Affinity Alkylation of the Trp-B4 Residue of the β-Subunit of the Glutaryl 7-Aminocephalosporanic Acid Acylase of Pseudomonas sp. 130*

Received for publication, September 10, 2001, and in revised form, December 9, 2001

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Glutaryl 7-aminocephalosporanic acid acylase of Pseudomonas sp. 130 (C130) was irreversibly inhibited in a time-dependent manner by two substrate analogs bearing side chains of variable length, namely 7β-bromocrotyl aminocephalosporanic acid (BA-7-ACA) and 7β-3-bromopropionyl aminocephalosporanic acid (BP-7-ACA). The inhibition of the enzyme with BA-7-ACA was attributable to reaction with a single amino acid residue within the β-subunit proven by comparative matrix assisted laser desorption/ionization-time of flight mass spectrometry. Further mass spectrometric analysis demonstrated that the fourth tryptophan residue of the β-subunit, Trp-B4, was alkylated by BA-7-ACA. By ¹H-¹³C HSQC spectroscopy of C130 labeled by BA-2-¹³C-7-ACA, it was shown that tryptophan residue(s) in the enzyme was alkylated, forming a carbon-carbon bond. Replacing Trp-B4 with other amino acid residues caused increases in Kₚ, decreases in k_cat, and instability of enzyme activity. None of the mutant enzymes except W-B4Y could be affinity-alkylated, but all were competitively inhibited by BA-7-ACA. Kinetic studies revealed that both BA-7-ACA and BP-7-ACA could specifically alkylate Trp-B4 of C130 as well as Tyr-B4 of the mutant W-B4Y. Because these alkylations were energy-requiring under physiological conditions, it is likely that the affinity labeling reactions were catalyzed by the C130 enzyme itself. The Trp-B4 residue is located in the middle of a characteristic αβα sandwich structure. Therefore, a large conformational alteration during inhibitor binding and transition state formation is likely and suggests that a major conformational change is induced by substrate binding during the course of catalysis.

The compound 7-aminocephalosporanic acid (7-ACA),¹ a key intermediate in the production of many semisynthetic antibiotics, can be synthesized by the chemical decylation of cephalosporin C (CPC) employing iminoethers, nitroryl chloride, and methanol. Because these steps involve toxic compounds causing environmental contamination, enzymatic methods of CPC deacylation are of great interest. Since cephalosporin acylase (CA) has very low activity toward CPC, most of the bioprocess reported methods involve a two-step conversion of CPC to 7-ACA. First, CPC is oxidized by d-amino acid oxidase (1), and the product, glutaryl-7-ACA (GL-7-ACA), is then converted to 7-ACA by GL-7-ACA acylase (EC 3.5.1.11) (Fig. 1A).

The gene encoding GL-7-ACA acylase of Pseudomonas sp. 130 (C130) has been cloned (2) and overexpressed in Escherichia coli (3). The active enzyme consists of two dissimilar subunits, a β-subunit (58 kDa) and an α-subunit (18 kDa). The subunits are derived from one precursor polypeptide. The catalytic mechanism of CA was deduced from its sequence and that of the functionally analogous penicillin G acylase (4). The latter enzyme is a well known serine proteinase that carries out an N-terminal nucleophilic (Ntn) attack (5) via a single serine as its catalytic center (6).

The first crystal structure of a CA, from Pseudomonas diminuta (CAD), was solved at a resolution of 2.0 Å (7). A similar crystal structure of C130 at a resolution of 2.4 Å, was solved independently.² The sequence identity between CAD and C130 is >98%. The crystal structures revealed some similarities between the active centers of the CAs and penicillin G acylase, although their overall structures are quite different, as expected from the low sequence similarities of the two enzymes. The structure of CAD (and C130) bears a characteristic αβα motif. The N-terminal residue Ser-B1 of the β-subunit is the catalytic center, indicating that CA belongs to the Ntn hydrolase superfamily, as defined by the Structural Classification of Proteins data base (8). Recently, structures of the binary complexes of CAD with GL-7-ACA and glutarate were solved at 2.5- and 2.6 Å resolutions, respectively (9). Although the site of initial substrate binding can be inferred from these studies,

¹ This work was supported by National High Technology Development Program of China Grant 863-103-13-02-01. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

² Y. Ding, W. Jiang, X. Mao, H. He, S. Zhang, H. Tang, M. Bartlam, S. Ye, F. Jiang, Y. Liu, G. Zhao, and Z. Rao, unpublished data.

