Histidine Ammonia-Lyase Mutant S143C Is Posttranslationally Converted into Fully Active Wild-Type Enzyme. Evidence for Serine 143 To Be the Precursor of Active Site Dehydroalanine†

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ABSTRACT: Histidase [histidine ammonia-lyase (HAL); EC 4.3.1.3] from *Pseudomonas putida* is a homotetramer and contains one catalytically essential dehydroalanine residue per subunit. Since the mutant S143A was catalytically inert, it has been proposed that serine 143 is the precursor of the active site dehydroalanine [Langer et al. (1994) *Biochemistry 33*, 6462−6467]. To further define the role of serine 143, we prepared the mutants S143T and S143C by site-directed mutagenesis. The threonine 143 mutant was neither catalytically active (<0.01%) nor did it form with L-cysteine and oxygen a product absorbing at 340 nm. In contrast, the cysteine 143 mutant showed full catalytic activity and, after treatment with L-cysteine and oxygen, an increased absorbance at 340 nm similar to that of the wild-type enzyme. Also the kinetic constants (K_m and V_{max}) were identical with those of wild-type histidase. Titration with Ellman's reagent revealed that both wild-type and S143C mutant histidase contained seven thiol groups after exhaustive reduction. It must be concluded that posttranslational modification occurs with both serine 143 and cysteine 143 by elimination of water and hydrogen sulfide, respectively. In both cases dehydroalanine is formed and the resulting histidases are indistinguishable. In contrast, the threonine 143 mutant is not processed to active enzyme.

Histidase [histidine ammonia-lyase (HAL); EC 4.3.1.3] catalyzes the transformation of histidine to urocanate by the elimination of ammonia. It has been known for many years that histidase contains an electrophilic group that is essential for catalysis. Both Wickner (1969) and Givot et al. (1969) postulated that the prosthetic group is dehydroalanine, since reduction of histidase with NaB^3H_4 and subsequent hydrolysis led to [^{3}H]alanine. Moreover, treatment of histidase with nitro[^{14}C]methane followed by catalytic reduction and hydrolysis afforded, among others, radioactive 2,4-diaminobutyrate.

By the use of site-directed mutagenesis, we located serine 143 as the most likely precursor of dehydroalanine (Langer et al., 1994). Hernandez et al. (1993) treated histidase with L-cysteine in the presence of oxygen at high pH (>10), a procedure that leads to an increase of absorption at 340 nm (Klee, 1970). After proteolytic digestion they isolated an octapeptide that was chemically modified at serine 6. Fitting this peptide into the deduced amino acid sequence of histidase (Consevage & Phillips, 1990), it was concluded that the modification by an electrophilic cofactor occurred at serine 143.

To elucidate the nature of modification, we mutated the gene coding for histidase by exchanging the codon of serine 143 with those of threonine and cysteine, respectively. Here we report on the catalytic and chemical properties of the mutants, that provide further evidence for serine 143 being the precursor of the active site dehydroalanine.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions. Escherichia coli SURE cells [restriction-negative mcrA, D(mcrCB-hsdSMR-mrr)171, and endonuclease-deficient; purchased from Stratagene] were used for the isolation of single-stranded (ss) DNA from M13 phages to carry out site-directed mutagenesis.

E. coli BL21 (DE3) cells served for the expression of either wild-type or mutant histidase. The expression vector pT7-7 was generously donated by Dr. Stanley Tabor (Tabor & Richardson, 1985). The construction of pT7-7H containing the gene coding for histidase has been already described (Langer et al., 1994). The phage M13mp19 was from Boehringer Mannheim and pGEM 11-Zf-(−) from Promega.

Site-Directed Mutagenesis. Recombinant pT7-7H was used to subclone a *Xba*I−*Sal*I fragment into M13mp19. Exchanges from serine 143 into either cysteine or threonine were performed starting from this construct. Site-directed mutagenesis was performed as described by Sayers et al. (1988) following the protocol of the Amersham mutagenesis kit (Sculptor).

