Identification of Catalytic Nucleophile of *Escherichia coli* γ-Glutamyltranspeptidase by γ-Monofluorophosphono Derivative of Glutamic Acid: N-Terminal Thr-391 in Small Subunit Is the Nucleophile†

Makoto Inoue,‡ Jun Hiratake,*,‡ Hideyuki Suzuki,§ Hidehiko Kumagai,§ and Kanzo Sakata‡

Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan, and Graduate School of Biostudies, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan

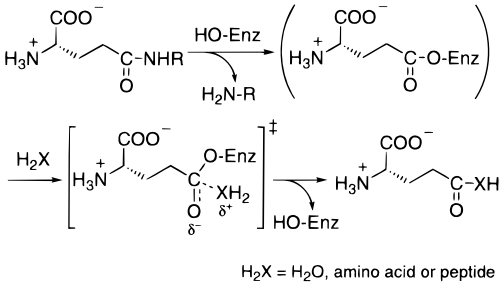
Received February 2, 2000; Revised Manuscript Received April 17, 2000

ABSTRACT: γ-Glutamyltranspeptidase (EC 2.3.2.2) is the enzyme involved in glutathione metabolism and catalyzes the hydrolysis and transpeptidation of γ-glutamyl compounds such as glutathione and its derivatives. The reaction is thought to proceed via a γ-glutamyl-enzyme intermediate where a hitherto unknown catalytic nucleophile is γ-glutamylated. Neither affinity labeling nor site-directed mutagenesis of conserved amino acids has succeeded so far in identifying the catalytic nucleophile. We describe here the identification of the catalytic nucleophile of *Escherichia coli* γ-glutamyltranspeptidase by a novel mechanism-based affinity labeling agent, 2-amino-4-(fluorophosphono)butanoic acid (**1**), a γ-phosphonic acid monofluoride derivative of glutamic acid. Compound **1** rapidly inactivated the enzyme in a time-dependent manner ($k_{on} = 4.83 \times 10^4$ M^{-1} s^{-1}). The inactivation rate was decreased by increasing the concentration of the substrate. The inactivated enzyme did not regain its activity after prolonged dialysis, suggesting that **1** served as an active-site-directed affinity label by phosphonylating the putative catalytic nucleophile. Ion-spray mass spectrometric analyses revealed that one molecule of **1** phosphonylated one molecule of the small subunit. LC/MS experiments of the proteolytic digests of the phosphonylated small subunit identified the N-terminal peptide Thr391-Lys399 as the phosphonylation site. Subsequent MS/MS experiments of this peptide revealed that the phosphonylated residue was Thr-391, the N-terminal residue of the small subunit. We conclude that the N-terminal Thr-391 is the catalytic nucleophile of *E. coli* γ-glutamyltranspeptidase. This result strongly suggests that γ-glutamyltranspeptidase is a new member of the N-terminal nucleophile hydrolase family.

γ-Glutamyltranspeptidase (EC 2.3.2.2) catalyzes the cleavage of the γ-glutamyl bond of glutathione and other related γ-glutamyl compounds to transfer the γ-glutamyl group either to water or to amino acids and peptides to complete hydrolysis or transpeptidation, respectively (*1*). This enzyme is widely distributed among living organisms from bacteria to mammals and plays important roles in the degradation of glutathione for the salvage of cysteine (*2*−*4*) and, hence, in the regulation of the intracellular levels of glutathione (*5*). In addition to the importance in glutathione metabolism, this enzyme also has clinical significance as a tumor marker because the enzyme is expressed strongly in hepatocarcinogenesis (*6*, *7*).

Despite the physiological importance of this enzyme, details of the catalytic mechanism still remain unclear. As shown in Scheme 1, the reaction catalyzed by γ-glutamyltranspeptidase is thought to proceed via a γ-glutamyl-enzyme intermediate (*8*−*10*) followed by nucleophilic substitution by water, amino acids, or peptides. Although a hydroxy nucleophile is proposed to undergo the acylation−deacylation double displacement (*11*, *12*), as observed with Ser hydrolases such as chymotrypsin and subtilisin (*13*), the catalytic nucleophile has remained unidentified.

Scheme 1: Proposed Reaction Mechanism of γ-Glutamyltranspeptidase



So far, the catalytic nucleophile has been probed mainly by affinity labeling and has been found to be located in the small subunit. Classical potent inhibitors, L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin)[1] (*14*−*17*) and 6-diazo-5-oxo-L-norleucine (DON) (*18*−*20*), inactivated the enzyme by forming a covalent bond to the small subunit. The labeling study on several γ-glutamyltranspeptidases with ^{14}C-acivicin identified Thr-523 (rat kidney

† This study was supported in part by Amano Enzyme Application Symposium Award to H.S. and by Grand-in-Aid for Scientific Research from Ministry of Education, Science, Sports and Culture of Japan to which we are grateful.

* To whom correspondence should be addressed. Fax: +81-774-38-3229. E-mail: hiratake@scl.kyoto-u.ac.jp.

‡ Institute for Chemical Research.

§ Graduate School of Biostudies.

10.1021/bi000220p CCC: $19.00
Published on Web 06/09/2000

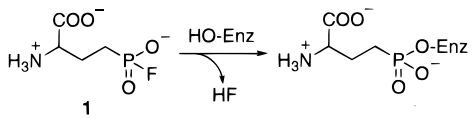
enzyme) (*16*), Ser-405 (pig kidney enzyme), and Ser-406 (human kidney enzyme, equivalent to Ser-405 in rat and pig enzymes) (*17*) as the labeled residue in the small subunit. However, the site-directed mutagenesis of the human enzyme has shown that neither of these hydroxy residues is essential for catalysis or for the inactivation by acivicin (*17*). Because acivicin was bound to the enzyme by an unstable hydroximic ester bond (*21*), it was suggested that acivicin was initially attached to the catalytic hydroxy residue but afterward was transesterified to the aforementioned nearby hydroxy residues.

Identification of the catalytic nucleophile was also approached by sequence comparison of several γ-glutamyltranspeptidases from different species. Among five well-conserved serine residues in the small subunit, two residues (Ser-451 and -452 in the human enzyme) were replaced with Ala to impair both the catalytic activity and the inactivation rate by acivicin (*22*). Although these two residues were likely to participate in the catalysis, this result did not lead to the conclusion that either of these residues was the catalytic nucleophile, due to the lack of direct evidence such as affinity labeling.