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this information may not reflect the dynamic status of the enzyme-substrate complex during catalysis.

In this study, a series of substrate analogs with side chains of variable length were synthesized. Two compounds, 7β-bromomethyl aminocephalosporanic acid (BA-7-ACA) and 7β-3-bromo-propionyl aminocephalosporinic acid (BP-7-ACA), could specifically function as affinity reagents. The enzyme peak was collected with a gradient of NaCl ranging from 0 to 500 mM on fast protein liquid chromatography (Amersham Biosciences). The enzyme peak was collected with a gradient of NaCl ranging from 0 to 500 mM on fast protein liquid chromatography (Amersham Biosciences).

Enzyme activity was determined as previously described (12). One unit of activity is that amount of enzyme that produces 1 μmol of 7-ACA/min (3). The specific activity of the purified C130 was 6.2 μmol/min/mg.

**MALDI-TOP Mass Spectrometric Studies**

All experiments were performed on a Bruker REFLEX mass spectrometer. Enzyme samples (1 mg/ml) were prepared using n-cyano-4-hydroxybutyric acid as matrix in solution with 50% acetonitrile, 0.1% trifluoroacetic acid in water. 1 μl of sample solution was mixed with 1 μl of matrix solution, and 1 μl of the mixture was applied to the SCOUT 384 target.

**Site-directed Mutagenesis**

The W-B4L mutant was conducted by PCR using plasmid pKKCA1 as template and the primer, 5'-TCC ACC TCC TTG GCG GTG GCC CGG-3'. This oligonucleotide corresponds to the coding sequence from Ser-B1 to Pro-B8 with the Trp-B4 codon replaced by a Leu codon (underlined). This mutated gene retained the characteristic BamHI site at the Ser-B1 codon and created a Small site by altering the Ala-B7 codon. Mutants of P-B8G, W-B4A, W-B4T, W-B4F, W-B4H, and W-B4Y were obtained by replacing this BamHI-Smal fragment using oligodeoxyribonucleotides that replaced Pro-B8 with Gly and Trp-B4 with Ala, Thr, Phe, His, and Tyr, respectively. Correctness of the mutant clones was confirmed by DNA sequencing.

**Preparation of Labeled Enzymes**

The acylases (0.5 mg/ml) were incubated with excess BA-7-ACA (8 mM) in 100 mM sodium phosphate buffer (pH 8.0) for 1 h at 37 °C. The course of the labeling reaction was followed by measuring residual enzymatic activity (lower than 1% of the original enzymatic activity, indicating the completion of labeling). After concentration, the reaction mixture was subjected to gel filtration (Superdex 75; Amersham Biosciences). The fractions containing modified enzyme were collected and concentrated to >1 mg/ml with Molecut II LGC™ (Millipore Corp.).

**Digestion of Denatured Enzymes**

Both the original enzyme (2 mg/ml) and the labeled enzymes (2 mg/ml) were denatured at 65 °C in 8 M urea for 45 min. The denatured enzymes were digested either with trypsin or Lys-endopeptidase (Roche Molecular Biochemicals) according to the protocol of the supplier.

**HPLC and MS-MS Analysis for Oligopeptides**

On-line HPLC separation was performed on a Hewlett-Packard model 1100 system at a flow rate of 0.05 ml/min. Solvent A was 0.01% trifluoroacetic acid, 1% HOAc in water, and solvent B was 0.01% trifluoroacetic acid, 1% HOAc in acetonitrile. A 2.1 × 30-mm C8 reverse phase column (Applied Biosystems) was used for oligopeptide separation after proteolytic digestion. The gradient was 0–100% solvent B over 60 min. The mass spectra were obtained on a Finnigan LCQ ion trap mass spectrometer (ThermoQuest, San Jose, CA) equipped with an electrospray ionization source. The MS spray voltage was 4.25 kV. The heated capillary was maintained at temperature of 200 °C. The spectrum was scanned from 400 to 2000 m/z. The ZoomScan, tandem MS (MS/MS) was performed in the data-dependent mode. The MS/MS collision energy value was 42.

