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Oxidative Modification of a Cephalosporin C Acylase from *Pseudomonas* Strain N176 and Site-Directed Mutagenesis of the Gene

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A cephalosporanic acid acylase from *Pseudomonas* strain N176 catalyzes hydrolysis of both glutarylcephalosporanic acid and cephalosporin C to 7-amino-cephalosporanic acid. Chemical modification of the enzyme with acidic hydrogen peroxide was performed to investigate residues which play important roles in enzymatic activity. The activity of the enzyme was reduced to 76% of the original by oxidation. From protein chemical analysis combined with site-directed point mutagenesis, modification of Met-164 was found to correspond to the reduction in activity. To study the effect of Met-164 on the enzymatic character, we prepared mutant acylases in which Met-164 was replaced with several other amino acids and obtained the following data: (i) there existed a trend of mutation to noncharged hydrophilic residues, resulting in an increase of activity against glutarylcephalosporanic acid; (ii) the mutation of Met-164 to Gly and Ala resulted in the lowering of both K_m values and the optimal pHs against glutarylcephalosporanic acid; (iii) the mutation to Leu enhanced cephalosporin C acylase activity; and (iv) the mutation to Gln improved the k_{cat} value for glutarylcephalosporanic acid. In particular, the mutation to Gln resulted in a high rate of conversion of glutarylcephalosporanic acid to 7-amino-cephalosporanic acid under conditions similar to those of a bioreactor system. These results may indicate that Met-164 is located in or near the cephalosporin compound binding pocket on the enzyme.

N176 cephalosporin C acylase, an enzyme isolated from *Pseudomonas* strain N176, is a two-chain protein consisting of α (Thr-1 to Gly-238) and β (Ser-239 to Ala-773) chains with no disulfide bond linkages (1, 3). It acts as a strong glutarylcephalosporanic acid acylase as well as a moderate cephalosporin C acylase (2). It appeared to be useful for the two-step enzymatic production of 7-amino-cephalosporanic acid, a key intermediate of cephem antibiotics, in combination with D-amino acid oxidase (10). It might also be a candidate for direct production of 7-amino-cephalosporanic acid from cephalosporin C. However, its optimal pH for activity is 9.0, at which β-lactam substrates are unstable, and the cephalosporin C acylase activity is not sufficient for large-scale production of 7-amino-cephalosporanic acid. Although it shows some similarity to other cephalosporin and penicillin acylases in the amino-terminal region of the β chain (1, 7), the tertiary structure of the acylase has not been elucidated. Modification of the enzyme with an affinity label reagent, 6-β-bromohexanoyl-7-amino-cephalosporanic acid, has revealed that Tyr-270 is responsible for the inactivation by the reagent (9). Tyr-270 is also the residue most reactive to nitration with tetranitromethane (16). These data suggest that Tyr-270 plays an important role in the enzymatic activity. Therefore, we mutated residues near Tyr-270 of the acylase and found that mutation of Met-269 to Phe or Tyr resulted in increasing cephalosporin C acylase activity (8). However, these Met-269 mutant acylases were inferior as glutarylcephalosporanic acid acylases. In order to search for residues relating to glutarylcephalosporanic acid acylase activity, another chemical modification was necessary. Therefore, we performed oxidative modification of N176 acylase with hydrogen peroxide and found that Met-164 was responsible for the reduction of the activity. We also studied the characters of the mutants in which Met-164 was altered to several other amino acids and the application by use of these mutant acylases for enzymatic production of 7-amino-cephalosporanic acid.

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MATERIALS AND METHODS

Materials. Glutarylcephalosporanic acid, cephalosporin C, and 7-amino-cephalosporanic acid were prepared by Central Research Laboratories of Fujisawa Pharmaceutical Co., Ltd. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, T4 DNA polymerase, and *Taq* polymerase were purchased from Takara Shuzo (Kyoto, Japan), Toyobo (Osaka, Japan), or New England Biolabs (Beverly, Mass.). *Escherichia coli* JM109 was from Nippon Gene (Tokyo, Japan), and *E. coli* CJ236 was from Bio-Rad Laboratories (Richmond, Calif.).

Oxidation reaction. The wild-type N176 acylase was prepared from *E. coli* JM109/pCK013 (8). The acylase (100 μl, 200 μg/ml in 40 mM phosphate buffer [pH 6.0]) was mixed with 20 mM H_2O_2 (100 μl), and the mixture was incubated for 2 h at ambient temperature. The resultant mixture was analyzed by reversed-phase high-performance liquid chromatography (HPLC) (Cosmosil 5C4-AR-300 column). Enzymatic activity was essentially determined by the method described previously (8, 16). To 500 μl of the substrate (10 mg/ml in 0.15 M Tris-HCl [pH 8.7]), 100 μl of sample acylase (approximately 25 μg/ml for glutarylcephalosporanic acid and 100 μg/ml for cephalosporin C) was added, and the mixture was incubated at 37°C for 5 min (glutarylcephalosporanic acid) or 10 min (cephalosporin C). The reaction was stopped by addition of 5% acetic acid (550 μl). After centrifugation (12,000 × *g*, 5 min), the 7-amino-cephalosporanic acid formed in the supernatant was determined by HPLC (column, LiChrospher RP-18 [4.0 by 250 mm; E. Merck, Darmstadt, Germany]; eluate, 100 mM citric acid, 5 mM *n*-hexane-1-sulfonate, 14.3% acetonitrile; detection, 254 nm). One unit was defined as the activity capable of liberating of 1.0 μmol of 7-amino-cephalosporanic acid from glutarylcephalosporanic acid or cephalosporin C per min.