Another well-conserved hydroxy residue in the small subunit is the N-terminal Thr (Thr-391 in *Escherichia coli* enzyme). This residue is invariably conserved among all the γ-glutamyltranspeptidases for which the primary sequences are known. Unfortunately, the replacement of the Thr with Ala in *E. coli* enzyme resulted in failure of posttranslational processing, thereby producing neither the small (20 kDa) nor the large (39 kDa) subunit with catalytic function, but instead yielding the unprocessed precursor peptide (59 kDa) with no enzymatic activity (*23*). Recently, from the preliminary X-ray structure of *E. coli* γ-glutamyltranspeptidase (*24*), we proposed that this enzyme was autocatalytically processed (*25*) where the hydroxy group of Thr-391 attacked the carbonyl carbon of the peptide bond between Gln-390 and Thr-391. This notion gave impetus to identify the catalytic nucleophile not only for understanding the catalytic mechanism but also for defining the role of this threonine residue in the processing of this class of enzymes.

An effective approach to identify the catalytic nucleophile is to use a mechanism-based inactivator. For this purpose, the mechanism-based inactivator must form a stable covalent bond with the catalytic nucleophile to avoid the postmodificational migration, as with the case with acivicin. Here, we designed 2-amino-4-(fluorophosphono)butanoic acid **1**, a γ-phosphonic acid monofluoride derivative of glutamic acid, as the mechanism-based affinity labeling agent to trap the catalytic nucleophile of *E. coli* γ-glutamyltranspeptidase. Because γ-glutamyltranspeptidase is proposed to catalyze the reaction in a manner similar to that of Ser hydrolases (Scheme 1), an electrophilic organophosphorus compound, a conventional active-site-directed inactivator of Ser hydrolases (*26*) should be effective also for trapping the catalytic nucleophile of γ-glutamyltranspeptidase. Thus, compound **1** is expected to bind covalently to the catalytic nucleophile

Scheme 2: Expected Inactivation Mechanism for **1**



1

in a mechanism-based manner, forming a transition-state-like adduct in the enzyme active site (Scheme 2). Unlike the conventional inactivators of Ser hydrolases, compound **1** has a negative charge on the phosphonate oxygen. The negative charge of **1** is expected not only to mimic the anionic transition state but also to stabilize the phosphonic acid monoester bond formed between the inactivator and the catalytic nucleophile. Such a stable phosphonic acid monoester bond would prevent the postmodificational transesterification as observed with acivicin, thus effecting the unambiguous identification of the labeled residue. In addition, the negative charge increases the hydrolytic stability of **1**, which enables a straightforward kinetic analysis.

E. coli γ-glutamyltranspeptidase was selected for this study, because *E. coli* enzyme has characteristics similar to those of well-studied mammalian enzymes, such as the amino acid sequence (especially of the small subunit) (*27*), the catalytic properties, and the inhibitor sensitivities (*28*). The major advantage of studying *E. coli* enzyme is the simplicity and homogeneity of the enzyme: *E. coli* γ-glutamyltranspeptidase is a soluble and nonglycosylated enzyme, while the mammalian ones are membrane-bound and heterologously glycosylated enzymes (*1*).

We describe here the mechanism-based affinity labeling of *E. coli* γ-glutamyltranspeptidase by the phosphonic acid monofluoride **1**. The enzyme was rapidly and irreversibly inhibited by **1**, and the inactivation rate was decreased by increasing the concentration of the substrate. Ion-spray mass spectrometric analyses of the inactivated enzyme revealed that Thr-391, the N-terminal residue of the small subunit, was the phosphonylated residue. This finding indicates that the Thr-391 is the catalytic nucleophile of *E. coli* γ-glutamyltranspeptidase.

EXPERIMENTAL PROCEDURES

Materials. 7-(γ-L-Glutamylamino)-4-methylcoumarin was purchased from Sigma. γ-L-Glutamyl-*p*-nitroanilide, glycylglycine (Gly-Gly), and endoproteinase LysC (from *Acromobacter lyticus* M 497-1) were obtained from Wako Pure Chemical Industries. 2-Amino-4-phosphonobutanoic acid was synthesized by a published procedure (*29*). *E. coli* γ-glutamyltranspeptidase was purified from the periplasmic fraction of a recombinant strain of *E. coli* K-12 (SH642) by lysozyme treatment, ammonium sulfate precipitation, and chromatofocusing as described previously (*30*). The protein concentration was determined by Bradford's method (*31*).

*Synthesis and Stability of Compound **1***. Compound **1** was synthesized from 2-amino-4-phosphonobutanoic acid, as reported previously (*32*). The detailed procedure is given in Supporting Information. The hydrolysis of **1** was monitored by ^{31}P NMR (81.0 MHz) on a Varian VXR-200 spectrometer after incubating 10.6 mM **1** in 1 M sodium succinate buffer (pH 5.5) containing D_2O (50% v/v) at 37 °C. The ratios of **1** and the hydrolyzed product, 2-amino-4-phosphonobutanoic acid, were calculated from the integration of ^{31}P NMR peak areas.

[1] Abbreviations: acivicin, L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; DON, 6-diazo-5-oxo-L-norleucine; LysC, lysyl endopeptidase; amu, atomic mass unit; LC/MS, liquid chromatography/mass spectrometry; MS/MS, tandem mass spectrometry; CID, collision-induced decay.

Enzyme Assay. The hydrolase activity was measured using 0.2 μM 7-(γ-L-glutamylamino)-4-methylcoumarin (*33*) as the substrate in 0.1 M sodium succinate buffer (pH 5.5) at 37 °C. The released 7-amino-4-methylcoumarin was monitored at 440 nm emission (excitation at 350 nm) on a Hitachi F-2000 spectrophotometer. Fluorescence changes (ΔF) were converted to concentration changes (ΔC) of the product by the correlation coefficient ($\Delta F/\Delta C = 1.03$ nM^{-1}) calibrated with 7-amino-4-methylcoumarin. The fluorescence intensity was proportional to the concentration of 7-amino-4-methylcoumarin up to 0.1 μM. The Michaelis constant (K_m) for 7-(γ-L-glutamylamino)-4-methylcoumarin was determined as 0.3 μM. Regain of the inactivated enzyme was examined by measuring the hydrolase and the transpeptidase activities using 0.5 mM γ-L-glutamyl-*p*-nitroanilide as the substrate in 0.1 M sodium succinate (pH 5.5) and 0.1 M Tris-HCl buffer (pH 8.3), respectively, at 37 °C. Gly-Gly (67 mM) was added for the measurement of the transpeptidase activity. The released *p*-nitroaniline was monitored at 410 nm ($\epsilon_{410} = 8800$ M^{-1} cm^{-1}) on a Shimadzu UV-3101PC spectrophotometer.