**NMR Study**

Heteronuclear correlation 1H-13C HSQC (13) spectrum was recorded on a Varian Inova 750-MHz spectrometer equipped with a z axis shielded triple resonance probe. The sample of C130 modified by BA-2,3,4,7-ACA was dissolved in 20 mM sodium phosphate buffer (pH 8.0) at a concentration of 40 mg/ml. BA-2,3,4,7-ACA was synthesized as described previously.

**RESULTS**

C130 Undergoes Stoichiometrically Labeling by BA-7-ACA in a Time-dependent Manner—Based on the structure of GL-7-ACA, we designed and synthesized a series of substrate analogs by replacing the side chain of GL-7-ACA with different length of bromoacetyl moieties (Fig. 1B). Although these compounds are structurally similar to the native substrate, GL-7-ACA, only...
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Marginal hydrolytic activities remained after incubating with C130 (Table I). Only BA-7-ACA and BP-7-ACA could inhibit the activity after prolonged incubation with C130. In addition, the inactivation was irreversible, because reactivated C130 could not be recovered even if the inhibitors were removed (data not shown). The other analogs and bromoacetic acid itself could not be recovered even if the inhibitors were removed (data not shown). The other analogs and bromoacetic acid itself could not be recovered even if the inhibitors were removed (data not shown).

The inhibition of C130 by BA-7-ACA proceeded in a time-dependent manner under physiological conditions. The kinetics of inactivation was pseudo-first-order. Each individual product of the irreversible inactivation was recovered immediately. The residual activity was then determined under standard assay conditions (see "Experimental Procedures"). To protect the C130 enzyme against inactivation, the reaction mixture was incubated individually with the addition of either glutaric acid (●) or 7-ACA ( ●), each at 8.0 mM.

indicating that this modification does not lead to a gross change in protein structure.

To confirm the equimolecular character of the alkylation of C130, a comparison of the native and labeled C130 was made by MALDI-TOF mass spectrometry to determine the stoichiometry of inhibitors binding. Unambiguously, the signals at m/z 17474.75 and m/z 17474.94 (Fig. 3, A and C) corresponding to the α-subunits (original and labeled) indicated that there was no labeling of the α-subunit of C130. On the other hand, signals corresponding to the β-subunits at m/z 58,315.54 (Fig. 3B) or m/z 58,614.26 (Fig. 3D) revealed an average mass increase of 299 on labeled C130. This result, within the instrumental error of MALDI-TOF, matches well enough to the expected molecular mass of 313 for -CH₂CO-7-ACA (-R), indicating that there was only one -R covalently bound to the β-subunit of C130.

**Table I.** Relative activities of Pseudomonas sp. 130 acylase (C130) in the presence of various substrate analogs

| Substrate analogue | Residual activity (toward GL-7-ACA) | Hydrolysis activity (toward substrate analogs) | % | % |
|--------------------|------------------------------------|-----------------------------------------------|----|----|
| None               | 100                                |                                               | 100| 100|
| Bromoacetyl-7-ACA  | 95.5                               |                                               | < 1| < 1|
| 3-Bromopropionyl-7-ACA | 97.2                         |                                               | < 1| < 1|
| 4-Bromobutyryl-7-ACA | 97.2                            |                                               | < 1| < 1|
| 5-Bromopentanoyl-7-ACA | 97.2                         |                                               | < 1| < 1|
| 6-Bromohexanoyl-7-ACA | 97.2                           |                                               | < 1| < 1|
| Bromoacetic acid   | 95.2                               |                                               | 95 | 95 |

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The inhibition of C130 by BA-7-ACA proceeded in a time-dependent manner under physiological conditions. The kinetics of inactivation was pseudo-first-order. Each individual product of the reaction, glutaric acid or 7-ACA, slowed down the rate of inactivation (Fig. 2). A semilogarithmic plot of inhibition rate k versus the concentration of BA-7-ACA yielded a straight line with a slope of 0.91. This implies that BA-7-ACA was functioning as an affinity labeling reagent, resulting in single hit modification of C130. In addition, far UV circular dichroism spectra of the original and modified C130 were almost identical (data not shown), indicating that this modification does not lead to a gross change in protein structure.

