Activation of an Asp-124→Asn mutant of haloalkane dehalogenase by hydrolytic deamidation of asparagine

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Abstract Haloalkane dehalogenase hydrolyses various 1-halo-*n*-alkanes to the corresponding alcohols by covalent catalysis with formation of an alkyl-enzyme intermediate. The carboxylate function of the nucleophilic aspartate (Asp-124) that displaces the halogen during formation of the intermediate was changed to an amide by site-directed mutagenesis (Asp-124→Asn). Activity measurements and analysis of peptides containing the nucleophilic residue showed that the mutant enzyme was inactive, but that the activity increased by rapid deamidation of the asparagine residue, yielding wild type enzyme. There was no indication for isoaspartate formation during this process. The results suggest that a water molecule that is located close to the carboxyl function of Asp-124 in the X-ray structure is highly reactive and is responsible for the observed deamidation.

Key words: Dehalogenase; Nucleophilic substitution; Deamidation; Active site mutant; Reactivation; Asparagine

1. Introduction

Haloalkane dehalogenase (DhlA) from *Xanthobacter autotrophicus* GJ10 hydrolyses terminally chlorinated and brominated *n*-alkanes to the corresponding alcohols [1]. The primary sequence of the *dhlA* gene [2] and the three-dimensional structure of the protein are known [3,4]. The enzyme belongs to a class of hydrolytic enzymes, that share a main domain of similar topology, the α/β hydrolase fold [5]. Three active-site residues form a catalytic triad and are located at conserved positions along this fold (Fig. 1). The hydrophobic active site is a buried cavity between the α/β hydrolase fold main domain and a cap domain, that is formed by a 75 amino acids sequence excursion between β-strand 6 and α-helix 9 of the main domain [3]. The halogen moiety of the substrate is bound in the active site by two tryptophans, one localized in the cap domain and the other in the main domain [6].

The reaction of haloalkane dehalogenase proceeds via a nucleophilic attack of a carboxylate oxygen of Asp-124 on the carbon atom to which the halogen is bound, leading to formation of an alkyl-enzyme ester (Fig. 1) [7,8]. The halide ion that is cleaved off is bound by the two tryptophan residues [6]. The alkyl-enzyme is subsequently hydrolyzed, releasing the alcohol. In the X-ray structure, there is a water molecule close to the carboxylate carbon of Asp-124 and His-289. The latter residue could enhance the nucleophilicity of this water molecule by acting as a base catalyst, probably assisted by Asp-260, the third residue of the catalytic triad. In agreement with this, replacing His-289 by a glutamine yielded a mutant enzyme in which the alkyl-enzyme intermediate is not hydrolyzed, causing accumulation of covalent intermediate in the presence of substrate [9]. The last step of the catalytic cycle is product release. Probably, the alcohol leaves the active site before the halide ion and the proton on His-289 [7].

In order to further understand the catalytic mechanism we constructed an Asp-124→Asn mutant enzyme. Surprisingly, mutant enzyme showed increasing activity upon storage or purification. Here we describe the properties of the mutant enzyme and show that activation is caused by direct hydrolytic deamidation of the asparagine residue introduced at position 124, leading to an enzyme with a primary sequence and catalytic activity similar to that of the wild type dehalogenase.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Merck (Darmstadt, Germany) or Sigma Chemie GmbH (Deisenhofen, Germany). Enzymes used for DNA manipulations were obtained from Boehringer (Mannheim, Germany). The mutagenesis primer was obtained from Eurosequence (Groningen, The Netherlands).

2.2. DNA manipulations

An Asp-124→Asn mutant haloalkane dehalogenase was constructed using plasmid pGELAF+, which has a f1 origin for production of ssDNA and a T7 promoter for expression [10]. Standard DNA manipulation techniques were used [11]. Site-directed mutagenesis was done as described by Kunkel et al. [12] with a primer having the sequence 5'CTGGTCGTACAGAACTGGGGCGGA3' (mutated codon underlined). The plasmids containing the wild-type gene or the mutant gene were introduced in host strains by electroporation [10]. The sequence of the construct was verified by dideoxy chain termination sequencing [13].