Time-Dependent Inhibition. To the preincubated mixture of **1** and 0.2 μM 7-(γ-L-glutamylamino)-4-methylcoumarin in 0.1 M sodium succinate buffer (pH 5.5) at 37 °C was added the enzyme to a final concentration of 0.26 nM to initiate the reaction. The time-dependent inhibition of the enzyme was monitored until the product release reached a plateau in the presence of various concentrations of **1**. Pseudo-first-order rate constants for the inactivation (k_{obs}) were determined by fitting each curve to a first-order rate equation (1):

$$[P] = [P]_\infty[1 - \exp(-k_{obs}\, t)] \quad (1)$$

where [P] and $[P]_\infty$ are the concentrations of product formed at time t and at the time approaching infinity, respectively (*34*). The second-order rate constant for the inactivation of the enzyme (k_{on}) was determined according to the following equation (2) derived from the mechanism described below:

$$\begin{array}{ccccc} E + S & \overset{K_m}{\rightleftharpoons} & ES & \longrightarrow & E + P \\ & & \downarrow k_{on}[I] & & \\ & & E\text{-}I & & \end{array}$$

$$k_{obs} = k_{on}[I]/(1 + [S]/K_m) \quad (2)$$

where [S] and K_m were 0.2 and 0.3 μM, respectively. Nonlinear regression analyses of kinetic data were performed using the KaleidaGraph program (Synergy Software).

Regain of Activity of Inactivated Enzyme. The enzyme (4.16 μM) was incubated with 1 mM **1** in 0.1 M sodium succinate buffer (pH 5.5) at 25 °C for 10 min. The excess inhibitor was removed by rapid gel filtration at 4 °C through a Bio-Spin column (BioRad) equilibrated with 0.05 M Tris-HCl buffer (pH 8.3). The inactivated enzyme was dialyzed against 0.05 M Tris-HCl buffer (pH 8.3) at 4 °C for 10 days. The hydrolase and the transpeptidase activities were measured using 0.5 mM γ-L-glutamyl-*p*-nitroanilide as described above. The control experiment was conducted by using the same procedure without **1**.

Isolation of Small and Large Subunits and Limited Proteolysis. The enzyme (10 nmol, final concentration of 0.5 mM) was inactivated by incubating with 2 mM **1** in duplicate in 20 μL of 0.05 M Tris-HCl buffer (pH 7.0) at 37 °C for 20 min. The small and large subunits of the modified enzyme were isolated by reversed-phase HPLC (COSMOSIL 5C18-AR-300, 4.6 × 150 mm) and lyophilized as described previously (*35*). The lyophilized samples were used directly for the mass spectrometric analyses (see below). The lyophilized small subunit was dissolved in 50 μL of 0.2 M Tris-sulfate buffer (pH 7.2) containing 8 M urea and was incubated at 37 °C for 1 h. To the mixture was added 75 μL of 1 M Tris-sulfate buffer (pH 9.0), followed by the addition of 2.5 μL of 33 pmol/μL LysC dissolved in 2 mM Tris-HCl buffer (pH 8.0). The digestion was carried out at 37 °C for 12 h. The mixture of the digest was vacuum-evaporated to dryness and was analyzed by LC/MS (see below). The control experiments were performed by using the same procedure without inactivation by **1**.

Ion-Spray Mass Spectroscopy. All the mass spectra were recorded on a Sciex API-3000 mass spectrometer (PE Sciex) interfaced with an ion-spray ion source. Each of the isolated small and large subunits (ca. 10 nmol) from the unmodified and modified enzymes was dissolved in a mixture of 50 μL of 0.1% formic acid and 50 μL of acetonitrile and was injected directly into the mass spectrometer using a syringe (1.46 mm inside diameter) at a flow rate of 2.5 μL/min. The quadrupole was scanned over a range of 500−2000 atomic mass units (amu) with a step size of 0.1 amu and a dwell time of 0.2 ms. Ion-spray voltage was set at 5 kV, and orifice potential was 60 V. Data analyses were performed using the BioMultiView program (PE Sciex).

LC/MS analyses of the proteolytic digest were performed by loading 10 nmol of the digest onto a Shiseido Capcellpak C18 column (4.6 × 250 mm) connected to the mass spectrometer. The column was eluted for 5 min with 2% acetonitrile containing 0.1% formic acid and then over 40 min with a linear gradient of 2−82% acetonitrile containing 0.1% formic acid at a flow rate of 1 mL/min. The postcolumn splitter sent the sample into the mass spectrometer as well as into the fraction collector for MS/MS analyses (see below). The total ion current was recorded in a single-quadrupole scan mode to produce an HPLC chromatogram for the digests. The quadrupole was scanned over a range of 150−2500 amu with a step size of 0.1 amu and a dwell time of 0.05 ms. Ion-spray voltage and orifice potential were set in the same manner as described above.

For MS/MS analyses, the HPLC fractions eluted at 12.60 min (unmodified enzyme) and at 13.11 min (modified enzyme) were collected, concentrated, and injected directly into the mass spectrometer. MS/MS product ion spectra were obtained in the triple-quadrupole scan mode by selectively introducing the *m*/*z* 350.7 (unmodified enzyme) and 405.8 (modified enzyme) precursor ions from the first quadrupole (Q1) into the collision cell (Q2), and the product ions in the third quadrupole (Q3) were observed. Q3 was scanned over a range of 5−1250 amu with a step size of 0.1 amu and a dwell time of 0.2 ms. Ion-spray voltage was set at 5 kV. The orifice potential was 35 and 40 V for the *m*/*z* 350.7 and 405.8 precursor ions, respectively. The collision energy (RO2 − RO1) was 15 eV.