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**Substrate analogue** | **Residual activity (toward GL-7-ACA)** | **Hydrolysis activity (toward substrate analogs)** | % | % |
|----------------------|---------------------------------------|-----------------------------------------------|----|----|
| None                 | 100                                   |                                               | 100| 100|
| Bromoacetyl-7-ACA    | 95.5                                  |                                               | < 1| < 1|
| 3-Bromopropionyl-7-ACA | 97.2                               |                                               | < 1| < 1|
| 4-Bromobutyryl-7-ACA | 97.2                                  |                                               | < 1| < 1|
| 5-Bromopentanoyl-7-ACA | 97.2                               |                                               | < 1| < 1|
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**The T1 Tryptic Fragment of the C130 β-Subunit Contains the Alkylation Site**—Theoretically, complete digestion of C130 with trypsin should produce 65 peptides. A precise comparison of the LC/MS tryptic fingerprints of native C130 and the alkylated enzyme was made. More than 30 peptides in each of the two digests, covering about 80% of the total amino acid residues, were identified and confirmed by tandem mass spectrometry (data not shown). Among the peptides identified, we found only one [M + 2]⁺ signal at m/z 509 corresponding to SNSWAVPK (T1) from C130 that was not present in the peptide spectrum of the labeled C130. In the peptides from the alkylated species, a new [M + 2]⁺ signal at m/z 665 (T1-R) appeared. The T1 tryptic fragment originates from the N terminus of the C130 β-subunit. Within this segment, the first serine residue (Ser-B1) is known to be the Ntn catalytic center (4, 7). The additional molecular mass of 312 corresponds to that of the inhibitor residue (-R). Thus, the alkylation of the C130 β-subunit by BA-7-ACA is likely to form a stable enzyme-CH₂CO-7-ACA complex, and the labeling site is likely to lie within the T1 peptide.

**Trp-B4 of the β-Subunit of C130 Is the Alkylation Site**—Further investigation of MS/MS spectra of both T1 and T1-R was carried out to elucidate the sequence of T1 and to identify the chemically modified amino acid residue within T1-R. As shown in Fig. 4, most of the B ions and Y ions (14) from the MS/MS spectrum of T1 were identified and matched well with the proposed sequence of SNSWAVPKG. However, the pres-
FIG. 3. MALDI-TOF spectra of α- and β-subunit of C130 before (A and B) and after labeling (C and D) with BA-7-ACA.

FIG. 4. MS/MS spectrum of the doubly charged T1 ion at m/z 509. T1 was separated from the tryptic digest of C130 by LC/MS (see "Experimental Procedures"). The Y series ions of Y2 through Y8 are consistent with the sequence of the peptide SNSWAVPGK, which is the N-terminal tryptic fragment of the β-subunit of C130.
This hypothesis was substantiated by the presence of the Lys-endoproteinase, and the first peptide fragment of the [M-60]2 minus, which was analyzed by LC/MS, giving a [M/H11001/2]2 signal at 528, corresponding to Trp-R1 and [Trp-Ala]-R1, respectively (Fig. 6), as indicated by the presence of [Y9-OH]2. The signal at m/z 835 corresponding to WAVAPG plus R1 (782 + 253) allowed an assignment of Trp-B4 as the labeling residue within the T1-R peptide fragment.

A similar investigation of the BA-7-ACA-modified P-B8G mutant further supported the above conclusion. It has been reported that the proline imide bond (15) might interfere with the normal collision-induced dissociation in MS. In addition, based on our observations, replacing Pro-B8 with glycine is likely to be a stable mutant maintaining most of the original enzymatic properties. Thus, Lys-endoproteinase, and the first peptide fragment of the β-subunit, from Ser-B1 to Lys-B10, containing the R residue within it, was analyzed by LC/MS, giving a [M + 2]2+ signal at m/z 645. The signals at m/z 439 and m/z 510 in the MS/MS spectrum, corresponding to Trp-R1 and [Trp-Ala]-R1, respectively, directly identified the labeling site of BA-7-ACA.