2.3 Expression and purification of the Asp-124→Asn mutant enzyme

The mutated enzyme was expressed in *Escherichia coli* BL21(DE3) [14]. The cells were grown in LB medium containing 100 μg/ml ampicillin at 30°C to an OD_{600} of 1.0. The temperature was subsequently lowered to 17°C, and isopropyl-thio-galactoside-pyranoside (0.4 mM) was added as inducer. At an OD_{600} of 2.0, the cells were harvested by centrifugation (6,000 × *g* for 5 min), washed with TEMAG buffer (10 mM Tris·SO_4, pH 7.5, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.02% sodium azide, and 20% (v/v) glycerol), and sonicated during 10 s/ml cell suspension. The cell debris was removed by ultracentrifugation for 1 h at 150,000 × *g*. The supernatant was fractionated on a DEAE cellulose anion exchange column as described previously [1]. The enzyme fractions containing the most purified enzyme, as estimated by SDS-polyacrylamide gel electrophoresis, were pooled and used for further experiments.

2.4. Reactivation measurements

An enzyme solution (4 mg/ml) was split in 6 parts. Each part was dialyzed for 12 h at 4°C against a different buffer, all containing 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM sodium azide, and 20%

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glycerol. The buffer components were: pH 5.0, 25 mM sodium acetate; pH 6.0 and 7.0, 25 mM potassium phosphate; pH 7.5 and 8.0, 25 mM Tris · H_2SO_4; pH 9.0, 25 mM glycine · NaOH. The enzyme solutions were subsequently incubated at 4°C.

Haloalkane dehalogenase activities were measured colorimetrically as described before [2] at 30°C, using 5 mM 1,2-dibromoethane as the substrate.

2.5. Peptide isolation and characterization

Peptides (Asn-117–Arg-140) containing Asp-124 or Asn-124 were isolated as described before by trypsin digestion and HPLC purification [8]. Determination of the amino acid sequence of the isolated peptides was done by automated Edman degradation [15], which was performed by Eurosequence BV (Groningen, The Netherlands).

3. Results and discussion

3.1. Reactivation of Asp-124→Asn enzyme

A mutant of haloalkane dehalogenase carrying an Asp-124 to Asn substitution was constructed by site-directed mutagenesis using the Kunkel procedure. Transformants of *E. coli* BL21(DE3) harboring the Asp-124→Asn mutant dehalogenase were easily recognized as dehalogenase negative with a colony assay. Initial purification of the Asp-124→Asn mutant enzyme resulted in an enzyme solution which had 60% of the activity of wild type enzyme with 1,2-dibromoethane, however. During purification, the activity of the enzyme increased to a value higher than the amount of activity present in crude extract.

In order to directly study the activation process, we used partially purified enzyme, obtained within one day after induction of dehalogenase synthesis and within 10 h after harvesting the cells. The rapidly isolated Asp-124→Asn enzyme was at least 60% pure, as determined by polyacrylamide gelelectrophoresis and showed a specific activity of 0.43 U/mg of protein. The solution was split in six parts and fractions were incubated at pH values of 5, 6, 7, 7.5, 8 and 9 at 4°C. The activity was followed in time. The measurements showed that the dehalogenase activity indeed increased in time at pH values 5–8 and initially also at pH 9.0 (Fig. 2).

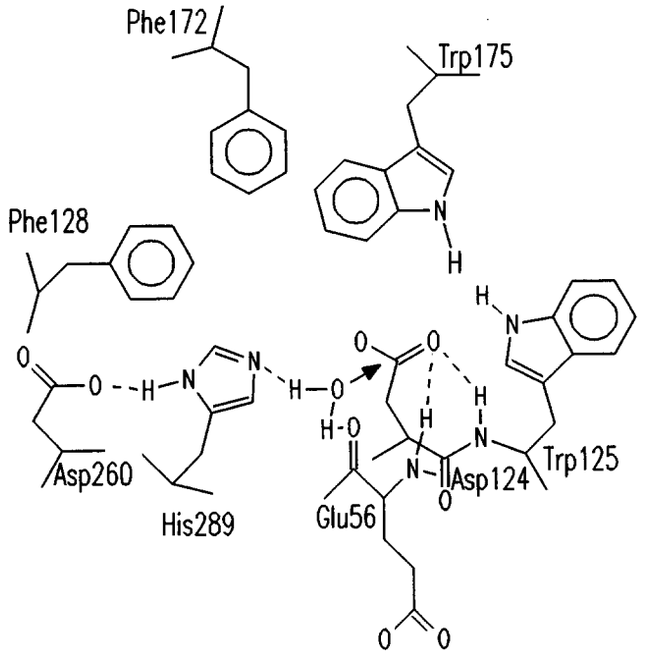

Fig. 1. Schematic view of the active site of haloalkane dehalogenase. The Cγ carbon atom of residue 124 can be attacked by a nearby water molecule, which is hydrogen bonded to $N_{\epsilon 1}$ of His-289. This water molecule is responsible for hydrolysis of the alkyl-enzyme intermediate during conversion of substrate by the wild type enzyme.