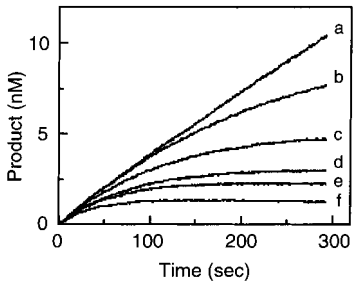


FIGURE 1: Time-dependent inhibition by **1**. The enzymatic reaction was carried out in the presence of the following concentrations of **1**: (a) 0, (b) 0.17, (c) 0.33, (d) 0.5, (e) 0.67, and (f) 1.0 μM.

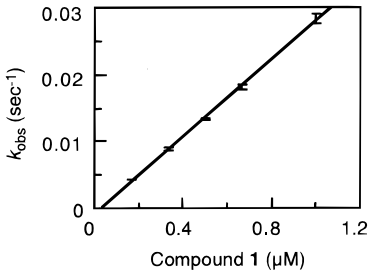


FIGURE 2: Plot of k_{obs} against the inhibitor concentration.

RESULTS

Inhibition of the Enzyme. Prior to the inhibition experiment, the hydrolytic stability of **1** was examined. Compound **1** was relatively stable in 1 M sodium succinate buffer (pH 5.5) at 37 °C and was hydrolyzed slowly to 2-amino-4-phosphonobutanoic acid with a half-life of 21.6 h, while **1** was hydrolytically unstable in neutral to alkaline media ($t_{1/2}$ = 20 min at pH 7.5) (*32*). Thus, the effective concentration of **1** remained essentially constant during the inhibition experiments at pH 5.5, at which the enzyme exhibited the maximal hydrolase activity.

Compound **1** inhibited *E. coli* γ-glutamyltranspeptidase in a time-dependent manner (Figure 1). The rate of enzyme inactivation increased with increasing concentration of **1**, and the enzymatic activity was completely lost in the end at each inhibitor concentration. The time-dependent inhibition curves were fit to the first-order rate equation (1) to determine the pseudo-first-order rate constants for the inactivation of the enzyme (k_{obs}) at each inhibitor concentration. The value of k_{obs} was decreased by increasing the concentration of the substrate. The plot of k_{obs} against the inhibitor concentrations was found to be linear up to 1.0 μM **1**, and no saturation kinetics was observed (Figure 2). With higher inhibitor concentrations, rapid enzyme inactivation did not allow the initial reaction rate to be measured by the conventional assay method. The second-order rate constant for the inactivation of the enzyme (k_{on}) was calculated according to eq 2, and k_{on} was calculated as 4.83×10^4 M^{-1} s^{-1}.

The regain of the enzyme activity was examined to see if the enzyme was inhibited irreversibly. The enzyme was incubated with 1 mM **1** at 25 °C for 10 min, and the excess compound was removed by rapid gel filtration. The hydrolase and the transpeptidase activities were lost completely. The inactivated enzyme was dialyzed against Tris-HCl buffer (pH 8.3) at 4 °C for 10 days, but neither the transpeptidase nor the hydrolase activity was regained, suggesting that the inhibitor was covalently attached to the enzyme.

Mass Spectrometric Analysis. The molecular masses of the small and large subunits of γ-glutamyltranspeptidase were determined first. Each unmodified and modified enzyme was subjected to reversed-phase HPLC to isolate the small and large subunits as described previously (*35*). The isolated subunits were analyzed separately by ion-spray mass spectrometry. With the unmodified enzyme, the reconstructed mass spectrum showed one major peak with a molecular mass of 20 014 and 39 209 Da for the small and large subunits, respectively (Figure 3A,B). These values agreed well with those predicted from the amino acid sequence (average mass of 20 010 and 39 196 Da for the small and large subunits, respectively) and also agreed with those reported previously (*35*). The reconstructed mass spectrum of the modified enzyme gave a single major peak with a molecular mass of 20 178 and 39 209 Da for the small and large subunits, respectively (Figure 3C,D). The mass of the small subunit increased by 164 Da by the treatment of the enzyme with **1**, while the mass of the large subunit remained unchanged. The observed mass increase of the small subunit corresponded well to the expected mass increase of 165 Da caused by the phosphonylation with **1**, indicating that one molecule of **1** was attached covalently to the small subunit.

Knowing that one molecule of **1** was incorporated into the small subunit, we determined the amino acid residue phosphonylated by **1** by LC/MS and MS/MS analyses. First, the small subunit of each unmodified and modified enzyme was digested by LysC, and the resulting proteolytic digests were analyzed by reversed-phase HPLC using the ion-spray mass spectrometer as the detector to produce a chromatogram (Figure 4). Both chromatograms were almost identical except for one peak: the peak eluted at 12.60 min from the unmodified enzyme disappeared, but instead, a new peak appeared at 13.11 min in the chromatogram of the modified enzyme. The ion-spray mass spectrum of the peptide eluted at 12.60 min from the unmodified enzyme showed $(M + 3H)^{3+}$, $(M + 2H)^{2+}$, and $(M + H)^{+}$ ions at *m*/*z* 350.8, 526.3, and 1050.0, respectively (Figure 5A). The molecular mass of this peptide was calculated as 1049.7 Da, which corresponded well to the predicted mass of the N-terminal peptide Thr391-Lys399 (theoretical mass of 1048.5 Da). As for the peptide eluted at 13.11 min from the modified enzyme, $(M + 3H)^{3+}$, $(M + 2H)^{2+}$, and $(M + H)^{+}$ ions were observed at *m*/*z* 405.9, 608.4, and 1215.2, respectively (Figure 5B). The molecular mass of this peptide was calculated as 1214.6 Da, larger by 164.9 Da than that from the unmodified enzyme (1049.7 Da). The difference corresponded exactly to the expected mass increase of 165.0 Da caused by the phosphonylation with **1**. These results indicated that the N-terminal peptide Thr391-Lys399 was phosphonylated by **1**. The mass spectrum of this peptide (t_R = 13.11 min) gave additional peaks at *m*/*z* 184.1 and 517.1 (Figure 5B). The formation of these ions is discussed later.