**Carbon Bond**—To confirm that a chemical reaction involving the bromoacetyl group of BA-7-ACA and Trp-B4 of C130 had taken place, a 13C-labeled inhibitor, BA-2-13C-7-ACA, was synthesized and used in the affinity labeling of C130. The 1H-13C COSY spectrum of BA-7-ACA (see "Experimental Procedures") indicated that the 13C chemical shift of its C-2‘ was assigned at δC 30.1 ppm in PBS, pH 8.0 (or δC 28.5 ppm in CD3OD), correlated to the two C-2‘ protons (at δ 3.98 ppm in PBS, pH 8.0). Only one signal of 1H-13C correlation resonance was clearly presented in the two-dimensional HSQC spectroscopy of the 13C-labeled enzyme-inhibitor complex (Fig. 7A). This, only one kind of amino acid residue specifically alkylated. Meanwhile, this alkylation was confirmed by the obvious change of the 13C chemical shift of the 13C labeled carbon, which was assigned at δC 43.4 ppm.

From the known increments of substituted alkanes (16), we can predict the 13C chemical shift of the 13C labeled carbon when an amino acid residue is alkylated by BA-2-13C-7-ACA. For tryptophan, it is believed that C-3 of the indole ring is initially attacked by the bromide and then a C-2 alkylated product would be formed after rearrangement (17, 18). Therefore, C-2 of the indole ring is considered to be the only possible position to be alkylated (19) (Fig. 7B). Here, we predict the chemical shift by calculation with the following increments: δC = -2.3 (CH4) + 22 (-CONH2) + 23 (phenyl) = 42.7 (ppm), where the -CONH2 and phenyl groups are approximate substitutes for -CO-7-ACA and Trp residue, respectively. This predicted shift is close to the measured shift (δC 43.4 ppm) assigned in Fig. 7A. With a similar chemical environment, the C-2’ of penicillin G (Fig. 7B) gives a measured shift at δC 42.8 ppm (20), very close to the predicted one. Since there are no cysteine residues in C130, other possible alkylated functional groups on the residues are -OH, -COOH, and -NH2 (or =NH), giving predicted shifts at δC of 76.7, 71.7, and 56.2 (60.2) ppm, respectively. These shifts are significantly different from those observed, excluding the possible alkylation of Ser, Tyr, Thr, Asp, Glu, His, Lys, Arg, Asn, and Gln residues, as well as N-1 of the indole ring of Trp. Therefore, C-2 of the indole ring is the

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3 X. Huang, R. Zeng, X. Ding, X. Mao, Y. Ding, Z. Rao, Y. Xie, W. Jiang, and G. Zhao, unpublished observation.
most likely position for alkylation with BA-7-ACA; this event would lead to the formation of a carbon-carbon bond between C130 and the inhibitor. This covalent bond was stable enough to generate useful information from MS/MS spectra for the determination of modification of Trp-B4 (Fig. 5).

Analysis of Trp-B4 Mutants Indicates Possible Mechanism of Affinity Labeling

In order to further confirm the specific reaction of BA-7-ACA with Trp-B4 of the C130/H9252 subunit, six site-directed mutants were generated, replacing Trp-B4 with Ala (W-B4A), Leu (W-B4L), Thr (W-B4T), Phe (W-B4F), His (W-B4H), and Tyr (W-B4Y) respectively. Each of the mutant proteins retained significant activity toward its natural substrate, GL-7-ACA, with moderately decreased values of \( k_{cat}/K_m \) (Table II), indicating that the Trp-B4 of the C130 subunit is essential for neither substrate binding nor catalytic activity, despite the fact that this residue can be specifically alkylated by BA-7-ACA.

Both BA-7-ACA and BP-7-ACA irreversibly inhibited W-B4Y but not the other mutants in a time-dependent manner. On the other hand, the activities of all of the mutants were readily inhibited by BA-7-ACA (Tables II and III and Fig. 8). It is obvious that the irreversible inhibition of W-B4Y is attributable to the presence of the nucleophilic phenolic group, while the lack of such groups on the mutated amino acid residues makes W-B4A, W-B4L, and W-B4F resistant to modification by these inhibitors. Unlike the alkylation of tryptophan, where an electrophilic addition reaction on C-3 of indole ring is the first reaction step, the modification of other amino acid residues substituted with an active proton is considered to be an SN2 nucleophilic substitution reaction. For an SN2 reaction, deprotonation of the substituted residue is essential for anion formation. The \( pK_a \) values of the relevant residues are as follows: Thr-OH (18) > Trp-NH (16) > His-NH2-NH (7.0, 14.4) > Tyr-OH (10.2).