Genetic engineering techniques. Oligodeoxyribonucleotides were prepared by a 381A DNA synthesizer (Applied Biosystems, Foster City, Calif.), and the reagents for the synthesis were obtained from Cruachem (Glasgow, United Kingdom). pCC013A and pCK013, ampicillin- and kanamycin-type expression vectors for the wild-type N176 acylase, respectively (8, 9, 16), were used as starting plasmids. All DNA manipulations were performed essentially according

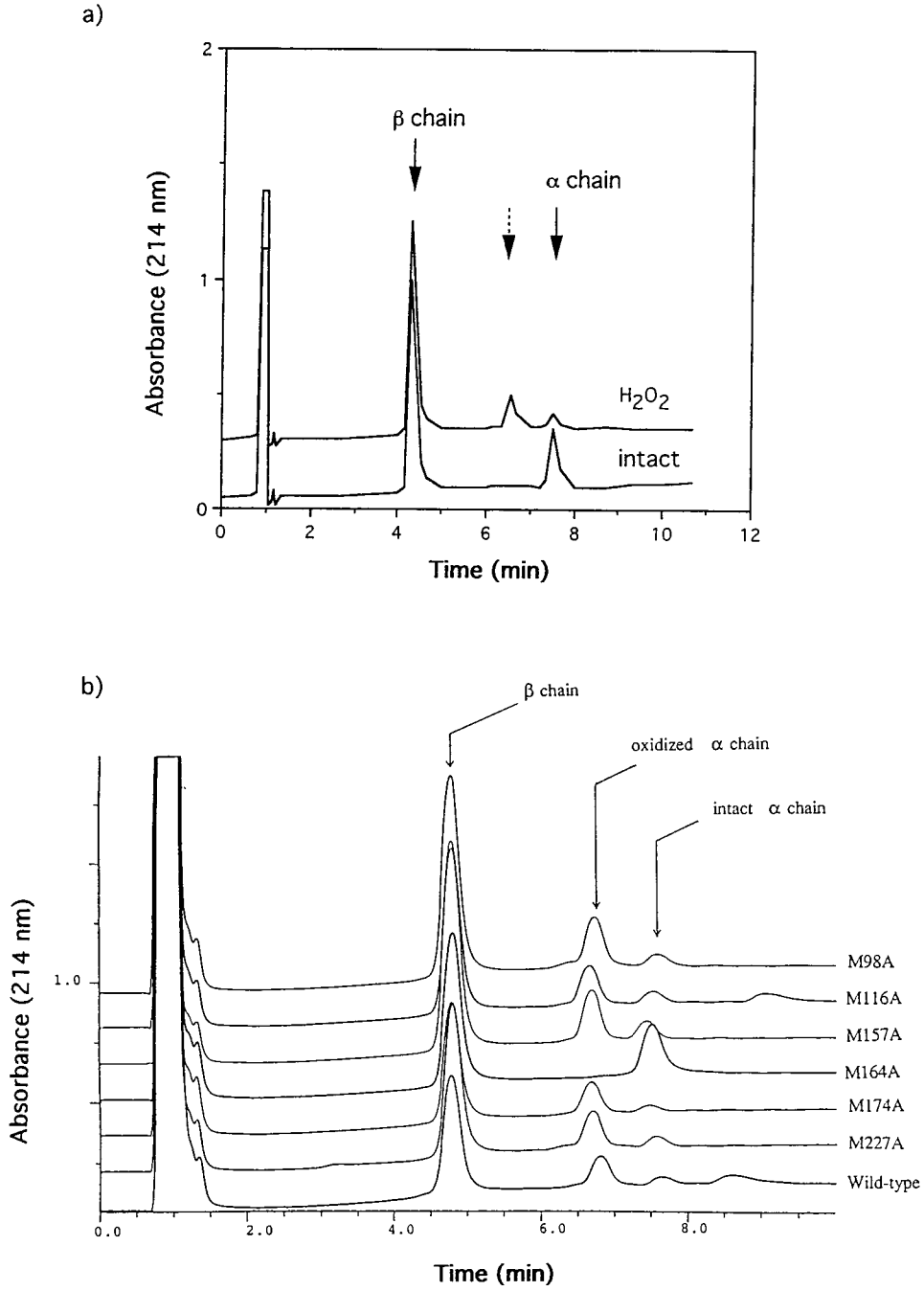

FIG. 1. Oxidation of wild-type and mutant N176 acylases. (a) HPLC analysis of N176 acylase treated with hydrogen peroxide. Dashed arrow indicates a peroxide-dependent peak. (b) HPLC analysis of methionine-to-alanine mutants treated with hydrogen peroxide.

to the method of Sambrook et al. (17). Sequencing of mutated DNAs was performed with a 373A DNA sequencer (Applied Biosystems).