Finally, the phosphonylated residue in the N-terminal peptide was identified. The $(M + 3H)^{3+}$ ions at *m*/*z* 350.7 (unmodified peptide) and 405.9 (phosphonylated peptide) were subjected to collision-induced decay (CID) on the triple-quadrupole mass spectrometer. The resulting CID spectrum and the amino acid sequence of the N-terminal peptide Thr391-Lys399 are depicted in Figure 6. The predicted monoisotopic masses for product ions of type y are shown below the sequence (Figure 6A). The CID spectrum for the

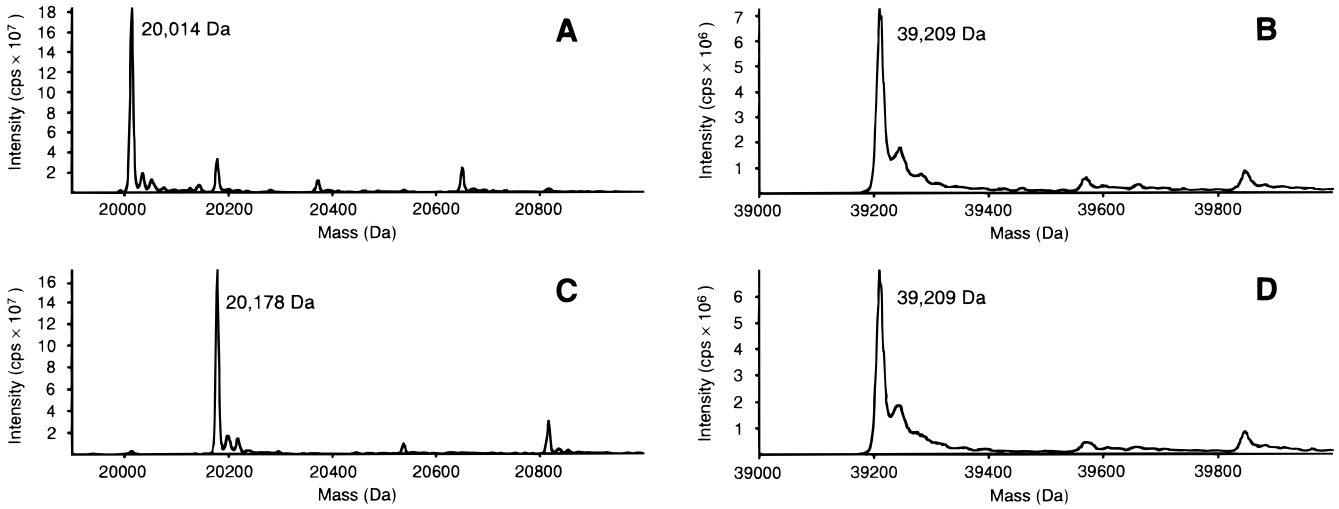


FIGURE 3: Reconstructed mass spectra of the small and large subunits. (A) Small subunit from the unmodified enzyme. (B) Large subunit from the unmodified enzyme. (C) Small subunit from the modified enzyme. (D) Large subunit from the modified enzyme.

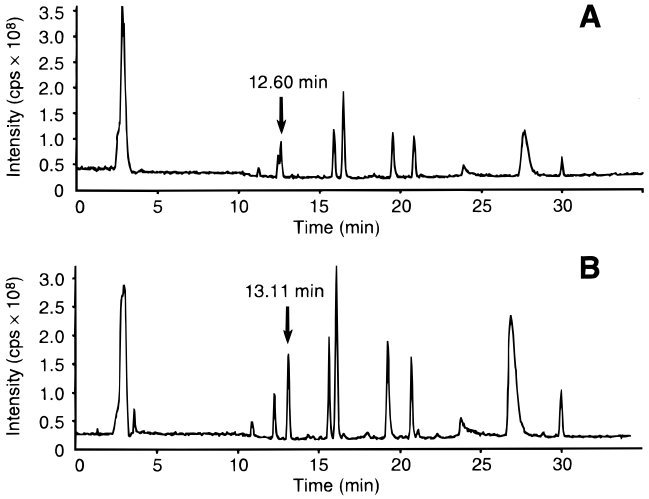


FIGURE 4: HPLC elution profiles of the proteolytic digests of the small subunit. Proteolytic digests were loaded onto an ODS column, and the column was eluted for 5 min with 2% acetonitrile containing 0.1% formic acid, followed by a linear gradient of 2−82% acetonitrile containing 0.1% formic acid over 40 min at a flow rate of 1 mL/min. (A) Digest from the unmodified enzyme. (B) Digest from the modified enzyme. The peak with a different elution time between A and B is indicated by an arrow.

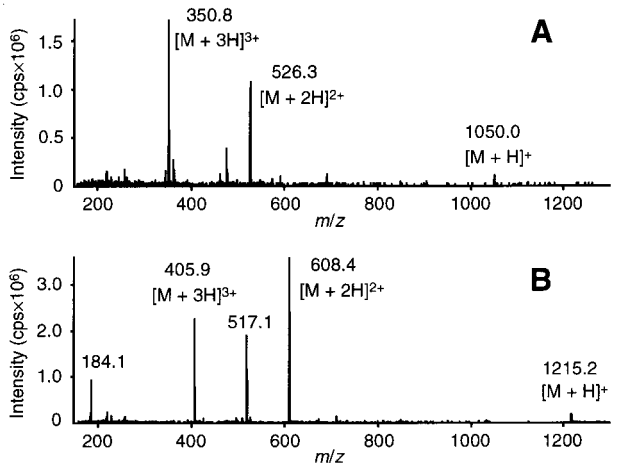


FIGURE 5: Mass spectra of the isolated peptides of the proteolytic digest. (A) The peptide eluted at 12.60 min from the unmodified enzyme. (B) The peptide eluted at 13.11 min from the modified enzyme.

$(M + 3H)^{3+}$ precursor ion at *m*/*z* 350.7 (unmodified peptide) gave all of the possible product ions of type y, along with the precursor ion itself (Figure 6B). The CID spectrum of the $(M + 3H)^{3+}$ precursor ion at *m*/*z* 405.8 (phosphonylated peptide) was the same as that of the unmodified peptide, except for the precursor ion (Figure 6C). Thus, a mass increase of 165 Da was not observed in the y_1-y_8 product ions but instead was observed only in the precursor ion. This result clearly indicated that **1** phosphonylated the N-terminal residue of the small subunit, that is, Thr-391. The CID spectrum of the phosphonylated precursor peptide also showed additional ions at *m*/*z* of 184.1 and 516.4 (Figure 6C), as observed with the LC/MS experiment of the phosphonylated peptide Thr391-Lys399 (Figure 5B).

