The Enethiolate Anion Reaction Products of EpiD

pKₐ VALUE OF THE ENETHIOL SIDE CHAIN IS LOWER THAN THAT OF THE THIOL SIDE CHAIN OF PEPTIDES

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Thomas Kupke‡ and Friedrich Götz

From the Mikrobielle Genetik, Universität Tübingen, Waldhäuserstrasse 70/18, 72076 Tübingen, Germany

One of the steps involved in the biosynthesis of the lantibiotic epidermin is the oxidative decarboxylation reaction of peptides catalyzed by the flavoenzyme EpiD. EpiD catalyzes the formation of a (Z)-enethiol derivative from the C-terminal cysteine residue of the precursor peptide of epidermin and related peptides. The UV-visible spectra of the reaction products of EpiD are pH-dependent, indicating that the enethiol side chain is converted to an enethiol anion. The pKₐ value of the enethiol group was determined to be 6.0 and is substantially lower than the pKₐ value of the thiol side chain of cysteine residues. The increased acid strength of the enethiol side chain compared with that of the thiol group is attributed to the resonance stabilization of the negative charge of the anion.

Several new posttranslational modification reactions, such as dehydration of serine and threonine residues, thioether formation, and formation of α-alanine residues from α-serine residues, are involved in the biosynthesis of the ribosomally synthesized lantibiotics (reviewed in Refs. 1 and 2). The lantibiotic epidermin contains the α,β-unsaturated amino acid dihydroxyaminobutyric acid, S-(Z)-2-aminovinyl)-D-cysteine, 3-methyl-lanthionine, and meso-lanthionine (3). The formation of epidermin from the precursor peptide EpiA (4) includes the oxidative decarboxylation of the C-terminal cysteine residue of EpiA to a (Z)-enethiol catalyzed by the FMN-containing enzyme EpiD (5, 6). Two reducing equivalents from the C-terminal cysteine residue are removed, a double bond is formed, and the coenzyme FMN is reduced to FNMH₂. The decarboxylation occurs spontaneously or is also catalyzed by EpiD. The (Z)-enethiol derivative is the intermediate in the formation of the C-terminal S-(Z)-2-aminovinyl)-D-cysteine residue of epidermin (6). The unusual enethiol structure has been confirmed by using mass spectrometry, tandem mass spectrometry, UV-visible spectroscopy, two-dimensional NMR spectroscopy, and conversion of the enethiol to a mixed disulfide with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman’s reagent (7, 8)). EpiD has a low substrate specificity, and most of the peptides with the sequence [V/I/L/M/P/Y/W]-[A/S/V/T/I/L]-C at the C terminus are substrates of EpiD, as elucidated by analysis of the reaction of EpiD with single peptides and peptide libraries, respectively (7).

Here we report the further characterization of the reaction products of EpiD. We demonstrate that at physiological pH, the dominant form of the reaction product is the enethiol anion and that the pKₐ value of the enethiol group is 6.0. We assume that the enethiolate anion is strongly nucleophilic and is the substrate for the Michael addition-like formation of S-(Z)-2-aminovinyl)-D-cysteine. Furthermore, we discuss the possibility that enzymes exist in nature that catalyze the formation of enethiol groups from non-C-terminal cysteine residues.

EXPERIMENTAL PROCEDURES

Synthesis and Purification of SFNSYV–NH–CH=CH–SH and SFNSYTV–NH–CH=CH–SH—SFNSYVC and SFNSYTC were synthesized as described previously (7). For generation of the enethiol derivative of SFNSYVC, approximately 25 mg of peptide were dissolved in 50 ml of 20 mM Tris/HCl (pH 8.0) containing 4 mM DTT and 1 mg of MBP-EpiD (5). For generation of the enethiol derivative of SFNSYTC, approximately 12 mg of peptide and 500 μg of MBP-EpiD were used. After 25 min of incubation at 37 °C, 500 μl of trifluoroacetic acid was added. The reaction mixture was then separated by reversed-phase chromatography using a ProRPC HR10/10 column (Pharmacia Biotech Inc.). Peptides were eluted with a linear gradient of 0–80% acetonitrile, 0.1% trifluoroacetic acid in 150 ml at a flow rate of 2 ml/min. Peptides with a high absorbance at 260 nm were collected and diluted with H₂O, 0.1% trifluoroacetic acid. DTT was added to 3 mM, and the samples were subjected to a second reversed-phase chromatography using a μRPC C2/C18 SC 2.1/10 column (Pharmacia). Peptides were eluted with a linear gradient of 0–50% acetonitrile, 0.1% trifluoroacetic acid in 5.8 ml at a flow rate of 200 μl/min. The absorbance was measured simultaneously at 214, 260, and 280 nm. The modified peptide was identified by the high A₅95/A₂₆₀ ratio and collected by the peak fractionation method using the SMART™ system (Pharmacia, Freiburg, FRG). Samples were dried with a vacuum concentrator (yields: approximately 4.4 mg of SFNSYV–NH–CH=CH–SH and 1 mg of SFNSYTV–NH–CH=CH–SH, respectively) at 70 °C. The modified peptides were analyzed by electrospray mass spectrometry as described recently (7).