Site-directed mutagenesis of Met to Ala was performed according to the method described by Kunkel et al. (12) with an appropriate oligodeoxyribonucleotide on a single-stranded M13 phage template, prepared from *E. coli* CJ236 (Dut^- Ung^-), carrying a part of the wild-type acylase gene (from pCC013A). For example, the mutated DNA for M164A N176 acylase, in which Met-164 was changed to Ala, was constructed with the following oligonucleotide: 5′-G GGC CTG CTT **GCG** GG**A** TCC GTG TGG TTC A. The mismatches are shown in boldface, with 5′-**GCG** directing the mutation of Met-164 to Ala. The change of 5′-GGTTCG to 5′-GG**A**TC**C** (a silent mutation for Gly-165 to Ser-166; numbers correspond to the work of Nobbs et al. [16]) introduces a *Bam*HI site at position 736 in the nucleotide sequence (numbers correspond to the work of Aramori et al. [1]) which was used for the selection of the mutant DNA. PCR was used for the mutagenesis of Met-164 to every other amino acid except Ala. The plasmid pCKM164L for M164L acylase was constructed from pCK013 and oligodeoxyribonucleotides 5′-ATG GAG CTG *ACG CGT* CGC AA**A** GCG CTG GGA CG (SO-MluFor [*Mlu*I site on the wild-type sequence, italicized], positions 442 to 473, 5′-AA**A** introducing a silent mutation [*Eco*47III site, underlined] on the sequence for Lys-73) and 5′-CAC GGA TCC CA**G** AAG CAG GCC CA**G** TCG ACG CAT CAC CG (SO-M164L, positions 707 to 744, 5′-CA**G** directing mutation of Met-164 to Leu and 5′-GG**A**TC**C** and 5′-**T**CG**A**CG introducing silent mutations [*Bam*HI and *Sal*I sites, underlined] for Gly-165 to Ser-166 and Arg-158 to Arg-159, respectively). pCK013 (template DNA, 0.5 fmol) and oligodeoxyribonucleotides SO-MluFor (125 pmol) and SO-M164L (125 pmol) were mixed with *Taq* DNA polymerase (1 U) in 100 μl of a buffer consisting of 10 mM

TABLE 1. Activities of methionine-to-alanine mutants[a]

Acylase	Sp act (% of that of the wild type)			Remaining activity (% of that of the intact type), GL-7ACA, pH 8.7
	GL-7ACA		CC, pH 8.7	
	pH 7.5	pH 8.7		
Wild type	100	100	100	76.0
M98A	126	83.1	91.1	77.0
M116A	76.9	95.8	108	78.1
M157A	70.9	58.0	65.0	61.3
M164A	167	104	86.6	94.5
M174A	96.5	109	122	76.6
M227A	106	74.2	105	65.9

[a] Specific activity was determined from the enzymatic activity and the concentration of each acylase. Assays were performed as described in Materials and Methods. Values for the wild-type acylase are 26.8, 46.3, and 1.55 U/mg of protein with glutarylcephalosporanic acid (GL-7ACA, pHs 7.5 and 8.7) and with cephalosporin C (CC, pH 8.7), respectively. Remaining activity represents the activity of each acylase after treatment with acidic hydrogen peroxide (assayed with glutarylcephalosporanic acid, pH 8.7; see Materials and Methods).

Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, and 0.2 mM deoxyribonucleoside 5′-triphosphate. The mixture was overlaid with mineral oil, and PCR was carried out as follows. After an initial denaturation (95°C for 0.5 min), the reaction was performed for 25 cycles of amplification (95°C for 1.5 min, 50°C for 2.5 min, and 72°C for 2.5 min) and was followed by a final extension (72°C for 7 min). The resultant DNA was digested with *Bam*HI and *Mlu*I and introduced to pCKM164A to obtain the desired plasmid pCKM164L.

Protein chemistry. Native and mutant acylases were purified with Fractogel EMD DEAE-650M (Merck Ltd.) in a Superformance chromatography column (12-mm internal diameter by 13.5-cm length) linked to an HPLC system (Millipore, Tokyo, Japan). Elution was performed with a linear gradient of NaCl (0.1 to 0.4 M) in 25 mM Tris-HCl (pH 8.0). Purity was assayed with reversed-phase HPLC (column, Cosmosil 5C4-AR-300 [4.6-mm internal diameter by 5-cm length; Nacalai Tesque, Kyoto, Japan]); elution, a linear gradient of 15 to 60% acetonitrile in 0.05% trifluoroacetic acid over 30 min; detection, 214 nm). The concentration of purified enzymes was determined by a spectrophotometric method (20). The amino-terminal sequence was determined with a 470A protein sequencer (Applied Biosystems), and mass measurement was performed with Mat TSQ-70 (Finnigan, San Jose, Calif.).

