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Inactivation of epoxide hydrolase by catalysis-induced formation of isoaspartate

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Abstract Epoxide hydrolases catalyze hydrolytic epoxide ring-opening, most often via formation of a covalent hydroxyalkyl-enzyme intermediate. A mutant of *Agrobacterium radiobacter* epoxide hydrolase, in which the phenylalanine residue that flanks the invariant catalytic aspartate nucleophile is replaced by a threonine, exhibited inactivation during conversion when the (*R*)-enantiomer of *para*-nitrostyrene epoxide was used as substrate. HPLC analysis of tryptic fragments of the epoxide hydrolase, followed by MALDI-TOF and TOF/TOF analysis, indicated that inactivation was due to conversion of the nucleophilic aspartate into isoaspartate, which represents a novel mechanism of catalysis-induced autoinactivation. Inactivation occurred at a lower rate with the (*S*)-enantiomer of *para*-nitrostyrene epoxide, indicating that it is related to the structure of the covalent hydroxyalkyl-enzyme intermediate.

Keywords: Epoxide hydrolase; Isoaspartate; Covalent modification; Enzyme inactivation

1. Introduction

Epoxide hydrolases catalyze conversion of epoxides to diols via a covalent hydroxyalkyl-enzyme intermediate [1–3]. This reaction is of great importance in the mammalian metabolism and detoxification of xenobiotic compounds [4]. Epoxide hydrolases can also be applied in industrial biocatalysis [5]. Most known epoxide hydrolases belong to the α/β-hydrolase fold family, which implies that they have a catalytic triad composed of a nucleophile, a histidine base, and an acidic amino acid. In epoxide hydrolases, haloalkane dehalogenases, and fluoroacetate dehalogenases the nucleophile is an aspartate, instead of the more common serine that is present in lipases and other esterases of this protein family [6].

During our studies with some mutant epoxide hydrolases that were found in a site-saturation library of *Agrobacterium radiobacter* epoxide hydrolase (EchA) at position 108, we observed that substrate conversion progress curves leveled off before complete conversion was achieved, and that the curves could not be described by Michaelis–Menten kinetics with product inhibition. This phenomenon indicated inactivation of the enzyme during catalysis (van Loo et al., unpublished results). Previous results showed that EchA was irreversibly inactivated during prolonged incubation with some diols that can be produced from epoxides, most notably 1,2-octanediol [7]. This inactivation could be prevented by addition of a competitive inhibitor, suggesting that inactivation was caused by interaction of the 1,2-diol in the active site. Although inactivation was mostly irreversible, mass spectrometry measurements indicated no net change in molecular mass during the inactivation process. Furthermore, circular dichroism (CD) measurements showed that no unfolding of the enzyme occurred, and only in the near-UV regions significant changes were observed, which were correlated to changes in the active site by comparison of spectra of wild-type and mutant enzymes [7].

In this paper, we address the mechanism of inactivation of an F108T mutant of EchA that is particularly sensitive to inactivation upon incubation with its substrate (*R*)-*para*-nitrostyrene oxide. Residue F108 distally flanks the nucleophilic aspartate and is conserved as a bulky group in α/β-hydrolase fold epoxide hydrolases. Kinetic measurements showed that inactivation occurred on average once in every 119 catalytic cycles and biochemical analysis of the inactivated enzyme suggests that the active-site nucleophilic aspartate is converted into isoaspartate.

2. Materials and methods

2.1. Materials

We used mutant EchA F108T that was obtained from a site-saturation library at position 108 (van Loo et al., manuscript in preparation). Production and purification of this mutant enzyme were done essentially as described by Rink et al. [3]. (*R*)-*para*-nitrostyrene epoxide ((*R*)-pNSO) and (*S*)-pNSO were a gift of Enzis. The purity was checked by HPLC (>98%). 1-(*R*)-*para*-nitrophenyl-1,2-ethanediol was prepared by hydrolysis of a solution of (*R*)-pNSO with epoxide hydrolase, followed by heat inactivation (10 min, 80 °C) and removal of protein by filtration. The preparation contained 90% (*R*)-diol and 10% (*S*)-diol as judged by chiral HPLC [8]. *Para*-nitrophenyl glycidylether was obtained from Acros.