UV-visible Spectroscopy—UV-visible spectra were obtained with a Beckman DU 7500 spectrophotometer. Spectral data were recorded in the range of 200–600 nm at 21 °C at various pH values in quartz cuvettes (1-cm light path; the reference cuvette contained the used buffers and on occasion 4 mM DTT). For determination of the pKₐ, 0.6-ml aliquots of a stock solution of the oxidatively decarboxylated peptide SFNSYV–NH–CH=CH–SH were mixed with 0.15 ml of 50 mM sodium phosphate (pHₐ = 2.15, 7.20, and 12.38), 50 mM sodium citrate buffer (pKₐ = 3.13, 4.76, and 6.40) (final buffer concentration was 10 mM each for phosphate and citrate). The pH of the buffers was adjusted by adding HCl. pH values of the 10 mM phosphate/citrate buffers and of the buffered peptide solutions were determined using a Knick pH meter (781 Calimatic).

RESULTS

pH Dependence of UV-visible Spectra of Oxidatively Decarboxylated Peptides—The reaction products of the flavoenzyme EpiD are oxidatively decarboxylated peptides with a C-terminal enethiol side chain (6–8). Recently, we demonstrated that the reaction products of EpiD have an absorption maximum at 260 nm when measured in 0.1% trifluoroacetic acid, 30% ace
The pH dependence of the UV spectrum of the oxidatively decarboxylated peptide indicates the ionization of the enethiol form to the enethiolate anion form (Fig. 1). From the UV spectra, the apparent pKₐ value of the enethiol group was determined to be pH 6.0 (Fig. 1B). The molar extinction coefficient (ε) of SFNSYV–NH–CH–SH at 259 nm and of SFNSYV–NH–CH–S⁻ at 283 nm was determined to be at least ε = 8,600 and ε = 7,200 M⁻¹ cm⁻¹, respectively. However, this value may even be higher due to error in weighing out the small amounts of available peptide and due to the presence of sodium chloride and water in the peptide sample. Assuming that the peptides SFNSYVC and SFNSYV–NH–CH–S⁻ have the same ε₅₂₀ value, ε₅₂₀ of SFNSYV–NH–CH–SH and ε₇₂₃ of SFNSYV–NH–CH–S⁻ were calculated to be approximately 10,000 M⁻¹ cm⁻¹.

**FIG. 2.** Analysis of the reaction products of SFNSYTC by reversed-phase chromatography using acidic and neutral buffer systems. The reaction of MNP-EpiD with SFNSYTC was investigated by separation of the reaction mixture on a Pharmacia μRPC C2/C18 SC 2.1/10 column under acidic (A1, no enzyme; A2, with MNP-EpiD) (buffer A, 0.1% trifluoroacetic acid, H₂O; buffer B, 0.1% trifluoroacetic acid, acetonitrile) and neutral buffer conditions (B1, no enzyme; B2, with MNP-EpiD) (buffer A, 25 mM of TEAA/H₂O; buffer B, 25 mM of TEAA/20% H₂O, 80% acetonitrile). The assay (total volume of 2 ml) was carried out in 20 mM Tris/HCl (pH 8.0) containing 3.5 mM DTT, 100 μg of the peptide, and approximately 10 μg of MNP-EpiD. The reaction was stopped after 30 min of incubation at 37°C by adding 20 μl of trifluoroacetic acid. Aliquots of the reaction mixture were applied to reversed-phase chromatography using acid and neutral buffer conditions, respectively. After washing the reversed-phase column with 2 ml of buffer A, the peptides were eluted with a linear gradient of 0–50% buffer B in 5.8 ml at a flow rate of 200 μl/min. The elution was followed by absorbance at 214 nm (upper thin line), 260 nm (thick line), and 280 nm (lower thin line), and the peak fractions were analyzed by electrospray mass spectrometry (determined m/z values are indicated in the panels: substrate peptide, 821 m/z; oxidatively decarboxylated peptide, 775 m/z).
**DISCUSSION**

The determined $pK_a$ value of 6.0 for the enethiol group of SFNSYY–NH–CH=CH–SH is significantly (3 pH units) lower than the reported $pK_a$ value for the thiol group of cysteine residues in small peptides. Peptides with a C-terminal cysteine residue in which the negative charge of the carboxyl group is removed (cysteine amides, a negative charge in the vicinity of the thiol group decreases the acidity of the thiol group (11)), as is also the case for the investigated oxidatively decarboxylated peptides SFNSYV–NH–CH=CH–SH and SFNSYT–NH–CH=CH–SH, are reported to have a $pK_a$ value in the range of pH 8.8 (12). The increased acid strength of the enethiol group compared with the acid strength of the thiol group is explained by a resonance effect in which the negative charge on the sulfur anion is delocalized in the enethiolate. Delocalization of the negative charge is the reason for stabilization of enolate ions so that the acidity of enols is increased compared with alcohols (13).

