Use of Adenosine(5′)polyphospho(5′)pyridoxals To Study the Substrate-Binding Region of Glutathione Synthetase from *Escherichia coli* B†

Takao Hibi,‡ Hiroaki Kato,‡ Takaaki Nishioka,‡ Jun'ichi Oda,*,‡ Hiroshi Yamaguchi,§ Yukiteru Katsube,§ Katsuyuki Tanizawa,∥ and Toshio Fukui∥

Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan, Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan, and Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka 567, Japan

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ABSTRACT: Adenosine(5′)polyphospho(5′)pyridoxals (AP_n-PLs, n = 2, 3, 4) were examined for affinity labeling of glutathione synthetase (EC 6.3.2.3) from *Escherichia coli* B. When the enzyme was incubated with an AP_n-PL or pyridoxal phosphate in the presence of Mg^{2+} and then reduced with sodium borohydride, it was most rapidly inactivated by AP_4-PL. AP_4-PL had a high affinity to the enzyme. The dissociation constant of AP_4-PL in the inactivation process was 23 μM. The enzyme was almost completely protected from inactivation by addition of either ATP or γ-glutamylcysteine. Complete inactivation corresponded to the incorporation of 1 mol of AP_4-PL/mol of subunit of the tetrameric enzyme. Proteolytic digestion and sequence analysis of the AP_4-PL-labeled enzyme revealed that only Lys-18 was modified. In contrast, the less efficient AP_3-PL was found attached to Lys-17, Lys-18, Lys-144, and Lys-148. In the three-dimensional structure of the enzyme, Lys-18 is located close to the putative γ-glutamylcysteine-binding site, but Lys-17, Lys-144, and Lys-148 are in the mouth of the inner-solvent region, at the bottom of which is the active-site cleft. Furthermore, difference Fourier analysis with the AP_4-PL-soaked crystal of the enzyme showed that the adenosine moiety of the bound AP_4-PL was in the crevice, which is the ATP-binding site of the enzyme. These results demonstrate the bivalent binding of AP_4-PL lying across the γ-glutamylcysteine- and ATP-binding sites.

Glutathione synthetase [γ-L-glutamyl-L-cysteine:glycine ligase (ADP-forming), EC 6.3.2.3] catalyzes the synthesis of glutathione from γ-L-glutamyl-L-cysteine and glycine in the presence of ATP and Mg^{2+} (Meister, 1974, 1985). The enzyme is an important example of the rare ATP-dependent enzymes that synthesize peptide bonds and occur in both procaryotes and eucaryotes. Little is known, however, of its structure and reaction mechanism. The complete amino acid sequence is known only for the enzyme from *Escherichia coli* B, and this was deduced from a nucleotide sequence of the *gsh* II gene (Gushima et al., 1984). The polypeptide chain of the *E. coli* B enzyme is composed of 316 amino acid residues with a calculated molecular mass of 35 561 daltons and corresponds to each subunit of the tetrameric enzyme (Gushima et al., 1983). Chemical modifications of the enzymes from rat kidney (Oppenheimer et al., 1979) and from *E. coli* B (Gushima et al., 1983) with sulfhydryl reagents have suggested that at least one cysteinyl residue is essential for catalytic activity. However, in the *E. coli* B enzyme, replacement of all four cysteines with alanine has shown that none of the cysteinyl residues is involved in the catalysis even though a cysteinyl residue (Cys-289) is close to the active site (Kato et al., 1988).

Recently, we obtained single crystals of the *E. coli* B enzyme suitable for X-ray crystal structure analysis (Kato et al., 1989) and determined the three-dimensional structure at 2.0-Å resolution (Yamaguchi et al., 1990; Yamaguchi et al., in press). The subunit is composed of three dissimilar domains: N-terminal (residues 1–121), central (134–201), and C-terminal (122–133, 212–316). The putative active site is along a cleft at the interface of the three domains. When ATP or γ-glutamylcysteine, or both, binds to the enzyme, the cleft is presumably covered with a disordered loop (residues 226–242), which is not visible on the electron density map. The disordered loop is extremely susceptible to limited proteolysis, and binding of substrates prevents the loop from being digested (Tanaka et al., 1992). Nevertheless, the precise locations of the substrate-binding sites remain to be studied.