RESULTS AND DISCUSSION

Oxidation of native N176 acylase. N176 acylase was treated with hydrogen peroxide at pH 6.0, and the remaining glutarylcephalosporanic acid acylase activity was assayed. The activity of the enzyme was reduced to 76% of that of the intact wild-type acylase. In a chromatogram of reversed-phase HPLC of the oxidized acylase (Fig. 1a), the peak height of the α chain decreased and a new peak (dashed arrow) appeared, whereas the peak of the β chain remained unchanged. The peptide corresponding to the new peak was revealed to be a modified α chain by amino-terminal sequencing and lysylendopeptidase (EC 3.4.21.50) (Wako, Osaka, Japan) mapping. In an HPLC chromatogram of the digest with lysylendopeptidase and trypsin (Seikagaku Co., Tokyo, Japan), a peptide fragment from the oxidized α chain eluted at a more hydrophilic position than did that from the intact one. Both peptides showed the identical amino acid sequence (Leu-160 to Lys-170; numbers correspond to the work of Nobbs et al. [16]). However, molecular weights of the peptides from the oxidized and the intact α chains were 1,266.9 and 1,250.55, respectively. These data indicate that Met-164 in the sequence was oxidized to methionine sulfoxide. Although N176 acylase has eight free cysteines that are sensitive to hydrogen peroxide at basic pH, mutation of each Cys to Ser generated little change in the enzymatic activity (16). These data suggest that the reduction of the acylase activity was due to the oxidation of Met-164. Since it seems unusual that a single oxidation of Met-164 in the α chain resulted in such a drastic shift of the peak position in the HPLC chromatogram (Fig. 1a), we thought that Met-164 might be located at a site important for affecting the hydrophobicity of the acylase.

Methionine-to-alanine mutants. In order to complement the oxidation experiments, each of the eight methionines in the α chain was altered to Ala by site-directed point mutagenesis according to the method of Kunkel et al. (12). Cultivation of *E. coli* JM109 carrying expression vectors was performed according to the previously described method (16). Each mutant was purified to homogeneity by anion-exchange column chromatography linked to an HPLC system (see Materials and Methods). M2A and M67A were not isolated because they precipitated as inactive precursor proteins in *E. coli* cells.

Each mutant acylase was treated with acidic hydrogen peroxide, and its activity with glutarylcephalosporanic acid (pH 8.7) was compared with that of the wild-type enzyme (Table 1). Although mutant acylases M98A, M116A, M157A, M174A, and M227A showed 23.0, 21.9, 38.7, 23.4, and 34.1%, respectively, loss of activity after oxidation, the activity of M164A acylase was not reduced by the oxidation (remaining activity was 94.5%). Each oxidation mixture was analyzed by reversed-phase HPLC (Fig. 1b). The peak of the α chain of M164A remained at an identical position compared with that of the intact α chain; however, peaks of other mutants shifted to more hydrophilic positions. These results are consistent with the experiments with endopeptidase digestion and indicate that Met-164 is the residue responsible for the reduction of the activity. Hydrogen peroxide is a small molecule and may not show site specificity (6). However, small molecules, including reactive oxygen species, can show considerable specificity, depending on the environment and the conformation of the residues involved (5). Therefore, it is assumed that Met-164 is located at a much more reactive position than the other methionines.

The activities of mutant enzymes were determined with glutarylcephalosporanic acid at pHs 7.5 and 8.7 and with cephalosporin C at pH 8.7 (Table 1). All mutants retained acylase activity, indicating that methionines in the α chain are not critical to enzymatic activity. Interestingly, the glutarylcephalosporanic acid acylase activity of M164A acylase at pH 7.5 showed a 1.6-fold increase over that of the wild type, although activity at pH 8.7 was similar to that of the wild type. From these data, we thought that its optimal pH may shift to one

TABLE 2. Activities of mutant N176 acylases[a]

Acylase	Sp act (U/mg)		Specificity, CC vs GL-7ACA, pH 8.7	Thermostability, GL-7ACA, % of that of the intact acylase
	GL-7ACA, pH 7.5/8.7	CC, pH 8.7		
Wild type	26.8/46.3	1.55	0.0335	78.0
M164A	44.8/48.2	1.34	0.0276	74.0
M164G	43.4/48.6	1.29	0.0265	71.0
M164L	22.5/31.9	1.89	0.0707	80.0
M164N	43.7/55.6	1.31	0.0236	81.0
M164Q	34.0/56.5	0.357	0.00633	90.0
M164P	31.9/43.8	1.10	0.0252	ND
M164S	35.4/49.1	1.12	0.0228	ND
M164T	33.2/40.5	0.981	0.0242	ND
M164F	17.4/24.7	0.645	0.0261	ND

[a] Specific activity was determined from the enzymatic activity and the concentration of each acylase. Assays were performed as described in Materials and Methods. Specificity is the ratio of the specific activities with cephalosporin C (CC) to those with glutarylcephalosporanic acid (GL-7ACA) at pH 8.7. Thermostability represents the percentage of the remaining activity of each acylase after incubation in 25 mM Tris-HCl (pH 8.0) at 50°C for 6 h (assayed with GL-7ACA, pH 8.7). ND, not determined.