2.2. Enzymatic conversions

Conversion of epoxides and activity assays were carried out with purified wild-type or F108T mutant epoxide hydrolase at 30 °C in 50 mM Tris · SO_4 buffer, pH 7.5, and followed at 310 nm (pNSO) or 350 nm (*para*-nitrophenyl glycidylether, pNPGE), as described previously [9]. In order to keep the absorbance of substrate at the start of the conversion below 1, the substrate concentration was lower than 250 μM in all experiments. Amounts of diol formed and activities were calculated from the decrease in absorbance (for pNSO, $\varepsilon_{310epoxide} = 4289\ M^{-1}\ cm^{-1}$, $\varepsilon_{310diol} = 3304\ M^{-1}\ cm^{-1}$ and for pNPGE, $\varepsilon_{350epoxide} = 4218\ M^{-1}\ cm^{-1}$, $\varepsilon_{350diol} = 4889\ M^{-1}\ cm^{-1}$). Enzyme reactivation was followed in the same Tris buffer.

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2.3. Circular dichroism measurements

Circular dichroism (CD) spectra of EchA F108T before and after incubation with (*R*)-pNSO were measured on an AVIV circular dichroism spectrometer (62ADS). Far-UV spectra were recorded at 25 °C from 190 to 250 nm in a 0.1 cm quartz cuvette using a bandwidth of 1 nm. Spectra obtained from three scans were averaged and were corrected for absorbance caused by the incubation solution. Near-UV CD spectra of wild-type and mutant epoxide hydrolases were recorded from 250 to 310 nm using a 0.5 cm quartz cuvette. The spectra of three scans, each recorded using a band width of 1 nm and steps of 1 nm, were averaged.

2.4. Tryptic cleavage of EchA F108T and HPLC analysis of digests

Purified EchA F108T, before and after incubation with (*R*)-pNSO, was dialyzed against 100 mM $NaHCO_3$, pH 8, and subsequently digested with 1% (w/w) of trypsin for 18 h at 37 °C. The digest was subjected to HPLC, which was carried out on a Nucleosil C18 column (5 μm, 250 × 2.1 mm) that was operated with a linear gradient of acetonitrile (0–70% (v/v), 1 ml min^{-1}, 60 min) in 0.1% (v/v) trifluoroacetic acid. Elution was followed at 214 nm and the target peptides were manually collected, lyophilized and used for mass spectrometric analysis.

2.5. Mass spectrometry of EchA F108T peptides

The isolated peptides were dissolved in 50% acetonitrile containing 0.1% (v/v) trifluoroacetic acid and mixed with an equal volume of 10 mg ml^{-1} α-cyano-4-hydroxycinnamic acid in the same solvent. Samples of 1.5 μl were spotted on the target and the samples were dried in air. MALDI-TOF and TOF/TOF mass spectra were recorded using a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA). Spectra were calibrated externally. Peptide fragmentation spectra were produced with a collision energy of 1 keV and air as collision gas at 1×10^{-6} Torr.

3. Results

3.1. Inactivation behavior of EchA F108T

Kinetic resolution experiments with purified F108T mutant epoxide hydrolase indicated that the enzyme became inactivated during conversion of *para*-nitrostyrene oxide (pNSO), although the protein was stable upon storage under the same conditions in the absence of substrate. The F108T enzyme exhibited enantioselectivity, but the conversion slowed down over time and stopped after 91% of the preferred (*R*)-enantiomer and 49% of the (*S*)-enantiomer of pNSO were hydrolyzed (Fig. 1). When conversion stopped, the enzyme had performed only 197 turnovers.