The oligonucleotides used in mutagenesis reactions were as follows:

S143C: 5′-GTGGGTGCTTGCGGCGACCTG-3′

S143T: 5′-GTGGGTGCTACCGGCGACCTG-3′

The mutations were confirmed by sequence analysis using the dideoxynucleotide chain-termination method of Sanger et al. (1977).

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The mutant DNA was patched up with the missing 3′ fragment *Sal*I−*Eco*RI by subcloning it into the vector pGEM 11-Zf-(−). Each mutant was then inserted into the expression vector pT7-7 by the use of the restriction sites 5′-terminal *Nde*I and 3′-terminal *Eco*RI. Transformation either of *E. coli* BL21 (DE3) cells or of *E. coli* SURE cells was performed as described elsewhere (Langer et al., 1994).

Double-strand DNA sequence analysis was performed with the pT7-7S143C DNA to confirm the genotype of this mutant after several transformation steps. The analysis was carried out following the *fmol*DNA Sequencing instructions from Promega by direct incorporation of [α-^{35}S]dATP using Taq DNA polymerase.

Purification, SDS/PAGE, Western Blot Analysis, and Protein Assays. Purification of recombinant histidase was carried out as described in detail by Langer et al. (1994).

SDS/PAGE was performed according to Laemmli (1970) using 10% polyacrylamide gels. For staining of the gels Coomassie Brillant Blue R 250 was used. Western blotting was carried out as described by Symingteon et al. (1981) using nitrocellulose as blotting filter. For blotting a Bio-Rad wet-blotting system was used. Histidase was detected with a polyclonal antibody that was raised in rabbits against wild-type histidase isolated from *Pseudomonas putida* (the antibody was a generous gift from Dr. G. Münscher, Behringwerke AG, Marburg, Germany).

Protein determinations were performed by the microbiuret method (Itzhaki & Gill, 1964) or by measurement of A_{260} and A_{280} according to Warburg and Christian (Lane, 1957).

Enzyme Assay. The assay used in this work was described by Mehler and Tabor (1953). For evaluation of the kinetic parameters, a standard assay described by Langer et al. (1994) was used. Measurements were performed at 25 °C at 277 nm, and each value was calculated from 6−8 individual measurements. The extinction coefficient (ϵ_{277}) of urocanic acid is 18 800 L mol^{-1} cm^{-1}. K_m and V_{max} values were determined using the double-reciprocal plot (Lineweaver & Burk, 1934).

Quantification of Thiol Groups in Wild-Type and Mutant S143C Histidase. The method described here is a modification of that of Ellman (1959) and was first applied for histidase from *Pseudomonas* (ATCC 11299) by Klee (1970). The assay is divided into three steps: The first step consists of the reduction and denaturation of histidase with dithiotreitol (DTT) and urea: 500 μg of histidase or mutant S143C was added to 800 μL of denaturation buffer, pH 8.0, containing 20 mM potassium phosphate, 8 M urea, 150 mM NaCl, and 1 mM EDTA. The solution was then treated with 10 mM DTT at 75 °C for 30 min in the case of complete reduction or 90 min at 25 °C as has been described by Klee (1970).

Second, excess DTT was removed by centrifugation of the enzyme solution in Centricon 30 at room temperature. The volume was repeatedly (five times) reduced to 40 μL and refilled to 2 mL with denaturation buffer. In a last step the enzyme was brought to a reaction volume of 1 mL. Finally, the enzyme solution was incubated with 5 μL of Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 3.96 mg/mL 0.1 M potassium phosphate buffer, pH 7.0], and its absorption was measured at 412 nm and 25 °C until a maximum absorption was reached. The values were corrected for a blank solution containing no enzyme.