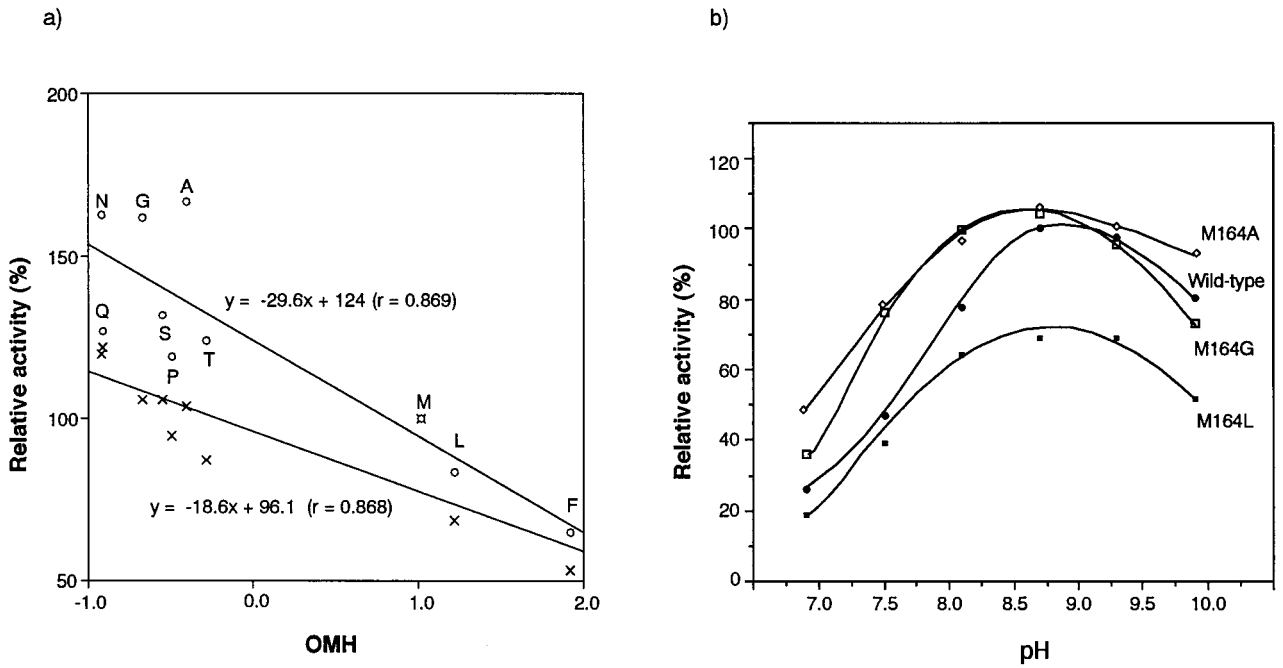

FIG. 2. Characters of M164 mutant acylases. (a) Relationship between OMH value and acylase activity. Activities at pHs 7.5 (○) and 8.7 (×) are represented as percentages of the wild type at both pHs (26.8 and 46.3 U/mg, respectively). (b) pH profile of Met-164 mutants. Each enzyme (4.2 μg/ml) was assayed with glutarylcephalosporanic acid (8.3 mg/ml, 21.5 mM) in 125 mM Tris-HCl (pHs 6.9, 7.5, 8.1, 8.7, 9.3, and 9.9) at 37°C for 5 min. The activity of the wild type at pH 8.7 (46.3 U/mg) is represented as 100%. ●, wild type; ◇, M164A mutant; □, M164G mutant; ■, M164L mutant.

more acidic and that the mutation of Met-164 changes the character of the acylase. As for cephalosporin C acylase activity, M174A acylase showed a weakly but definitely higher cephalosporin C acylase activity ([122 ± 8.1]% of that of the wild type).