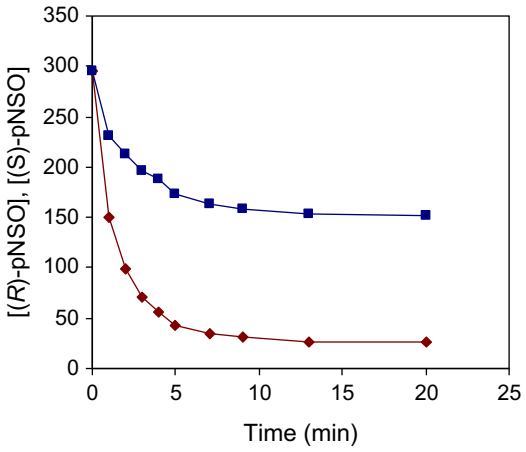

Fig. 1. Kinetic resolution of *rac*-pNSO and concomitant enzyme inactivation during turnover with F108T epoxide hydrolase (2.1 μM). Symbols: ◆, (*R*)-pNSO; ■, (*S*)-pNSO concentration.

To monitor the inactivation in more detail, varying amounts of F108T epoxide hydrolase were incubated with (*R*)-pNSO (170 μM), and hydrolysis of epoxide to diol was followed at 310 nm in a spectrophotometer. The amount of epoxide that was converted was proportional to the amount of enzyme added, indicating that only a limited number of substrate molecules was turned over per enzyme molecule. When more than 1.5 μM enzyme was added, all of the (*R*)-pNSO (>95%) was converted to its corresponding diol in 10 min (Fig. 2A). At enzyme concentrations below 1.5 μM, the amount of substrate converted was incomplete and proportional to the amount of enzyme added. The slope of the fitted curve, which is 119, is equal to the average number of conversions an enzyme molecule performs before it is inactivated, which can be called the transformation capacity.

The inactivation occurred faster with the preferred (*R*)-enantiomer of pNSO than with (*S*)-pNSO, and the calculated transformation capacity of F108T epoxide hydrolase with (*S*)-

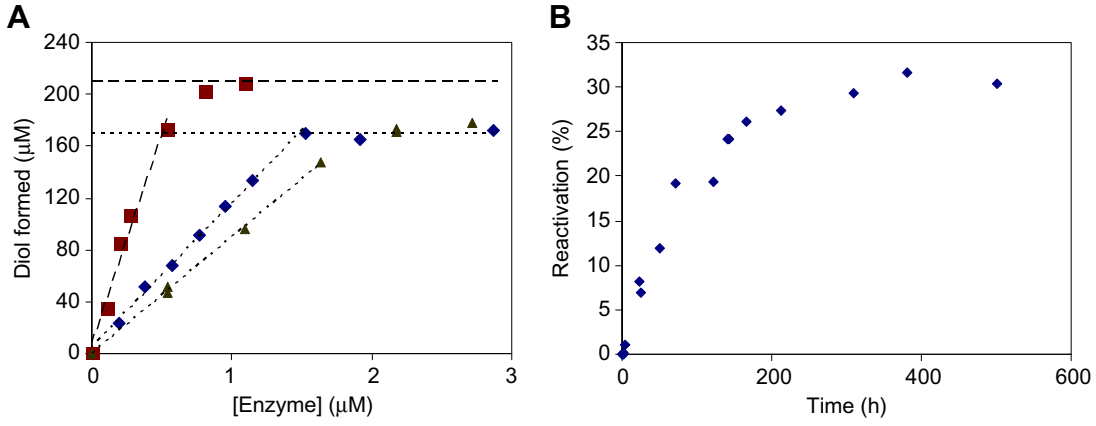

Fig. 2. Inactivation of F108T EchA by pNSO during substrate turnover. (A) Concentration of converted epoxide vs. the concentration of added enzyme for: (■), F108T EchA with 215 μM (*S*)-pNSO; (◆), F108T EchA with 170 μM (*R*)-pNSO; (▲), F108T EchA with 170 μM (*R*)-pNSO and 200 μM (*R*)-1-*p*-nitrophenyl-1,2-ethanediol. The slopes of the fitted curves (◆, 119; ■, 404) represent the average maximal turnovers per enzyme molecule (transformation capacity). Horizontal dashed and dotted lines indicate the level of diol formed in case of complete turnover of (*S*)-pNSO and (*R*)-pNSO, respectively. (B) Recovery of activity during storage of F108T EchA (5 μM) at 4 °C after removal of excess (*R*)-pNSO by dialysis.