production

Gel electrophoresis of cell extracts detected a protein specifically transaminating histidine only in nikkomycin-producing *S. tendae* Tü901 (Fig. 1). During cultivation in SP medium Tü901/8c produced about 400 mg nikkomycin X plus I l⁻¹, and in SM medium 750 mg l⁻¹. Crude extracts of the cells contained a protein band with HisAT activity and, in addition, AAT proteins that also reacted with tyrosine, tryptophan and histidine (data shown for SP medium). Only AAT proteins with broad substrate specificity were present in Tü901/8c cultivated in the

complex HA medium that did not support nikkomycin production (data not shown). In cell extracts of the non-producing mutants Tü901/NP9, NP47 and NP55 (Borrmann *et al.*, 1989) grown in production medium, only the multispecific AAT proteins were detected (data shown for NP47). Furthermore, a protein band transaminating histidine as sole substrate was not found in cell extracts of *S. lividans* cultivated in various media (YEME, CRM and SP) and harvested at different growth phases (Fig. 1; data shown for SP medium). The dark-stained bands on gels also appeared after treatment in the absence of a 2-oxo acid, and thus were not due to aminotransferase activity.

Purification of HisAT from *S. tendae* Tü901/hut-11

S. tendae Tü901/hut-11 was cultivated in production medium and harvested at highest HisAT activity; the culture conditions and time of harvest have been described by Roos *et al.* (1992). HisAT activity, which was found to be rather unstable in cell extracts, could be stabilized in the presence of 20% glycerol, 10 mM-EDTA, 1 mM-PMSF and 30 µM-pyridoxal phosphate (Fig. 2). The steps of the purification procedure are listed in Table 1 and illustrated in Fig. 3. After precipitation of nucleic acids by protamine sulphate, fractionation was performed with ammonium sulphate at 40–60% saturation. The desalted enzyme fraction was applied to Q-Sepharose and chromatographed with a linear NaCl gradient; HisAT activity eluted at approximately 0.15 M-NaCl with a sixfold purification. The active fractions, adjusted to 1 M-ammonium sulphate, were applied to butyl-Sepharose. Using a decreasing linear ammonium sulphate gradient, the active fractions eluted at about 0.3 M-(NH₄)₂SO₄ with a purification of about 7.5-fold. Fractionation by FPLC on Mono Q using a linear NaCl gradient eluted HisAT activity at approximately 0.15 M-

Fig. 2. Stabilization of HisAT activity in crude extract. Crude extract was prepared in 100 mM-Tris/HCl, pH 8.0 and stored at 4 °C in the presence of the following: ○, no further additions; □, 20% glycerol; △, 20% glycerol, 10 mM-EDTA, 1 mM-PMSF; ▽, 20% glycerol, 10 mM-EDTA, 1 mM-PMSF, 30 µM-pyridoxal phosphate. (100% activity corresponded to 6 mU HisAT.)

Table 1. *Purification of histidine aminotransferase*

Purification step	Vol. (ml)	Total activity (mU)	Total protein (mg)	Specific activity [mU (mg protein) ⁻¹]	Purification (-fold)	Yield (%)
Crude extract	330	12018	2640	4.5	1	100
Ammonium sulphate	86	5830	709	8.2	1.8	48.5
Q-Sepharose	52	3093	64.3	48.4	10.7	25.7
Butyl-Sepharose	21	1499	4.05	372	83	12.5
Mono Q	1.5	604	1.46	415	92	5.0
Superose-12	1.0	145	0.17	866	192	1.2

Fig. 3. Polyacrylamide gel electrophoresis of HisAT at different stages of purification. (a) SDS-PAGE stained with Coomassie blue. (b) Non-denaturing gradient PAGE stained for HisAT activity. Lanes: 2(a), 1(b) crude extract; 2(b) after ammonium sulphate precipitation; 3(a, b) after Q-Sepharose; 4(a, b) after butyl-Sepharose; 5(a, b) after Mono Q; 6(a, b) after Superose gel filtration; 7(a) HisAT band cut out from a native polyacrylamide gradient gel. (a) Lane 1, SDS-PAGE standards; lanes 1–5, 5 µg protein was separated; lane 6, 0.5 µg protein. (b) HisAT (1 mU) was applied in each lane. HisAT is indicated by an arrow.