DISCUSSION

The purpose of this study is to identify the key catalytic nucleophile responsible for the catalysis of *E. coli* γ-glutamyltranspeptidase. Considering that the chemistry of the reaction catalyzed by γ-glutamyltranspeptidase (Scheme 1) is essentially the same as that of the well-studied Ser hydrolases such as chymotrypsin and subtilisin, the same strategy should be applicable to trap the catalytic nucleophile of γ-glutamyltranspeptidase as that used for the active-site-directed modification of Ser hydrolases by electrophilic organophosphorus compounds (*26*). We, therefore, selected phosphonic acid monofluoride **1** as a mechanism-based affinity labeling agent. Compound **1**, however, has a negative charge on the phosphonate oxygen, unlike the conventional organophosphorus inactivators of Ser hydrolases. The negative charge might decrease the reactivity of the electrophilic phosphorus, but it is expected to stabilize the putative phosphonylated enzyme, if formed, toward hydrolysis or transesterification to other hydroxy residues after modification. The negative charge would also increase the hydrolytic stability of **1**. Indeed, compound **1** was relatively stable in an aqueous solution ($t_{1/2}$ of 21.6 h at pH 5.5), and the kinetic analyses of the enzyme inhibition were done without difficulty.

Compound **1** inhibited the enzyme in a time-dependent manner (Figure 1). Increasing the concentration of the substrate decreased the inactivation rate (k_{obs}), suggesting that

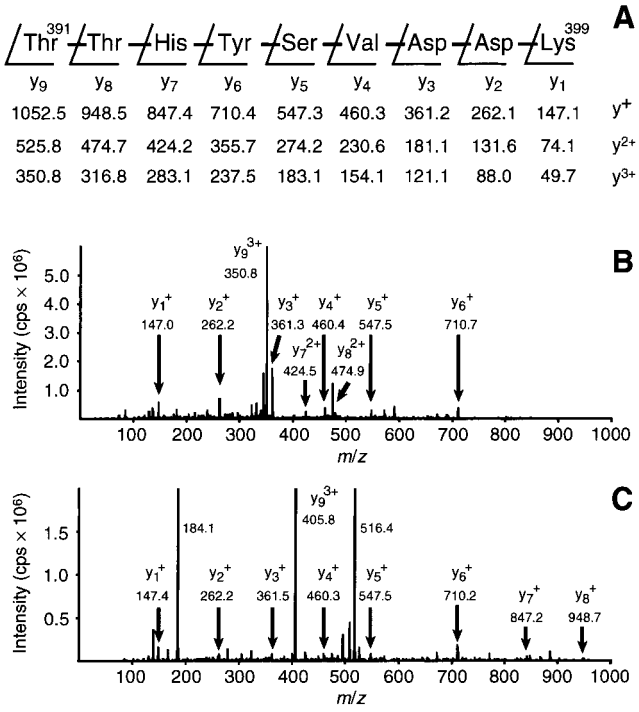
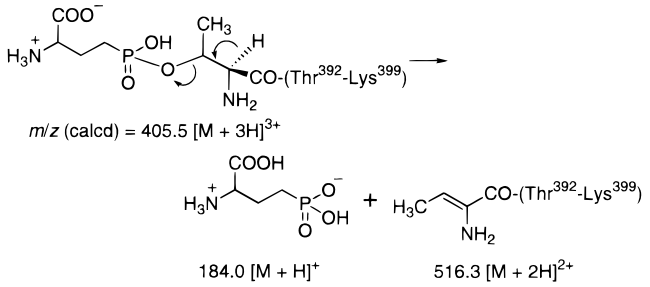


FIGURE 6: MS/MS analysis of the peptide Thr391-Lys399. (A) Predicted monoisotopic masses for product ions of type y derived from the sequence shown above. (B) CID spectrum of the *m/z* 350.7 precursor ion from the unmodified enzyme. (C) CID spectrum of the *m/z* 405.9 precursor ion from the modified enzyme.

compound **1** was competing with the substrate for the enzyme active site. Despite the considerable stability of **1**, the inactivation of the enzyme was very rapid: the second-order rate constant for the inactivation (k_{on}) was 4.83×10^4 M^{-1} s^{-1}, only 37-fold less than the second-order rate constant (k_{cat}/K_m) for the enzymatic hydrolysis of 7-(L-γ-glutamylamino)-4-methylcoumarin (1.79×10^6 M^{-1} s^{-1}). This inactivation rate was 3 orders of magnitude larger than that of acivicin (*17, 22*) or DON (*36*). This is probably because **1** recruited the catalytic power of the enzyme to phosphonylate the catalytic nucleophile (*37*), while acivicin and DON rely solely on their reactive groups to form a covalent bond with the nearby nucleophile. The inactivated enzyme did not regain the activity after prolonged dialysis, indicating that the inactivation most likely reflected the formation of a stable covalent bond between the inactivator and the enzyme. The phosphonic acid monoester linkage is known to be quite stable, as evidenced by the "aged" adduct in Ser hydrolases inactivated by diisopropyl phosphorofluoridate (DFP) or other related organophosphorus compounds, in which spontaneous dealkylation of the phosphonylated Ser residue forms the phosphonic acid monoester, a truly irreversible adduct (*38, 39*). The stability of the phosphonylated adduct of γ-glutamyltranspeptidase thus enabled the unambiguous identification of the catalytic nucleophile with ion-spray mass spectrometry.

Ion-spray mass spectrometry of the modified enzyme revealed that **1** was covalently and stoichiometrically attached to the small subunit (Figure 3). This result is in accord with the notion, from the studies of chemical modification of the enzyme with ^{14}C-acivicin (*14*−*17*) or ^{14}C-DON (*18*−*20*), that the small subunit is the catalytic subunit. Protease digestion and LC/MS analysis of the labeled small subunit identified the N-terminal peptide Thr391-Lys399 as the phosphonylation site (Figure 5), and the subsequent MS/MS experiment revealed that the phosphonylated amino acid residue was Thr-391, the N-terminal residue of the small subunit (Figure 6).