Activities of Met-164 mutant acylases. In order to investigate the role of Met-164 of the acylase for enzymatic activity, we prepared several mutant acylases in which Met-164 was altered to other amino acids by PCR (Table 2). For the activity with glutarylcephalosporanic acid at pH 7.5, the mutation to Asn, Gln, Gly, Ser, Pro, or Thr resulted in an increase of acylase activity and the mutation to Leu or Phe caused a decrease of activity. These data suggest that the activity tends to correlate with the hydrophobicity of each residue in the reverse direction. In fact, there seems to exist a weak linear correlation {relative activity = 124 − [29.6 × optimal matching hydrophobicity (OMH) value], $r = 0.869$} between the enzymatic activity and OMH determined by Sweet and Eisenberg (19) (Fig. 2a). The trend was also seen in the case of the activity at pH 8.7; however, the absolute value of the slope (−18.6) was smaller than that at pH 7.5. These data may indicate that the mutation of Met-164 to a noncharged hydrophilic residue results in an increase of glutarylcephalosporanic acid acylase activity and that Met-164 is located at an important site involved in exerting the catalytic activity of the acylase. In general, hydrophobicity of a residue in the interior of a protein correlates with thermostability (11, 15, 21). However, the mutation of Met-164 of N176 acylase was independent of thermostability at 50°C (Table 2). These experiments suggest that Met-164 is not buried in the interior of the molecule but exists near (or in) the substrate binding pocket at the active site of the enzyme. On the other hand, no distinct correlation was observed between the cephalosporin C acylase activity of the mutants and their OMH values (data not shown). Interestingly, the M164L mutant showed a small but a definite increase in cephalosporin C acylase activity ([122 ± 6.2]% compared with that of the wild type). In particular, its specificity ratio with cephalosporin C to glutarylcephalosporanic acid is approximately 2.6- to 11-fold higher than those of the hydrophilic mutants M164A and M164Q, respectively (Table 2). These

TABLE 3. Kinetic parameters and rate constants of M164 mutant acylases[a]

Acylase	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	Rate constant (0–120/10–60 min) (10^{-4} s^{-1})	Final yield of 7ACA (% of theoretical value)
Wild type	5.85 ± 0.50 (100)	49.8 ± 1.4 (100)	8.35 ± 0.53 (100)	3.1 ± 0.2/3.1 ± 0.7 (100/100)	89 ± 0.4
M164A	2.50 ± 0.06 (42.7)	45.2 ± 0.35 (90.8)	18.1 ± 0.55 (216)	3.4 ± 0.1/3.8 ± 0.1 (110/123)	93 ± 0.8
M164G	2.57 ± 0.02 (43.9)	47.4 ± 0.85 (95.1)	18.6 ± 0.15 (223)	ND	ND
M164N	3.68 ± 0.32 (57.8)	55.6 ± 10.9 (112)	15.1 ± 1.65 (181)	3.4 ± 0.2/3.1 ± 0.7 (110/100)	94 ± 1.8
M164Q	9.32 ± 0.67 (159)	76.8 ± 5.2 (154)	8.29 ± 1.16 (99.3)	4.1 ± 0.1/5.0 ± 0.4 (132/161)	96 ± 0.1

[a] Kinetic parameters were calculated from Lineweaver-Burk plots of the primary velocity of 7-amino-cephalosporanic acid (7ACA) formed from glutarylcephalosporanic acid (1.67, 2.5, 3.33, 5.0, and 10.0 mM) in the presence of acylases (26 nM) at 37°C for 5 min. Rate constants were calculated from the amounts of 7-amino-cephalosporanic acid formed by incubation with each enzyme (100 μg/ml) with glutarylcephalosporanic acid (4 mg/ml) at 25°C in 0.15 M Tris-HCl (pH 7.5) at intervals. ND, not determined. Values in parentheses after the parameter values are the percentages of the wild-type values.

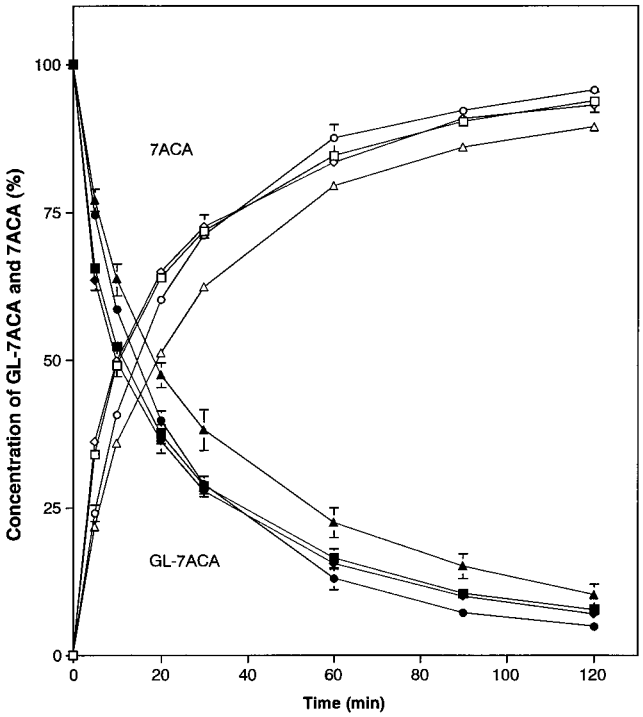

FIG. 3. Formation of 7-amino-cephalosporanic acid (7ACA) with the wild-type and mutant acylases. Each enzyme (100 μg/ml) was incubated with glutaryl-cephalosporanic acid (GL-7ACA) (4 mg/ml) at 25°C in 0.15 M Tris-HCl (pH 7.5). At intervals, an aliquot of the reaction mixture was withdrawn and analyzed by HPLC to determine the concentration of remaining glutarylcephalosporanic acid (closed symbols) and 7-amino-cephalosporanic acid formed (open symbols). Symbols represent means ± standard deviations. △ and ▲, wild type; ◇ and ◆, M164A mutant; □ and ■, M164N mutant; ○ and ●, M164Q mutant.

results indicate that mutation of Met-164 causes a characteristic change in the enzymatic activity and that hydrophobicity of the residue relates to the catalytic character.