Table 2. *Oxo acid specificity of partially-purified HisAT*

Oxo acid (50 mM)	Relative activity* (%)
Pyruvate	100
2-Oxovalerate	106
2-Oxobutyrate	101
2-Oxocaproate	95
2-Oxoglutarate	44
Glyoxylate	26
2-Oxoisovalerate	16

*100 mM-L-histidine was used as amino substrate. Activity on pyruvate (23 mU) taken as 100 %.

NaCl. This step resulted in a 2.5-fold loss of enzyme activity but separated HisAT from the multispecific AAT proteins (Fig. 3b). After gel filtration by FPLC, HisAT had been purified about 190-fold from the crude extract with a recovery of 1%. The final HisAT preparation

showed two major contaminating protein bands in SDS-PAGE gels stained with Coomassie blue (Fig. 3a).

The molecular mass estimated by gel filtration was approximately 85 kDa for the native enzyme. The HisAT protein, which was identified by activity staining on a non-denaturing gradient gel, was excised from a parallel lane on the same gel upon Coomassie blue staining and subjected to SDS-PAGE. As a single protein band was obtained with a molecular mass of approximately 45 kDa (Fig. 3a), the native enzyme is presumed to be a dimer of two identical subunits.

pH and temperature optima

The activity of the partially purified HisAT obtained after Mono Q chromatography was determined in borate buffer adjusted with HCl to pH 5.5–8.8. The optimum activity was at pH 7.2. The optimum temperature for HisAT activity was 37 °C.

Substrate specificity

In aminotransferase assays using the borate assay system with pyruvate as amino acceptor HisAT was highly specific for L-histidine and did not react with L-phenylalanine, L-tyrosine, L-tryptophan and D-histidine. In addition, HisAT did not react with L-histidinol phosphate using the assay of Ames & Horecker (1956) for the formation of imidazolylacetol.

On gels HisAT had no activity towards L-alanine, L-leucine, L-lysine, L-glutamate and L-aspartate using pyruvate as amino acceptor, or towards L-aspartate with 2-oxoglutarate. In contrast, HisAT exhibited a broad specificity towards amino acceptor substrates. Similar activities were obtained with pyruvate, 2-oxobutyrate, 2-oxovalerate and 2-oxocaproate, but other 2-oxo acids could also serve as substrates (Table 2).

Kinetic parameters

Double-reciprocal plots of the initial velocity against the concentration of one substrate at a series of fixed concentrations of the second substrate gave a series of parallel straight lines. These Lineweaver-Burk plots indicated a ping-pong mechanism involving only binary substrate complexes (Cleland, 1963). Double reciprocal plots of the apparent K_m constants and apparent maximum velocities against the concentration of the second substrate gave straight lines, from which the concentration-independent K_m values for L-histidine and pyruvate were determined to be 25 mM and 10 mM, respectively.

Discussion

In primary metabolism of *Streptomyces* the first step in histidine catabolism is its non-oxidative deamination to urocanate and ammonia, which is catalysed by histidine ammonia-lyase (histidase; EC 4.3.1.3; Kendrick & Wheelis, 1982). In addition to histidase, an amino-transferase with a narrow substrate specificity for L-histidine as amino donor has been found in nikkomycin-producing *S. tendae* (Roos *et al.*, 1992). Since histidine is known to be the precursor of the imidazolone base of nikkomycins (Schmidt & Pape, 1985), we investigated whether there is a correlation between formation of HisAT and nikkomycin production. While HisAT protein was detected in *S. tendae* strains under nikkomycin production conditions in the early production phase, it was not found in non-producing mutants. As these strains are unable to form any of the known biosynthetic intermediates and cannot complement each other to form nikkomycin structures (unpublished results), they are suggested to be regulatory mutants or

mutants with extensive genomic alterations. Therefore, enzymes involved in nikkomycin biosynthesis are not expected in these non-producers. Furthermore, a HisAT protein could not be detected in *S. lividans* grown under various conditions. This species is unable to synthesize nikkomycins. Although we cannot exclude that a HisAT activity band in gel electrophoresis gels was masked by one of the dark bands given by *S. lividans* extracts, and which are probably due to dehydrogenase activity, we conclude that HisAT is specific for nikkomycin producers.