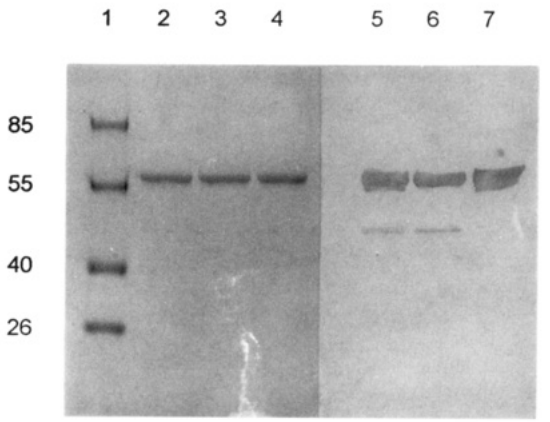

FIGURE 1: SDS/PAGE (lanes 1−4) and Western blot (lanes 5−7) of histidase and mutants. Lane 1: molecular mass standard; lane 2: wild-type histidase; lane 3: mutant S143C; lane 4: mutant S143T; lane 5: wild-type histidase; lane 6: mutant S143C; lane 7: mutant S143T. The proteins were purified to homogeneity as described by Langer et al. (1994). Each lane was loaded with 2 μg of protein. The gel was stained with Coomassie Brillant Blue. The Western blot was performed with a polyclonal antibody against histidase isolated from *P. putida*. The molecular masses of the markers are indicated.

The content of free SH groups per subunit of the enzyme was calculated as follows:

$$\text{SH content} = \frac{(\text{maximum } A_{412})(V_{\text{cuvette}})(M_r)}{(\epsilon_{412})(4)}$$

The molar extinction coefficient of the thiobenzoate anion (ϵ_{412}) is 13 600 L mol^{-1} cm^{-1}; the molecular mass of the histidase homotetramer is 220 000.

Inactivation of Histidase by L-Cysteine. Histidase was inactivated by L-cysteine as described earlier (Klee, 1970, 1974; Hernandez et al., 1993). Histidase or one of its mutants (2.25 mg) was dissolved in 2.9 mL of 0.05 M $NaHCO_3/Na_2CO_3$ buffer, pH 10.5. The reaction was initiated by the addition of 100 μL of 0.3 M L-cysteine, giving a final L-cysteine concentration of 10 mM. The reaction was considered complete when the increase in absorption at 340 nm ceased, usually after 150−180 min.

RESULTS

Expression and Kinetic Constants of Wild-Type and the Mutant S143C and S143T Histidases. It has been previously described that expression of histidase from *P. putida* in *E. coli* BL21 (DE3) cells leads to a high level of recombinant active histidase (Langer et al., 1994). In Figure 1 the SDS/PAGE gel and Western blot of wild-type histidase and the mutants S143C and S143T are shown. It can be seen that both mutants are expressed in similar quantities as wild-type histidase and, within the limit of error, do not differ in mass. The Western blot shows that about 3−5% of the total histidase is degraded when stored at 4 °C for several weeks. In the first two lines wild-type histidase and mutant S143C are shown. These two proteins were stored at 4 °C for 2 months. In the seventh lane one can see freshly isolated protein of mutant S143T. Here the band at about 42 kDa is missing, indicating that the additional band arises from partial degradation of old enzyme preparations.

The kinetic constants K_m and V_{max} of recombinant wild-type histidase were estimated and compared to those of

Table 1: Summary of Kinetic Constants of Histidases Isolated from *Pseudomonas* ATCC 11299b (Klee et al., 1975) and *P. putida* (Consevage & Phillips, 1985), Overexpressed Recombinant *P. putida* Histidase in *E. coli* (Hernandez & Phillips, 1993), and Overexpressed Recombinant *P. putida* Expressed in *E. coli* BL21 (DE3) Cells (Langer et al., 1994)[a]

histidases	kinetic constants of enzyme	
	K_m (mM)	V_{max} (IU/mg)
wild-type *Pseudomonas* ATCC 11299b	4.0	28
wild-type *P. putida*	5.3	25
recombinant wild-type *E. coli*	5.6	16.7
recombinant wild-type *E. coli* BL21	5.2[b]	22.3
recombinant mutant S143C	5.4	22.2
recombinant mutant S143T	not accessible	less than 0.01%

[a] All proteins were purified to electrophoretic homogeneity and tested in a standard assay described elsewhere (Langer et al., 1994). [b] K_m values for recombinant wild-type histidase in our laboratory vary between 3.5 and 5.2 mM depending on the experimenter.

histidase isolated from *Pseudomonas* ATCC 11299b (Klee et al., 1975) and histidase from *P. putida* (Consevage & Phillips, 1985) and overexpressed *P. putida* histidase in *E. coli* (Hernandez & Phillips, 1993; Langer et al., 1994). It can be seen from Table 1 that the proteins exhibit nearly the same kinetic behavior even after different expression and purification procedures. The mutant S143C for instance showed exactly the same kinetic behavior as recombinant wild-type histidase. In contrast, mutant S143T showed no catalytic activity at all (less than 0.01% of that of the wild-type enzyme, as it has been described for mutant S143A; Langer et al.,1994).