To identify these sites, we used adenosine(5′)polyphospho(5′)pyridoxals (AP_n-PLs; n = 2, 3, 4).[1] Reagents with two to four phosphoryl groups linking the pyridoxal and the adenosine moieties have been successfully used for affinity labeling of a number of ATP-binding proteins [see Colman (1990) for a recent review]. We found that, among the reagents examined, AP_4-PL has the highest affinity for *E. coli* B glutathione synthetase and labels Lys-18 specifically in a stoichiometric fashion. In addition, X-ray diffraction analysis of the crystals soaked with AP_4-PL has revealed binding of its adenosine moiety in the active-site crevice where the adenosine moiety of ATP binds. This is the first example of binding of an AP_n-PL at a nucleotide-binding site in an X-ray crystallographic structure.

EXPERIMENTAL PROCEDURES

Materials. Glutathione synthetase from *E. coli* B was overproduced in *E. coli* JM109 carrying an expression vector pKGS00 and was purified to homogeneity as described

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* Address correspondence to this author.
‡ Kyoto University.
§ Institute for Protein Research, Osaka University.
∥ Institute of Scientific and Industrial Research, Osaka University.

[1] Abbreviations: AP_n-PL, AP_2-PL, AP_3-PL, and AP_4-PL, adenosine-(5′)poly-, di-, tri-, and tetraphospho(5′)pyridoxal, respectively; PLP, pyridoxal 5′-phosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; HPLC, high-performance liquid chromatography.

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previously (Kato et al., 1988, 1989). The following materials were obtained commercially: ATP, NADH, pyruvate kinase, and lactate dehydrogenase (Oriental Yeast Co., Ltd., Tokyo); *Achromobacter lyticus* protease I (lysyl endopeptidase) (Wako Pure Chemical Co., Ltd., Osaka); Coomassie Protein Assay Reagent (Pierce Chemical Co., Ltd., Rockford, IL); bacteriophage M13mp19 (Takara Shuzo Co., Ltd., Kyoto); and a Capcell Pak C_{18} column (Shiseido Co., Ltd., Tokyo). γ-Glutamylcysteine was a special gift from Kojin Co., Ltd. (Tokyo). AP_n-PLs ($n = 2, 3, 4$) were synthesized as described previously (Tagaya & Fukui, 1986).

Protein and Enzyme Assays. The protein concentration of the glutathione synthetase was determined from the absorbance at 280 nm with $A^{1\%} = 9.02$ cm^{-1} in 50 mM potassium phosphate buffer, pH 7.0 (Kato et al., 1987). A protein assay method with the Coomassie reagent (Bradford, 1976) was used to measure the concentration of AP_n-PL-modified peptides.

Glutathione synthetase activity was spectrophotometrically measured by coupling the formation of ADP with pyruvate kinase and lactate dehydrogenase (Bergmeyer, 1983). The assay system contained 100 mM Tris-HCl buffer, pH 7.5, 5 mM γ-glutamylcysteine, 15 mM glycine, 10 mM Na_2ATP, 10 mM $MgCl_2$, 100 mM KCl, 1 mM phosphoenolpyruvate, 0.24 mM NADH, 5.6 units of pyruvate kinase, 25 units of lactate dehydrogenase, and an appropriate amount of glutathione synthetase in a total volume of 1 mL. The reaction was carried out at 37 °C, and the decrease in absorbance at 340 nm was monitored. One unit of the enzyme was defined as the amount that catalyzed the formation of 1 μmol of product/min at 37 °C.

Kinetics of Inactivation by AP_n-PL. All experiments were carried out at 20 °C in 50 mM HEPES, pH 7.8, containing 5 mM $MgSO_4$, unless otherwise stated. The enzyme (5 μM) was incubated with various concentrations of AP_n-PL ($n = 2$, 3, 4) in a total volume of 1 mL. For reactions in the absence of Mg^{2+} ion, Mg^{2+} was removed from the enzyme solution by dialysis before use. At different times, aliquots were withdrawn from the reaction mixtures and the reaction was terminated by the addition of 1/20 volume of freshly prepared 0.1 M sodium borohydride. The quenched mixtures were kept at 0 °C for 10 min and then used to measure enzyme activity and protein concentration. Control experiments without AP_n-PL were also done. Since the time course of inactivation by AP_n-PL deviated from the first-order kinetics, apparent equilibrium constants for inactivation (K^{app}) were calculated from the extent of inactivation of the enzyme (0.5 μM) measured at equilibrium (at 30 min after starting the inactivation reaction) with the various concentrations of either reagent and the equation:

$$I = I_{max}[AP_n\text{-PL}]/(I_{max}K^{app} + [AP_n\text{-PL}])$$

where $[AP_n\text{-PL}]$ is the concentration of ligand, I is the fraction of the inactive enzyme at equilibrium, and I_{max} is the fraction of the inactive enzyme at the saturating concentration of the reagent for inactivation. A nonlinear least-squares method (Marquardt, 1963) was used to fit the data.

Stoichiometry of Inactivation with AP_n-PL. The enzyme (29 μM) was incubated with various concentrations of AP_n-PL (29–290 μM) at 20 °C for 40 min in a total volume of 100 μL, and the reaction was terminated by the addition of 5 μL of 0.1 M sodium borohydride. The reaction mixture was kept on ice for 10 min and then centrifuged on a Sephadex G-25 column prepared with 1-mL pipet tips to remove free AP_n-PL (Penefsky, 1977). The remaining activity and the protein concentration in each effluent were measured. The amount of AP_n-PL incorporated into the enzyme was measured by fluorescence (excitation at 330 nm and emission at 395 nm) on a Hitachi fluorescence spectrophotometer MPF-4, after denaturation in 0.5 M Tris-HCl, pH 8.5, containing 10 mM EDTA and 6 M guanidine hydrochloride (Furukawa et al., 1990).

Identification of Labeled Residues. The enzyme (36 or 70 nmol) was labeled with 0.4 mM AP_3-PL or 40 μM AP_4-PL as described above. The labeled enzyme was dialyzed against 25 mM Tris-HCl buffer, pH 8.0, containing 8 M urea and 0.1% (w/v) EDTA overnight. After carboxymethylation (Hirs, 1967), the labeled protein was digested at 37 °C for 24 h with lysyl endopeptidase at a protease-to-substrate ratio of 1:(150–250) (w/w), in 25 mM Tris-HCl buffer, pH 9.0, containing 2 M urea. For the AP_4-PL-labeled enzyme, the digestion was repeated with V8 protease at 37 °C for 48 h in 100 mM phosphate buffer, pH 8.0, containing 1 mM EDTA. Peptide fragments were purified by reverse-phase HPLC with a Capcell Pak C_{18} column in 0.1% (v/v) trifluoroacetic acid (solvent A). A linear gradient of acetonitrile was developed over 48 min from 0% to 70% solvent B (10% solvent A in acetonitrile) at 1 mL/min. The absorbance of peptides was continuously monitored at 215 nm. The label was detected by the fluorescence emission at 395 nm with excitation at 330 nm. The multiple labeled peptides obtained in the first HPLC of AP_3-PL-modified enzyme (numbered II–VII in order of elution, see below) were further purified to homogeneity by repeating reverse-phase HPLC under similar conditions but with narrower concentration ranges of acetonitrile gradient (48-min linear gradients of 5–35% solvent B for peak II peptide; 15–45% solvent B for peak III peptide; 25–55% solvent B for peak IV and V peptides; and 30–60% solvent B for peak VI and VII peptides). N-terminal amino acid sequencing of the labeled peptides was done with a gas-phase sequencer (Applied Biosystems Model 477A) linked with a phenylthiohydantoin analyzer (Applied Biosystems Model 120A).

Preparation of Crystals Soaked with AP_4-PL and X-ray Diffraction Analysis. Native crystals of the enzyme were prepared by microdialysis against 27% saturated ammonium sulfate in 50 mM potassium phosphate buffer, pH 6.0, containing 5 mM $MgCl_2$, as reported previously (Kato et al., 1989). The native crystals were immersed for 3 days in 50 mM potassium phosphate buffer, pH 6.0, containing 5 mM AP_4-PL, 40% saturated ammonium sulfate, 10 mM $MgSO_4$, 10 mM 2-mercaptoethanol, and 0.02% (w/v) NaN_3. Diffraction data were collected on a computer-controlled Rigaku C-5 four-circle diffractometer, with Ni-filtered CuKα radiation from a Rigaku RU300 rotating-anode X-ray generator (40 kV, 300 mA). Intensity data sets were merged and scaled according to the method of Hamilton et al. (1965).