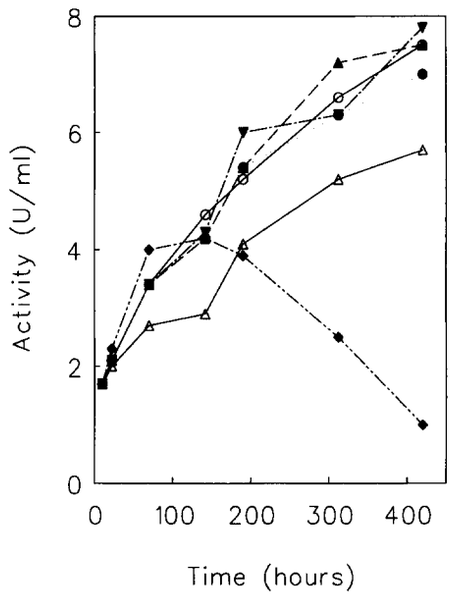

Fig. 2. Reactivation of Asp-124→Asn haloalkane dehalogenase. The enzyme solution (4 mg/ml) was divided in six parts and incubated in different buffers. Incubation conditions and assays were as described in section 2. Symbols: △, pH 5.0; ○, pH 6.0; ▲, pH 7.0; ●, pH 7.5; ▼, pH 8.0; ◆, pH 9.0. The activity of wild type haloalkane dehalogenase with DBE is 4.0 U/mg protein.

The rate of activation was pH dependent. At pH 6.0 to 8.0, a rapid activation was found, until dehalogenase levels similar to those of the wild type enzyme were reached, i.e. 1.6 U/mg protein with 1,2-dibromoethane. The initial rate of reactivation corresponds to a rate constant of about 4×10^{-3} h^{-1} (half-life of 170 h). At pH 5.0, reactivation was about 60% slower ($k = 1.7 \times 10^{-3}$ h^{-1}). Enzyme incubated at pH 9.0 showed a more rapid activation during the first 100 h, but the activity decreased after 150 h due to instability at high pH.

3.2. Isolation of the Asp/Asn-124 containing peptides

To determine the nature of the reactivation reaction, peptides containing the nucleophilic residue were isolated. Trypsin cleavage of haloalkane dehalogenase is expected to produce a 24-meric polypeptide (Asn-116–Arg-139), starting with the sequence Asn-Ile-Thr-Leu-Val-Val-Gln-Asp-Trp-Gln-Gln (position of the nucleophile underlined). Comparison of the HPLC elution profile a preparation of trypsin cleaved Asp-124→Asn dehalogenase with that of wild type enzyme showed that an additional polypeptide was produced from mutant enzyme. The peptide had a retention time (48 min) longer than the peak at retention time 43 min, which is the Asp-124 containing 24-mer of the wild type enzyme (Fig. 3). The first 10 N-terminal amino acid residues of both peptides were determined with automated gas phase Edman degradation. The earlier eluting peptide had the wild type sequence, whereas the peptide at 48 min had the mutant sequence with Asn at position 8.

The deamidation of mutant enzyme as a function of time was followed by HPLC analysis of tryptic peptides obtained from Asp-124→Asn haloalkane dehalogenase incubated at pH 7.5. The ratio of Asp-124 containing peptide to Asn-124 peptide at different timepoints was determined by peak integration. The data showed that reactivation was accompanied by gradual deamidation of Asn-124 (Fig. 4).

3.3. Mechanism of deamidation

Deamidation occurs in many enzymes during aging, generally leading to a decrease of activity. Usually, deamidation proceeds via nucleophilic attack on the amide carbon by the peptide bond nitrogen that is located C-terminal of the asparagine. This yields a cyclic imide peptide, which is subsequently hydrolyzed to aspartate or isoaspartate, generally at a ratio of 1:3 [16–18]. There are several observations suggesting that deamidation of Asn-124 in the mutant dehalogenase takes place in a different way. The reactivation of the mutant is almost complete, which is unlikely to be the case if isoaspartate is formed. There is also no indication for the presence of isoaspartate in any of the tryptic peptides that appeared during deamidation. Edman degradation stops at isoaspartate, and no such stops were encountered. Furthermore, deamidation with a cyclic imide as intermediate is most rapid with asparagines located on the N-terminal side of glycine residues; it has never been found with an asparagine preceding a tryptophan, as present in haloalkane dehalogenase [17]. The rate of deamidation of Asn-124 is also much faster than deamidation rates observed in other proteins, since it can be measured within hours instead of days or years [16].