To confirm that the mutation in the serine 143 codon indeed occurred, double-strand DNA of the recombinant mutant S143C which was cloned in pT7-7 and transformed into *E. coli* BL21 (DE3) cells was isolated and partially sequenced as described under Materials and Methods. The sequencing revealed that the clone used for the kinetic studies was indeed mutated in the expected manner.

Quantification of Free Protein Thiol Groups in Wild-Type Histidase and Mutant S143C. To test the presumption that mutant S143C is posttranslationally converted into wild-type histidase, its cysteine content was compared with that of the wild-type. Ellman's reagent (DTNB) was used to quantify the free thiol groups as described under Materials and Methods. Klee (1970) described the titration of free thiol groups in histidase isolated from *Pseudomonas* ATCC 11299b. She applied Ellman's (1959) original conditions; i.e., the denaturation and reduction temperature was 25 °C. Under these conditions the thermostable histidase is not completely reduced even in 8 M urea. At this low temperature Klee found four free thiols per subunit. In this work we could confirm this value for wild-type histidase and establish it for mutant S143C. On the other hand, the deduced sequence of histidase (Consevage & Phillips, 1990) reveals seven cysteines per subunit. So we decided to raise the denaturation and reduction temperature drastically from 25 to 75 °C. As a result of the higher temperature histidase was completely reduced, leading to 6.8 SH groups per subunit titratable with Ellman's reagent both in the wild-type and in the mutant S143C. This result suggests that mutant S143C is indistinguishable from wild-type histidase by the number of SH groups. The titration experiments are summarized in Table 2.

Table 2: Titration of the Thiol Groups of Wild-Type Histidase and Mutant S143C per Monomer at Different Denaturation and Reduction Temperatures [Compared to the Values of Klee (1970)][a]

histidases	denaturation and reduction conditions	
	25 °C, 90 min	75 °, 30 min
recombinant wild-type	4.1	6.8
recombinant mutant S143C	3.8	6.8
Pseudomonas ATCC11299b wild-type	3.6	–

[a] 0.5 mg of pure wild-type histidase or mutant S143C was treated as described under Materials and Methods.

As there are four free thiol groups per monomer that can be detected under mild reducing conditions, three SH residues are missing to give the correct number of seven cysteine residues per monomer. Assuming one disulfide bridge that cannot be reduced at 25 °C, the missing third SH residue seems to be hidden inside the folded structure of histidase, being inaccessible for DTNB.

Inactivation of Histidase and Mutants with L-Cysteine. L-Cysteine can inactivate histidase in three different ways (Klee, 1970, 1974; Hernandez et al., 1993). First, at pH below 9.0 without the addition of M^{2+}, L-cysteine reacted as a competitive inhibitor. Second, at the same pH in the presence of M^{2+} (Mn^{2+}, Zn^{2+} or Cd^{2+}) L-cysteine reacts in a mixed type of inhibition. These two inhibitions are reversible. A third inhibition of histidase by L-cysteine occurs at pH above 10.0 and in the presence of oxygen. This inhibition is concomitant with an increase in absorbance at 340 nm which is stable even in 6 M guanidinium hydrochloride. Both Klee (1974) and Hernandez et al. (1993) showed, with [^{14}C]- and [^{35}S]-L-cysteine, respectively, that approximately 4 mol of cysteine is bound per mole of native enzyme. This indicates that one L-cysteine binds per monomer of histidase. After proteolytic digests of the modified histidase with trypsin and V8 protease Hernandez et al. (1993) showed that some modified residue was bound to serine 143. They postulated the binding of an "electrophilic center" at this position of the enzyme.