The histidine side chain is readily protonated, with a \( pK_a \) value near 7, but it is difficult to deprotonate with an apparent \( pK_a \) value of about 14.4. Obviously, nucleophilic substitution at the N-H of the Trp indole ring is also impossible. Therefore, the inability to covalently modify W-B4H and W-B4T can be explained by the much higher \( pK_a \) values of their active protons.
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Wild type and mutant enzymes were partially purified (>50%). The concentration of enzyme was determined on the basis of chromatic scanning of Coomassie Blue-stained SDS-PAGE gels. Enzyme kinetics employed standard procedures (see “Experimental Procedures”) with approximately 10 μg/ml enzyme and various concentrations of GL-7-ACA. The kinetic constants $K_m$ and $v_{max}$ were determined by the Lineweaver-Burk plots. Inhibition kinetics employed the same procedure with the addition of various concentrations of BA-7-ACA (2.0, 4.0, and 8.0 mM), giving competitive patterns. The $K_i$ values were determined as competitive inhibition constants by standard methods. The loss of enzyme activity was assumed to proceed via a first-order reaction. Enzymes (1 mg/ml) were incubated in sodium phosphate buffer (pH 8.0) at 37°C. At suitable time intervals, 20 μl of the enzyme was withdrawn, and the residual enzymatic activity was immediately determined under standard assay conditions. The $t_{1/2}$ values were measured from the plot of In(residual activity) versus time (min). All of the experiments were repeated three times, and the S.D. values were calculated with $n = 3$.

**TABLE II**

Kinetic parameters of C130 β subunit mutants and half-life of their enzymatic activities

| Enzyme | $K_m$ | $v_{max}$ | $K_i$ | $t_{1/2}$ |
|--------|-------|-----------|-------|-----------|
| C130   | 0.21 ± 0.03 | 7.8 ± 0.5 | 3.7 x 10^4 | 600 ± 31 |
| W-B4A  | 0.33 ± 0.03 | 3.4 ± 0.2 | 1.1 x 10^4 | 61 ± 8  |
| W-B4L  | 0.37 ± 0.03 | 3.8 ± 0.3 | 1.2 x 10^4 | 98 ± 7  |
| W-B4T  | 0.36 ± 0.03 | 3.3 ± 0.2 | 0.92 x 10^4 | 12 ± 3  |
| W-B4F  | 0.37 ± 0.03 | 5.0 ± 0.2 | 1.4 x 10^4 | 16 ± 3  |
| W-B4H  | 0.40 ± 0.03 | 5.5 ± 0.4 | 1.4 x 10^4 | 307 ± 25|
| W-B4Y  | 0.33 ± 0.03 | 5.9 ± 0.3 | 1.8 x 10^4 | 50 ± 7  |

a Competitive inhibition constant for BA-7-ACA.

b These enzymes can be affinity labeled by BA-7-ACA.

**TABLE III**

Kinetic parameters for specific modification of C130 and W-B4Y by BA-7-ACA and BP-7-ACA

| Inhibitor | $K_i$ | $v_{max}$ | $K_i$ | $v_{max}$ |
|-----------|-------|-----------|-------|-----------|
| C130      | 5.5 ± 0.3 | 0.21 ± 0.02 | 0.87 ± 0.1 | 0.17 ± 0.02 |
| W-B4Y     | 14 ± 1.2 | 0.014 ± 0.002 | 20 ± 2.3 | 0.014 ± 0.002 |

Consider the characteristics of BA-7-ACA labeling or inhibition of C130 and its missense mutants, it is further confirmed that Trp-B4 in C130 is a site of alkylation for BA-7-ACA.

According to Huang and Colman (21), an affinity labeling event can be expressed as follows,

$$
\frac{k_1}{k_2} = \frac{[E]}{[I]} \rightarrow E + I \rightarrow E^* \rightarrow EI \\
$$

where $E$ represents the free enzyme, $I$ represents the inhibitor, $E^*$ is the reversible enzyme-inhibitor complex, and $EI$ is the covalently modified enzyme. The observed rate constant ($k$) at a particular concentration of the inhibitor is described as follows;

$$
\frac{1}{v} = \frac{1}{v_{max}} \frac{K_i}{K_{max}} \frac{1}{[I]} \\
(\text{Eq. 1})
$$