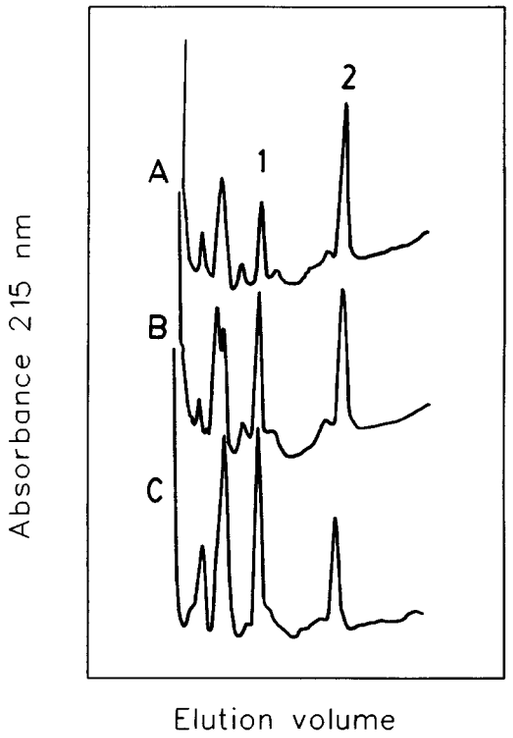

Fig. 3. Partial HPLC elution profiles of tryptic digests of Asp-124→Asn haloalkane dehalogenase. Enzyme was incubated at pH 7.5 and analyzed at different times after the cells were harvested. A: sample 10 h after cell harvesting; B: 22 h; C: 70 h. (1) Peptide with Asp at the 6th position in 24-meric tryptic peptide; (2) peptide with an Asn at that position.

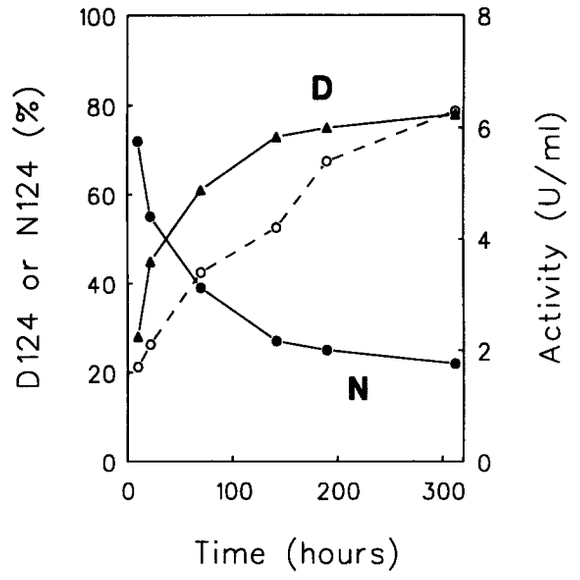

Fig. 4. Time course of reactivation and deamidation. The activity and relative amount of Asp-124 to Asn-124 of a solution of 4 mg/ml mutant dehalogenase was determined at different timepoints. The peak areas of the peptides appearing in the HPLC profiles were used to determine relative amounts of Asp-124 and Asn-124 containing dehalogenases. Symbols: ○, activity; ▲, amount of aspartate containing peptide; ●, amount of asparagine containing peptide.

The above indicates that the deamidation is caused by direct attack of water on the side-chain amide carbon of Asn-124. Two factors may elevate the unusual sensitivity of this amide bond to hydrolysis. First, a water molecule located in the proximity of the amide function may be highly nucleophilic. In the X-ray structure of the native enzyme, there is a water molecule whose oxygen is 3.11 Å away from the Cγ of Asp-124. It is hydrogen bonded to the $N_{\varepsilon 2}$ of His-289 which is 2.8 Å away. This water was proposed to be responsible for the cleavage of the covalent intermediate, and it is likely that it is also involved in the amide hydrolysis observed here. Another factor that could increase the sensitivity of Asn-124 to hydrolysis is the presence of an oxyanion hole that could stabilize the negative charge that develops on the $O_{\delta 1}$ of Asn-124 during hydrolysis. The stabilization can be provided by hydrogen bonding to the hydrogens of the peptide bond nitrogens of Trp-125 and Glu-56.

The active site of haloalkane dehalogenase thus appears to have evolved to optimize two different nucleophilic substitution reactions: one with a carboxylate oxygen of Asp-124 on the on the Cα of the substrate and a second one with water on the on the Cγ of Asp-124.

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