Characteristics of mutant acylases. Among Met-164 mutants, M164A and M164G acylases showed higher activity with glutarylcephalosporanic acid at pH 7.5 (Table 2). The ratios of the specific activities at pH 7.5 versus 8.7 were 0.58, 0.93, and 0.89 for the wild-type, M164A, and M164G acylases, respectively. To confirm the data, the activities of several mutants were assayed at various pHs (Fig. 2b). The pH profile curves of M164A and M164G acylases are broader compared with that of the wild-type acylase. These data indicate that the optimal pH of N176 acylase can be changed by mutation of Met-164. Next, kinetic parameters of some mutants with glutarylcephalosporanic acid at pH 7.5 were determined (Table 3). Although the k_{cat} values of M164A, M164G, and M164N mutants were unchanged, the K_m values of those mutants were approximately 1/2 of that of the wild type. These data indicate that the increase of specific activities of M164A, M164G, and M164N mutant acylases at pH 7.5 is due to lower K_m values of these mutants compared with the wild-type acylase. In the case of the M164Q mutant, both the K_m and k_{cat} values increased to more than 150% of those of the wild-type acylase. These results suggest that Met-164 is located at an important position for participating in the formation of the enzyme-substrate complex as well as the liberation of 7-amino-cephalosporanic acid formed.

Reaction of mutant acylase in a bioreactor system. To investigate the industrial application of M164A, M164N, and M164Q mutant acylases, formation of 7-amino-cephalosporanic acid from glutarylcephalosporanic acid by these acylases was monitored under conditions (enzymes, 100 μg/ml; substrate, 4 mg/ml; buffer, Tris-HCl [pH 7.5]; temperature, 25°C) which are similar to those in a bioreactor system (Fig. 3; Table 3). Each mutant acylase was more capable of converting glutaryl-7-aminocephalosporanic acid to 7-amino-cephalosporanic acid than the wild-type acylase. In particular, M164A acylase showed superior initial formation rates (0 to 5 min) of 7-amino-cephalosporanic acid compared with that of the wild type (Fig. 3). The data correspond to the specific activity (Table 1), suggesting that the high initial rate is caused by the low K_m values. On the other hand, the M164Q mutant showed a high overall rate constant as well as a high final yield ([96 ± 0.1]%) of 7-amino-cephalosporanic acid. In particular, the rate from 10 to 60 min was much superior to that of the wild type or other mutants (160% of the wild type). These results indicate that the M164Q mutant carrying both high k_{cat} and K_m values is less sensitive to the concentrations of the product compared with the wild-type or M164A (or M164N) mutant acylase. Our present results coincide with those for the experiments with M269Y acylase that has an improved k_{cat} value with cephalosporin C and shows superior activity as a cephalosporin C acylase in a bioreactor system (8). It also indicates that a high k_{cat} value correlates to the acceleration of the elimination of 7-amino-cephalosporanic acid formed from the activated complex. It seems that an enzyme with a high k_{cat} value is more suitable than that with a low K_m value in a bioreactor system for the production of 7-amino-cephalosporanic acid.

A model for N176 cephalosporin acylase. An assumed secondary structure of the enzyme is depicted in Fig. 4. This model was constructed on the basis of the algorithms of Chou and Fasman (4) and Kyte and Doolittle (13) and our experimental results. The enzyme is produced as an inactive single chain precursor protein in the cytoplasm (1) and converted to a two-chain type by cleavage between Gly-238 and Ser-239, followed by conformational change to an active form (8). No internal or external disulfide linkages exist in spite of carrying eight cysteine residues in the enzyme (16). This model predicts that Met-164 is located in a hydrophobic β-sheet structure. The affinity label experiment of the enzyme with 6-β-bromohexanoyl-7-amino-cephalosporanic acid revealed that Tyr-270 was responsible to the residue interacting with glutaryl-7-aminocephalosporanic acid (9). It is proposed that Ser-239, the amino-terminal residue of the β-chain, is a very possible candidate for an active residue from the results of inactivation by carbamoylation, mutation of Ser to Cys, and conservation with other cephalosporin and penicillin acylases (8). It corresponds to Ser-290, an active residue of penicillin G acylase reported by Slade et al. (18) and Martin et al. (14). Tyr-270 and Ser-239 are also located in the hydrophobic cluster predicted by the Kyte-Doolittle algorithm (13). From these data, we suggest that Met-164, Tyr-270, and Ser-239 are located in the interior surface of the protein and constitute a substrate binding pocket for recognition of cephalosporin compounds (Fig. 4).