where $K_i = (k_{-1} + k_2)k_1$. This represents the concentration of inhibitor giving a half-maximal rate of inactivation, and $k_{max}$ is the maximum rate of modification at saturating concentrations of the inhibitor. Accordingly, the inhibition kinetics of BA-7-ACA and BP-7-ACA on C130 and W-B4Y were analyzed by a double reciprocal plot of the pseudo-first-order rate constant ($k$) against the concentration of inhibitors ([I]). All four affinity alkylation reactions gave straight lines (Fig. 8, A and B). The respective $K_i$ and $k_{max}$ values were determined (Table III). As an affinity labeling reagent, BA-7-ACA is much better than BP-7-ACA for either enzyme, with lower $K_i$ values and higher $k_{max}$ values, indicating its higher affinity and reactivity. The relatively poor affinity of BP-7-ACA may well be due to its longer side chain, while the lower $k_{max}$ is certainly due to its poor electrophilicity. With the same inhibitor, the $K_i$ values of the two enzymes are quite different, while the $k_{max}$ values are almost the same. This result indicates that the fourth amino acid residue of the β-subunit has a stronger influence upon the binding of the inhibitor rather than its reactivity. This argument can be substantiated by the characteristics of the other five mutant enzymes, W-B4A, W-B4L, W-B4T, W-B4F, and W-B4H, which failed to react with BA-7-ACA but were inhibited reversibly and competitively by the inhibitor, with different competitive inhibition constants $K_i$ (Table II).

Significantly, none of the mutant enzymes with one residue replaced were stable under normal enzyme assay conditions, although the mutant W-B4H was much more stable than the others (Table II). This indicates that the Trp-B4 is essential for the enzymatic stability and may thus play an important role in catalysis.

**DISCUSSION**

To investigate the substrate binding site of GL-7-ACA acylase C130, we synthesized a series of bromide-substituted substrates with varying length of acyl-7-ACA. Only analogs with shorter acyl-side chains, BA-7-ACA and BP-7-ACA, were able to specifically bind and label the C130 β-subunit as affinity reagents. The labeling mechanism of BA-7-ACA was shown to occur via the alkylation of the C-2 on indole ring of Trp-B4. Mutational analysis of the site-directed mutants of the 4th residue of the C130 β-subunit further supported this conclusion.

Some of the above findings were unexpected. First, 7β-4-bromobutyrylamidocephalosporanic acid, 7β-5-bromopentanylamidocephalosporanic acid, and 7β-6-bromohexanoylamidocephalosporanic acid failed to affinity-modify C130 or to function as competitive inhibitors despite the fact that these chemicals, particularly the 7β-5-bromopentanoylamidocephalosporanic acid, seem to be better substrate analogs than BA-7-ACA. Second, there is steric hindrance of the 4th residue of the β-subunit, as shown in the C130 crystal structure. A significant energy barrier to the observed reaction makes the Trp-B4 labeling site unexpected. In addition, these results must be further considered with respect to their effect on the binding and hydrolysis of the native substrate, GL-7-ACA.

The affinity labeling of Trp-B4 by BA-7-ACA and BP-7-ACA described in this paper are unique cases of the specific alkylation of a tryptophan residue under physiological conditions. The activation energy that must overcome during this reaction
is substantial, because the C-Br bonds of these two analogs are much more stable than those of other affinity reagents, such as the oxidant N-bromosuccinimide (22) and the alkylation reagent 2-hydroxy-5-nitrobenzyl bromide (23–26). It follows that the affinity alkylations of C130 and W-B4Y are more likely to be suicide reaction-catalyzed by the enzyme rather than normal chemical modifications. In addition, the activation energy of catalysis is likely to be supplied by a dramatic conformational change associated with binding (see below).

With the length of the side chain of the inhibitor increased by one carbon, the $K_i$ of BP-7-ACA was greatly increased compared with that of BA-7-ACA (Table III). The other substrate analogs containing longer side chains (4–6 carbons) could neither inhibit nor modify C130 or its mutant W-B4Y. Because BA-7-ACA has the shortest side chain of all the analogs tested, BA-7-ACA is likely to be the analog of the core backbone of the substrate glutaryl moiety rather than the complete substrate (GL-7-ACA) or the side chain of the substrate (the glutaryl moiety).