RESULTS

Inactivation of Glutathione Synthetase with AP_n-PL. The purified glutathione synthetase was incubated with one of the AP_n-PLs ($n = 2, 3, 4$) or with PLP and was then reduced with sodium borohydride. There was substantial inactivation of the enzyme, particularly with AP_4-PL. At the same concentration (180 μM), AP_2-PL, AP_3-PL, and PLP inactivated the enzyme more slowly than AP_4-PL did and the extents of inactivation at equilibrium (after about 10 min) were much less than that with AP_4-PL (Figure 1). Incubation with AP_4-PL under these conditions caused the enzyme to lose its activity within 2 min. The inactivation was reversible before reduction but not after; i.e., there was no recovery of activity after removal

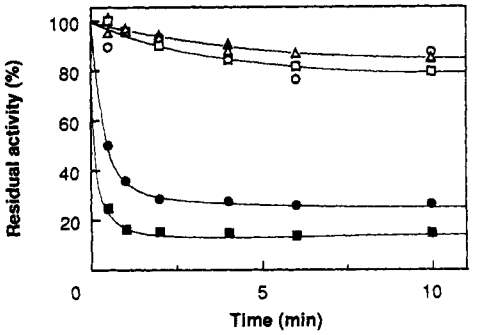

FIGURE 1: Time courses of inactivation of glutathione synthetase by AP_n-PL and PLP. The inactivation mixtures (1 mL) containing 5.0 μM enzyme, 50 mM HEPES (pH 7.8), 5 mM $MgSO_4$, and 180 μM PLP (△), 180 μM AP_2-PL (▲), 180 μM AP_3-PL (□), and 180 μM AP_4-PL (■) were incubated at 20 °C. In the presence (●) or absence (○) of $MgSO_4$, experiments with 40 μM AP_4-PL were also done. At the indicated times, aliquots of the mixture (0.1 mL) were withdrawn, reduced with 5 mM sodium borohydride, and diluted 100 times with 100 mM Tris-HCl, pH, 7.5, followed by the measurements of the remaining activity.

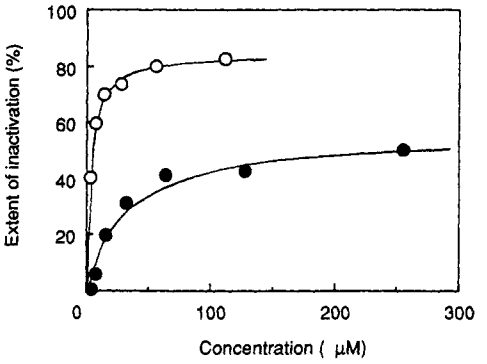

FIGURE 2: Effect of concentrations of AP_3-PL and AP_4-PL on the inactivation of glutathione synthetase. The reaction mixtures (400 μL) containing 50 mM HEPES, pH 7.8, 0.5 μM enzyme, and an indicated concentration of AP_4-PL (○) or AP_3-PL (●) were incubated at 20 °C for 30 min. The percentage extents of inactivation obtained from the remaining activity were plotted against concentrations of either reagent and analyzed as described under Experimental Procedures. The curves were obtained by the nonlinear least-squares best fit of the experimental data (Marquardt, 1963).

of the excess reagent by dialysis and coincubation with 10 mM ATP (data not shown). The inactivation by these reagents did not reach the complete inactivation in any case. However, when the inactivated enzyme with about 30% activity remaining was treated, after dialysis, again with 40 μM AP_4-PL and reduced with borohydride, the enzyme was further inactivated to a level less than 10% of the initial activity. These results show that the enzyme was inactivated through reversible formation of a Schiff base between amino group(s) of the enzyme and the aldehyde group of the reagent according to the reaction scheme (Fayat et al., 1979):

$$E + AP_n\text{-PL} \overset{K_d}{\rightleftharpoons} E{\cdot}AP_n\text{-PL} \overset{K}{\rightleftharpoons} E\text{–}AP_n\text{-PL}$$