Scheme 3: Possible Mechanism for the Formation of *m/z* 184 and 156 Ions



It is worth noting that two additional ions were observed for the phosphonylated peptide in the ionization of the LC/MS and the MS/MS analyses. One was *m/z* 184.1 (Figures 5B, 6C), and the other was *m/z* 517.1 (Figure 5B) or 516.4 (Figure 6C). The former ion at *m/z* 184.1 corresponded to the $(M + H)^{1+}$ ion of 2-amino-4-phosphonobutanoic acid whose monoisotopic mass is 183.0 Da. Bearing this in mind, the latter ion at *m/z* 517.1 or 516.4 was assigned to be $(M - 183 + 2H)^{2+}$, which was formed probably by the β-elimination of 2-amino-4-phosphonobutanoic acid (183.0 Da) from the phosphonylated N-terminal peptide Thr391-Lys399 (theoretical mass of 1213.5 Da) during the ionization process (Scheme 3). Similar fragment ions were reported with phosphorylated proteins, where the phosphothreonine and phosphoserine residues underwent β-elimination of a phosphoric acid (98.0 Da) to produce the (y − 98) product ions in the MS/MS experiment. This is often taken as proof that threonine or serine residues are phosphorylated (*40*). The present MS/MS analysis of the labeled peptide Thr391-Lys399 gave the (y − 183) ion only from the precursor ion (y_9), not from the other product ions (y_1−y_8). These results also supported that the N-terminus Thr-391 was the phosphonylated residue.

The N-terminal Thr-391 is most likely to be the catalytic nucleophile of *E. coli* γ-glutamyltranspeptidase, and several studies support this. First, Thr-391 of the *E. coli* enzyme is the only threonine or serine residue conserved among all the γ-glutamyltranspeptidases for which the primary sequences are known. Second, γ-glutamyltranspeptidase is predicted to be a member of N-terminal nucleophile hydrolases (*25*), a recently recognized new hydrolase family, because the preliminary crystal structure of *E. coli* enzyme has shown the presence of two antiparallel pleated β-sheets (*24*), which is unique to this family. γ-Glutamyltranspeptidase was predicted to be the N-terminal nucleophile hydrolase also from the fact that the enzyme is posttranslationally processed from the catalytically inactive precursor (*27, 41*−*44*), which is a characteristic of N-terminal nucleophile hydrolases. In all the N-terminal nucleophile hydrolases, the processing generates an N-terminal residue as the catalytic nucleophile, and this residue corresponds to Thr-391 with *E. coli* γ-glutamyltranspeptidase. Thus, we conclude that N-terminal Thr-391 is the catalytic nucleophile of *E. coli* γ-glutamyltranspeptidase.

N-Terminal nucleophile hydrolases were proposed to have a different catalytic mechanism from that of the Ser hydro-

lases with a Ser-His-Asp (Glu) catalytic triad (*13*). The crystal structures of penicillin acylase (*45*) and aspartylglucosaminidase (*46, 47*), typical N-terminal nucleophile hydrolases, revealed that no basic amino acid residues (as His in Ser hydrolases) were present near the catalytic nucleophile. Instead, the free α-amino group of the catalytic residue itself was considered to act as a base. In addition, N-terminal nucleophile hydrolases had no carboxylate counterpart (as Asp or Glu in Ser hydrolases) interacting with the base, and they were proposed to use a "nucleophile−amine" catalytic dyad in the catalysis (*48*). A similar active site environment was observed with class C β-lactamases (*49*) where a Ser nucleophile is facing toward a Lys ϵ-amino group without a carboxylate counterpart interacting with the Lys. In the present study, anionic phosphonic acid monofluoride served as a potent inactivator of *E. coli* γ-glutamyltranspeptidase. It should be noted that anionic phosphonic acid monoesters were also reported to be potent inactivators of class C β-lactamases, but they were poor inactivators of Ser hydrolases with a Ser-His-Asp (Glu) catalytic triad (*50, 51*). This might suggest that the catalytic mechanism of *E. coli* γ-glutamyltranspeptidase is more related to that of class C β-lactamases whose active site is proposed to have a greater positive potential than to that of Ser hydrolases with a Ser-His-Asp (Glu) catalytic triad (*49*). This positive potential was likely to allow the anionic phosphonate inactivators to effectively phosphonylate the catalytic nucleophile. In this regard, other N-terminal nucleophile hydrolases with a nucleophile−amine catalytic dyad might be inactivated effectively by anionic phosphonic acid monofluoride-based inhibitors.

Another interesting feature of N-terminal nucleophile hydrolases is the posttranslational autocatalytic processing (*25*). Although direct evidence of autocatalytic processing of γ-glutamyltranspeptidase is not available at present, Thr-391 is likely to be the key catalytic residue in this process. This is probably the reason the replacement of this residue of the *E. coli* enzyme with alanine resulted in failure of posttranslational processing (*23*).

In conclusion, we identified the N-terminal Thr-391 as the catalytic nucleophile of *E. coli* γ-glutamyltranspeptidase by using phosphonic acid monofluoride **1** as a novel mechanism-based affinity labeling agent. The detailed catalytic mechanism and autocatalytic processing should be available from the three-dimensional structure of *E. coli* γ-glutamyltranspeptidase, and this aspect of the study is in progress.

ACKNOWLEDGMENT

We thank Dr. Susumu Tsunasawa (Protein Analysis Center, Takara Shuzo Co., Ltd.) for his kind permission to use the ion-spray mass spectrometer. We are also grateful to Mr. Yoshifumi Kogure (Biomedical Group, Takara Shuzo Co., Ltd.) and Dr. Susumu Ichiyama (Institute for Chemical Research, Kyoto University) for technical assistance with the mass experiments and for fruitful discussions.