For several enzymes, such as the protein thiol-disulfide oxidoreductase DsbA from Escherichia coli, human glutathione transferase, and cysteine peptidases, it has been shown that the catalytic active cysteine thiol groups have a $pK_a$ value near 4.0 (14–16). This low $pK_a$ value is partially explained by stabilization of the thiolate anion by interaction with histidine or lysine side chains in the tertiary structure of the proteins. It is very unlikely that the peptides SFNSYV–NH–CH=CH–SH and SFNSYT–NH–CH=CH–SH have a defined three-dimensional structure and that this structure is responsible for stabilization of the enethiolate anion.

There is a strong correlation between the low $pK_a$ value of the thiol group of Cys$^{30}$ of DsbA and the redox potential of DsbA (17). The two cysteine residues of the active site Cys$^{30}$, Pro$^{31}$-His$^{32}$-Cys$^{33}$ form a disulfide bridge, and disulfide-bonded DsbA is a potent oxidant that generates protein disulfide bonds (18, 19). Because of the correlation between the $pK_a$ value of the thiol group and the redox potential, we are interested in determining the redox potential of peptides containing both a cysteine residue and a C-terminal enethiol group (e.g., oxidatively decarboxylated precursor peptide EpiA), and we are interested in investigating the reaction between protein thioldisulfide oxidoreductases and such substrates.

As known for thiols (20), we assume that enethiols are far more reactive as the enethiolate anions. Therefore, reasonable chemical intuition suggests that the intermediate in the Michael addition-like formation of the S-(Z)-2-aminovinyl]-l-cysteine residue of epidermin is the enethiolate and not the enethiol form of posttranslationally modified precursor peptide EpiA. Furthermore, we postulate that in the case of lanthanione formation the thiolate anions of the involved cysteine residues are stabilized by interaction with positive charges within the precursor peptide or by interaction with the enzyme responsible for the thiolate formation (EpiC and/or EpiB). Glutathione transferase catalyzes a similar reaction, the nucleophilic addition of glutathione to xenobiotic compounds containing an electrophilic center. It has been discussed that the $pK_a$ of the thiol group of glutathione is shifted from 9.0 in aqueous solution to 6.6 in the active site of glutathione transferase so that the predominant species in the active site is the thiolate anion of glutathione (21).

**FIG. 3. Alternative model for the thiazole backbone modification of microcin B17.** The recently postulated mechanism for the posttranslational modification of Gly-Cys-Gly- segments of the microcin precursor peptide to the thiazole backbone modification of microcin B17 involved the attack of the thiol group of cysteine on the peptide carbonyl of the preceding glycine residue, the elimination of water, and the oxidation (removal of H$_2$) (22, 23). Here we describe that, in principle, the oxidation can be the first reaction and that an enethiol is formed as the first intermediate. Due to the increased acidity of the enethiol, the reactive nucleophile is the enethiolate anion. A similar reaction is the equilibrium between thiamine (vitamin B$_2$) and its enethiolate form (26, 27).

A fascinating question is whether enzymes exist in nature that catalyze the formation of enethiols/enethiولاتe from cysteine residues that are not located at the C terminus. Principally, such a reaction can be involved in the formation of the thiazole backbone modification of the gyrase inhibitor microcin B17, whose structure has been elucidated recently (22, 23) (Fig. 3).

Enzymes involved in microcin B17 modification have not been characterized, and there is no experimental evidence for the existence of the proposed enethiol-forming activity in microcin B17 modification. By converting the thiol side chain of cysteine residues in peptides and proteins to enethiol/enethiولاتate the (pH dependence of the) activity and stability of proteins may be influenced to a large extent. However, nothing is known about the stability of these enethiol side chains. Peptides with a C-terminal enethiolate group are unstable, but they are stabilized in the presence of zinc ions (6–8). In addition to the flavoprotein EpiD, another enzyme catalyzing an α,β-dehydrogenation reaction of peptides has already been characterized; heme-containing L-tryptophan 2’,3’-oxidase from Chromobacterium violaceum is responsible for the synthesis of α,β-dehydrotryptophan derivatives (24, 25).

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