We have now compared the reaction of L-cysteine with wild-type histidase to that of the mutants S143A (Langer et al., 1994), S143C, and S143T. Wild-type histidase and mutant S143C were indistinguishable. In Figure 2 the increase in A_{340} is plotted versus reaction time. It is identical for wild-type histidase and for mutant S143C and shows the same behavior described by Klee (1974). A short lag phase at the beginning (first 5 min) is followed by a strong rise in A_{340}. After about 100 min the absorption at 340 nm rises very slowly, reaching a maximum after 180 min.

As expected, the mutant S143A when treated with L-cysteine and oxygen does not show the increase of absorption at 340 nm. Interestingly, the mutant histidase S143T also showed the same behavior, i.e., no increase of the 340 nm absorption upon the same treatment.

DISCUSSION

A comparison of the known amino acid sequences of histidases from various sources and of the closely related phenylalanine ammonia-lyases revealed four conserved serines. Recently, we showed by site-directed mutagenesis that among these only serine 143 is essential for catalysis (Langer et al., 1994). We proposed serine 143 to be the

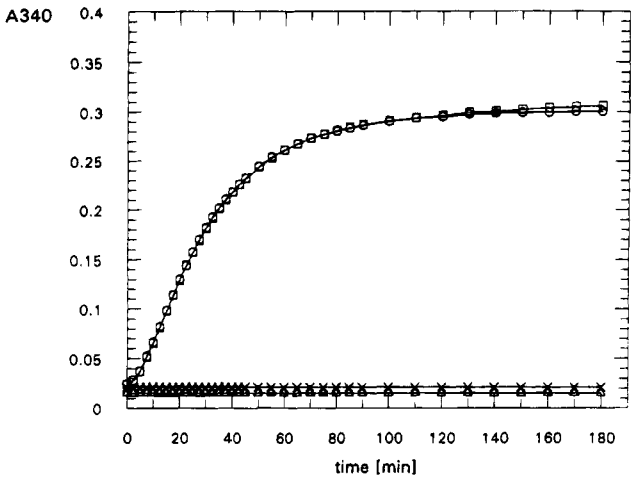

FIGURE 2: L-Cysteine inactivation of wild-type histidase and mutants. Absorbance of a new species at A_{340}. (□) Wild-type histidase; and (○) mutants S143C, (×) S143T, and (△) S143A). Measurements were performed as described in Materials and Methods.

precursor of dehydroalanine in the active site of histidase. Independently, Hernandez et al. (1993) found serine 143 to be "the binding site for an electrophilic cofactor required for histidase activity". Since both Klee (1974) and Hernandez et al. (1993) stated that histidase, previously modified and labeled with either [^{14}C]- or [^{35}S]cysteine, is devoid of radioactivity after denaturation, the material absorbing at 340 nm must come from elsewhere. According to Hernandez et al. (1993), their octapeptide, isolated from the modified histidase and containing amino acids 138−145, has an excess weight of 184 Da. Although the structure of this at 340 nm absorbing material could not be determined, the authors speculate that it might be attached to serine 143 by an ester linkage.

In this work we showed that mutation of the codon for serine 143 to a cysteine codon in the histidase gene leads to an enzyme that is indistinguishable from wild-type histidase by several criteria. The most simple and logical explanation for this unexpected finding is that in both cases the amino acid in position 143 is modified to dehydroalanine to afford fully active wild-type histidase. Cysteine is closely related to serine and was discussed as a possible precursor of dehydroalanine either in histidase or in phenylalanine ammonia-lyase (Hanson & Havir, 1981; Consevage & Phillips, 1985). Since β-elimination of water or hydrogen sulfide is the same type of reaction, it may be mediated by the same catalytic center responsible for the posttranslational modification.