We developed a purification procedure aimed at providing sufficient enzyme for partial characterization and amino acid analysis. The HisAT product obtained was purified from other aminotransferases present in the crude extract. However, two major contaminating proteins were still evident in SDS-PAGE gels. Attempts to remove them by chromatography on hydroxylapatite, ion-exchange and hydrophobic chromatography using various conditions were not successful. The low recovery of HisAT might be due to the fact that the HisAT activity measured in crude extract results from the combined action of HisAT and multispecific AAT proteins. Another possible reason is the instability of HisAT under the conditions used for chromatography, even with glycerol, EDTA and PMSF present.

Like AAT1 from *K. aerogenes* (Paris & Magasanik, 1981b), and the *aspC* and *tyrB* gene product, the so-called aspartate and aromatic aminotransferase from *E. coli* K12, HisAT from *S. tendae* is composed of two identical subunits of approximately 45 kDa. The molecular structure of the histidine transaminating enzyme from *E. coli* B (81 kDa), L-histidine-2-oxoglutarate aminotransferase from *Pseudomonas testosteroni* (70 kDa) and the AAT from *Rhizobium leguminosarum* (53 kDa) have not been described (Wickramasinghe, 1969a; Hacking & Hassall, 1975; Perez-Galdona *et al.*, 1992). In general, there is a wide variation in the molecular masses of microbial AAT proteins, ranging from 33 kDa (histidinol-phosphate aminotransferase from *Bacillus subtilis*; Weigent & Nester, 1976) to 260 kDa (AT-II from *Corynebacterium flavum*; Fazel & Jensen, 1979).

Studies on the amino substrate specificity of the partially purified enzyme indicated that HisAT from *S. tendae* exclusively functioned with L-histidine. No aminotransferase activity could be detected between histidinol phosphate and 2-oxoglutarate, the reaction catalysed by the imidazolylacetolphosphate-L-glutamate aminotransferase in histidine biosynthesis. Therefore, *S. tendae* HisAT activity is not attributed to this enzyme, as reported for *Salmonella typhimurium* (Albritton & Levin, 1969).

Purified histidine-2-oxoglutarate aminotransferase

from *Pseudomonas testosteroni* functioning in the degradation pathway of imidazolyl-L-lactate also exhibits high specificity for L-histidine, but it still retains slight activity towards phenylalanine, tyrosine and tryptophan (Hacking & Hassall, 1975). It is also specific for 2-oxoglutarate as the amino acceptor whereas HisAT from *S. tendae* utilized pyruvate and several other 2-oxo acids to the same extent, but exhibited only about 40% of the activity with 2-oxoglutarate.

The kinetic parameters suggested a typical ping-pong mechanism of enzyme reaction. The Michaelis constants determined for histidine (25 mM) and pyruvate (10 mM) are unusually high. Even higher values for histidine were reported for the multispecific AAT1 from *K. aerogenes* (70 mM) and the histidine-transaminating *E. coli* B enzyme (137 mM) (Paris & Magasanik, 1981*b*; Wickramasinghe, 1969*a*). In contrast, the apparent Michaelis constants for histidine determined for the AAT1 from *Rhizobium* and the HisAT from *Pseudomonas* were 1.86 mM and 4 mM (Perez-Galdona *et al.*, 1992; Hacking & Hassall, 1975). The instability of HisAT during the purification procedure implies that the *in vitro* conditions used were not optimal for enzyme activity. This might also be a reason for the high K_m values obtained. Further investigations are needed to establish whether HisAT functions in nikkomycin biosynthesis. This should be resolved by isolation of the corresponding gene and the construction of mutants having the gene inactivated.

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