Conclusions. The improvement of the enzymatic function of N176 cephalosporin acylase was achieved by the mutation of a residue which is responsible for the reduction of the activity by the oxidation with hydrogen peroxide. The residue, Met-164, is assumed to be located in a substrate binding pocket (Fig. 4), and the mutation of Met-164 may perturb the structure of the enzyme. Although further structural analysis of the acylase will be necessary, our present experiments may indicate that a combination of chemical modification and site-directed point mutagenesis is a convenient method for the improvement of an enzyme whose tertiary structure has not been elucidated.

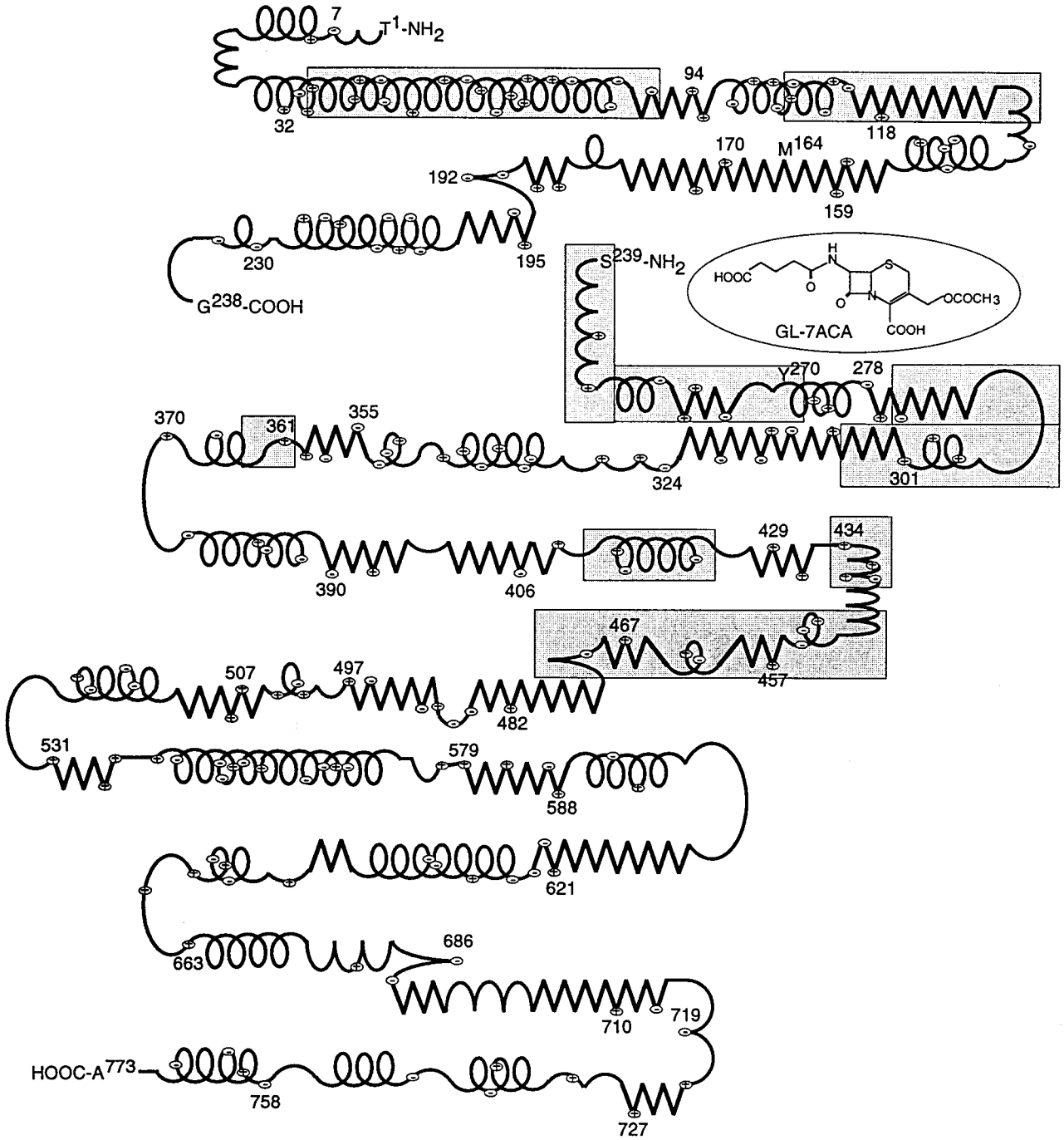

FIG. 4. An assumed secondary structure of N176 acylase. The model was assembled by the results of chemical modifications of the enzyme in addition to computer calculation of algorithms of Chou and Fasman (4) and Kyte and Doolittle (13). Helix, α-helix; notched line, β-sheet; arrowhead, β-turn; other symbols, random coil; boxed area, region homologous with other cephalosporin and penicillin acylases (7).

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