The affinity labeling of BA-7-ACA on C130 was protected by glutaric acid and 7-ACA, which implies the existence of overlapping binding sites. In addition, the competitive inhibitory effects of BA-7-ACA on mutant enzymes having amino acid switches at position 4 and the decrease in their $K_m$ values toward the natural substrate further support the contention that Trp-B4 is likely to be involved in substrate binding, although it is not essential. In fact, the involvement of a Trp residue in substrate binding for GL-7-ACA acylase was first indicated by the oxidation of a Trp residue of CA GK16 by N-bromosuccinimide, causing a moderate increase in its $K_m$ (22). Although the position of this Trp residue remains to be determined (22), we suggest that the modified tryptophan is Trp-B4, because this CA is almost identical in sequence with C130 (27). It is noteworthy that Trp-B4 is conserved in all of the known CAs and the majority of the PAGs.

Recently, Kim and Hol (9) solved the structures of binary complexes of CAD with GL-7-ACA and glutarate at 2.5- and 2.6-Å resolutions, respectively. Models of enzyme-ligand complex were inferred from these studies. Because these complex structures were determined by co-crystallizing ligands with the enzyme, no significant conformational changes were observed between the structure of apoenzyme and the complexes. In addition, based on the enzyme-substrate complex model, the distance between the O/H$_{9253}$ of Ser-B1 and the target carbonyl carbon of the substrate glutaryl side chain is 3.4 Å, which is too great to form a transition state tetrad. Therefore, these models may only reflect the initial stage of substrate binding but not the details of enzyme-substrate interaction during catalysis.

As expected, Trp-B4 was not involved in an obvious manner in either of the two ligand binding models. Because Trp-B4 is located in the first β sheet of the αββα sandwich structure with its indole ring facing the first α helix layer (Fig. 9), there is no space to accommodate ligand (Fig. 10) without a conformational alteration that would enlarge the space between the first αβ structure. Therefore, we propose that the binding of BA-7-
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ACA to GL-7-ACA acylase is accompanied by a conformational change that reduces the activation energy for the alkylation of Trp-B4 of C130 β-subunit. This proposed conformational change might involve a transition state similar to the substrate-induced conformational transition state of the enzyme-substrate complex (28). The proposed involvement of Trp-B4 in the process of substrate binding may well reflect the dynamic state of the enzyme-substrate complex during catalysis.

Replacing Trp-B4 with other amino acid residues reduced the stability of the enzyme, except in the case of histidine replacement. This is probably attributable to conformational instability of the mutated enzymes. W-B4H is probably more stable due to the formation of a hydrogen bond between its ε N-H (produced by tautomerization) with the hydroxyl group of Ser-B278, which is similar to the situation in Trp-B4. This in turn suggests that this hydrogen bond may be essential for the stability of C130 (Fig. 10) and might contribute up to 10–20 kcal/mol to the stabilization (29). On the other hand, during the substrate binding, the proposed ligand-induced conformational change may lead to the dissociation of this hydrogen bond as the enzyme enters a highly active transition state. The “unstable” active enzymes are likely to be protected efficiently by substrate, including BA-7-ACA (data not shown). The absence of this hydrogen bond may cause the proposed conformational transition and enzyme stability largely altered in most of the mutants except for His-B4, which retains the ε N-H required for hydrogen bond formation.

In conclusion, the affinity inhibition and alkylation of C130 by BA-7-ACA documented in this paper suggests a reaction process requiring significant energy that is most likely compensated by ligand-induced conformational change. Although BA-7-ACA is only an analog of the core backbone of the substrate, this conformational change may to a certain extent reflect the transition state of the natural reaction. In addition, the hydrogen bond between the Trp-B4 and Ser-B278 may also play an important role in maintaining the stability of the enzyme. Accordingly, we propose that the binding of substrate to C130 may result in a major conformational change whose reversal provides sufficient energy for the collapse of the transition state. This suggested model might also apply to other Ntn CAs and penicillin G acylases.

Acknowledgments—We thank Prof. Mingjie Zhang’s laboratory at Hong Kong University of Science & Technology for NMR analysis and Xiaoxia Shao for kind help in MS data collections. We particularly appreciate Dr. R. L. Somerville for fruitful discussions and proofreading of the manuscript.

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