SUPPORTING INFORMATION AVAILABLE

The detailed procedure of the synthesis of **1** is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Taniguchi, N., and Ikeda, Y. (1998) *Adv. Enzymol. Relat. Areas Mol. Biol. 72*, 239−278.
2. Hanigan, M. H., and Ricketts, W. A. (1993) *Biochemistry 32*, 6302−6306.
3. Lieberman, M. W., Wiseman, A. L., Shi, Z. Z., Carter, B. Z., Barrios, R., Ou, C. N., Barrios, P. C., Wang, Y., Habib, G. M., Goodman, J. C., Huang, S. L., Lebovitz, R. M., and Matzuk, M. M. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 7923−7926.
4. Suzuki, H., Hashimoto, W., and Kumagai, H. (1999) *J. Mol. Catal. B 6*, 175−184.
5. Harding, C. O., Williams, P., Wagner, E., Chang, D. S., Wild, K., Colwell, R. E., and Wolff, J. A. (1997) *J. Biol. Chem. 272*, 12560−12567.
6. Sawabu, N., Nakagen, M., Yoneda, M., Makino, H., Kameda, S., Kobayashi, K., Hattori, N., and Ishii, M. (1978) *Gann Monogr. Cancer Res. 69*, 601−605.
7. Taniguchi, N., Iizuka, S., Zhe, Z. N., House, S., Yokosawa, N., Ono, N., Kinoshita, K., Makita, A., and Sekiya, C. (1985) *Cancer Res. 45*, 5835−5839.
8. Tate, S. S., and Meister, A. (1974) *J. Biol. Chem. 249*, 7593−7602.
9. Elce, J. S., and Broxmeyer, B. (1976) *Biochem. J. 153*, 223−232.
10. Smith, T. K., and Meister, A. (1995) *J. Biol. Chem. 270*, 12476−12480.
11. Inoue, M., Horiuchi, S., and Morino, Y. (1978) *Biochem. Biophys. Res. Commun. 82*, 1183−1188.
12. Tate, S. S., and Meister, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4806−4809.
13. Dodson, G., and Wlodawer, A. (1998) *Trends Biochem. Sci. 23*, 347−352.
14. Gardell, S. J., and Tate, S. S. (1980) *FEBS Lett. 122*, 171−174.
15. Kozak, E. M., and Tate, S. S. (1980) *FEBS Lett. 122*, 175−178.
16. Stole, E., Seddon, A. P., Wellner, D., and Meister, A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1706−1709.
17. Smith, T. K., Ikeda, Y., Fujii, J., Taniguchi, N., and Meister, A. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2360−2364.
18. Tate, S. S., and Ross, M. E. (1977) *J. Biol. Chem. 252*, 6042−6045.
19. Tate, S. S., and Meister, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 931−935.
20. Takahashi, S., Zukin, R. S., and Steinman, H. M. (1981) *Arch. Biochem. Biophys. 207*, 87−95.
21. Stole, E., Smith, T. K., Manning, J. M., and Meister, A. (1994) *J. Biol. Chem. 269*, 21435−21439.
22. Ikeda, Y., Fujii, J., Anderson, M. E., Taniguchi, N., and Meister, A. (1995) *J. Biol. Chem. 270*, 22223−22228.
23. Hashimoto, W., Suzuki, H., Yamamoto, K., and Kumagai, H. (1995) *J. Biochem. 118*, 75−80.
24. Sakai, H., Sakabe, N., Sasaki, K., Hashimoto, W., Suzuki, H., Tachi, H., Kumagai, H., and Sakabe, K. (1996) *J. Biochem. 120*, 26−28.
25. Brannigan, J. A., Dodson, G., Duggleby, H. J., Moody, P. C. E, Smith, J. L., Tomchick, D. R., and Murzin, A. G. (1995) *Nature 378*, 416−419.
26. Oleksyszyn, J., and Powers, J. C. (1994) *Methods Enzymol. 244*, 423−441.
27. Suzuki, H., Kumagai, H., Echigo, T., and Tochikura, T. (1989) *J. Bacteriol. 171*, 5169−5172.
28. Suzuki, H., Kumagai, H., and Tochikura, T. (1986) *J. Bacteriol. 168*, 1325−1331.
29. Chambers, J. R., and Isbell, A. F. (1964) *J. Org. Chem. 29*, 830−836.
30. Suzuki, H., Kumagai, H., Echigo, T., and Tochikura, T. (1988) *Biochem. Biophys. Res. Commun. 150*, 33−38.
31. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
32. Inoue, M., Hiratake, J., and Sakata, K. (1999) *Biosci. Biotechnol. Biochem. 63*, 2248−2251.
33. Smith, G. D., Ding, J. L., and Peters, T. J. (1979) *Anal. Biochem. 100*, 136−139.

34. Tsou, C. L. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol. 61*, 381−436.
35. Hashimoto, W., Suzuki, H., Nohara, S., Tachi, H., Yamamoto, K., and Kumagai, H. (1995) *J. Biochem. 118*, 1216−1223.
36. Inoue, M., Horiuchi, S., and Morino, Y. (1977) *Eur. J. Biochem. 73*, 335−342.
37. Kovach, I. M., Larson, M., and Schowen, R. L. (1986) *J. Am. Chem. Soc. 108*, 5490−5495.
38. Kossiakoff, A. A., and Spencer, S. A. (1980) *Nature 288*, 414−416.
39. Millard, C. B., Kryger, G., Ordentlich, A., Greenblatt, H. M., Harel, M., Raves, M. L., Segall, Y., Barak, D., Shafferman, A., Silman, I., and Sussman, J. L. (1999) *Biochemistry 38*, 7032−7039.
40. Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J. H., Shabanowitz, J., Hunt, D. F., Weber, M. J., and Sturgill, T. W. (1991) *EMBO J. 10*, 885−892.
41. Nash, B., and Tate, S. S. (1982) *J. Biol. Chem. 257*, 585−588.
42. Capraro, M. A., and Hughey, R. P. (1983) *FEBS Lett. 157*, 139−143.
43. Barouki, R., Finidori, J., Chobert, M. N., Aggerbeck, M., Laperche, Y., and Hanoune, J. (1984) *J. Biol. Chem. 259*, 7970−7974.
44. Nash, B., and Tate, S. S. (1984) *J. Biol. Chem. 259*, 678−685.
45. Duggleby, H. J., Tolley, S. P., Hill, C. P., Dodson, E. J., Dodson, G., and Moody, P. C. E. (1995) *Nature 373*, 264−268.
46. Oinonen, C., Tikkanen, R., Rouvinen, J., and Peltonen, L. (1995) *Nat. Struct. Biol. 2*, 1102−1108.
47. Guo, H. C., Xu, Q., Buckley, D., and Guan, C. (1998) *J. Biol. Chem. 273*, 20205−20212.
48. Paetzel, M., and Dalbey, R. E. (1997) *Trends Biochem. Sci. 22*, 28−31.
49. Lobkovsky, E., Billings, E. M., Moews, P. C., Rahil, J., Pratt, R. F., and Knox, J. R. (1994) *Biochemistry 33*, 6762−6772.
50. Pratt, R. F. (1989) *Science 246*, 917−919.
51. Rahil, J., and Pratt, R. F. (1992) *Biochemistry 31*, 5869−5878.

BI000220P