In the light of our results, the attachment of an electrophilic group to serine 143 appears rather unlikely. In the S143C mutant a thiolester linkage would apply. It is questionable that this would survive the treatment at pH 10.5. Even the O-ester bond should hydrolyze under such conditions. Moreover, it would be difficult to understand why the S143T mutant neither shows catalytic activity nor does it afford the 340 nm material when treated with L-cysteine in the presence of oxygen. The most simple explanation for this is that the water eliminating catalytic center discriminates against threonine because of the extra methyl group. The possibility that threonine in this position prevents the correct folding of the polypeptide chain is unlikely since the circular dichroism (CD) spectra of the wild-type enzyme and of all our mutants (including the S143T mutant) are identical [the CD measurements have been carried out by Dr. Jennifer Reed; see also Langer et al. (1994)].

One might argue that β-elimination from threonine 143 occurs (without affecting the protein's structure), but the resulting dehydro-α-aminobutyrate is not catalytically functional and does not react to form the 340 nm chromophore. This is indeed possible but less likely, since the elimination must be catalyzed by a highly specific enzymatic center that is usually more sensitive to structural modifications than a nonenzymic reaction like the inhibiton by L-cysteine and oxygen. The definitive answer to this question, however, warrants further experimentation.

β-Eliminations can usually be facilitated by abstraction of an α-proton and protonation of the OH (or SH) group in a concerted or a stepwise manner. At this stage it is not

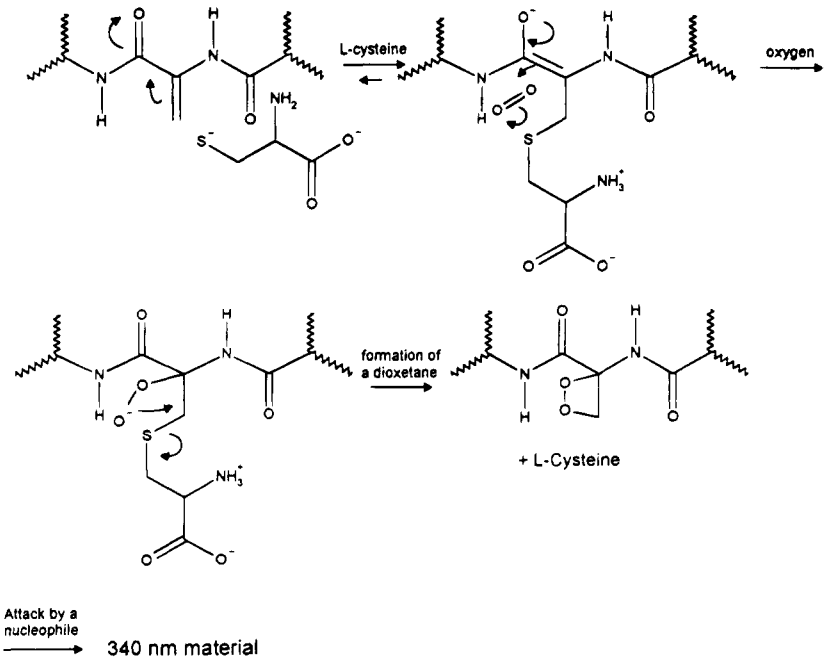

FIGURE 3: A possible mechanism for the irreversible inhibition of histidase by L-cysteine and oxygen.

possible to distinguish between these possibilities. It is also not known which amino acid residues of the protein are involved in this autocatalytic process.

The results of Hernandez et al. (1993) may be explained by considering the fact that cysteine in the absence of oxygen does not irreversibly modify histidase. Rather, its reaction may produce an extremely nucleophilic enolate at the active site that is trapped by oxygen to form a highly reactive species, e.g., a dioxetane, which may attack an amino acid near the active site (precedence for such a reaction is provided by the photorespiration catalyzed by ribulose-1,5-bisphosphate carboxylase). In this sense L-cysteine in combination with oxygen is a suicide inhibitor (Figure 3).

Conclusion. The results presented here support the view that serine 143 of histidase is the precursor of the active site dehydroalanine (Langer et al., 1994). The hypothesis that serine 143 serves as an anchor for an as yet unknown electrophilic cofactor (Hernandez et al., 1993) seems to be unlikely in the light of the behavior of the histidase mutants S143C and S143T.

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