In fact, the enzyme solution added with either reagent (AP_3-PL or AP_4-PL) exhibited a new absorption peak at 440 nm (with AP_3-PL) or at 432 nm (with AP_4-PL) due to the Schiff base formed. The increase in absorbance at these peaks reached a plateau at equilibrium within 5 min (with AP_4-PL) and 30–40 min (with AP_3-PL) (data not shown). The remaining activity at equilibrium depended on the concentrations of AP_3-PL and AP_4-PL (Figure 2). From the percentage of maximum inactivation at saturating concentrations of the reagents, the Schiff base equilibrium constants

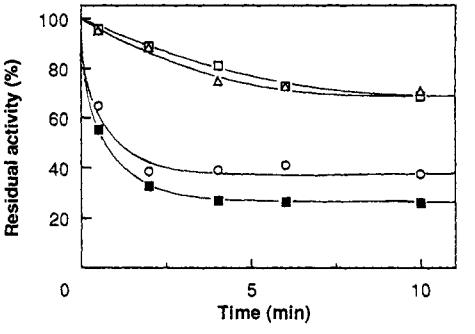

FIGURE 3: Effect of substrates on the inactivation of glutathione synthetase by AP_4-PL. The inactivation mixtures (1 mL) containing 50 mM HEPES, pH 7.8, 5.3 μM enzyme, and 40 μM AP_4-PL (■), plus 2 mM ATP (△), 2 mM γ-glutamylcysteine (□), or 7.5 mM glycine (○), were incubated at 20 °C. At the indicated times, aliquots of the mixture (0.1 mL) were withdrawn, reduced with 5 mM sodium borohydride, and diluted 100 times with 100 mM Tris-HCl, pH 7.5. Then, the remaining activity was assayed.

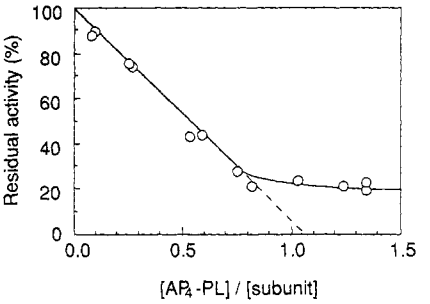

FIGURE 4: Relationship between inactivation of glutathione synthetase by AP_4-PL and the amount of label incorporated into the monomer of the tetrameric enzyme. Experimental details are given in the text.

(K = [E–AP_n-PL]/[E·AP_n-PL]) were calculated to be 1.4 for AP_3-PL and 5.7 for AP_4-PL. Since the apparent equilibrium constants for inactivation ($K^{app} = K_d/K$ = [E][AP_n-PL]/[E–AP_n-PL]), determined from the saturation curves (Figure 2), were 58 μM for AP_3-PL and 4.0 μM for AP_4-PL, the K_d values of AP_3-PL and AP_4-PL in the inactivation process were deduced to be about 81 and 23 μM, respectively. These results suggest that the efficient inactivation by AP_4-PL is brought about by the high affinity of AP_4-PL for the enzyme in combination with the equilibrium of its reaction in favor of Schiff base formation with the enzyme.

The effect of substrates on inactivation of the enzyme by 40 μM AP_4-PL was examined (Figure 3). When ATP (2 mM) or 2 mM γ-glutamylcysteine was added, there was little inactivation, but glycine, another substrate of the enzyme, had almost no such effect even at 7.5 mM. The effect of ATP or γ-glutamylcysteine was accompanied by a decrease in the amount of label incorporated into the enzyme (see below). It should be also noted that there was almost no specific inactivation by AP_4-PL when Mg^{2+} was omitted from the reaction mixture (Figure 1).

Stoichiometry of Inactivation. The relationship between inactivation of the enzyme by AP_4-PL and incorporation of the reagent into the enzyme was studied by incubating the enzyme with AP_4-PL at various concentrations (29–290 μM), reducing it with sodium borohydride, and then removing free AP_4-PL by centrifugal gel filtration (Penefsky, 1977). For each fraction that passed through, the remaining activity was measured and plotted against the amount of AP_4-PL per subunit of the tetrameric enzyme (Figure 4). Extrapolation of the linear portion of the plot to zero gave an x-axis intercept of about 1.05 mol of AP_4-PL/mol of enzyme subunit

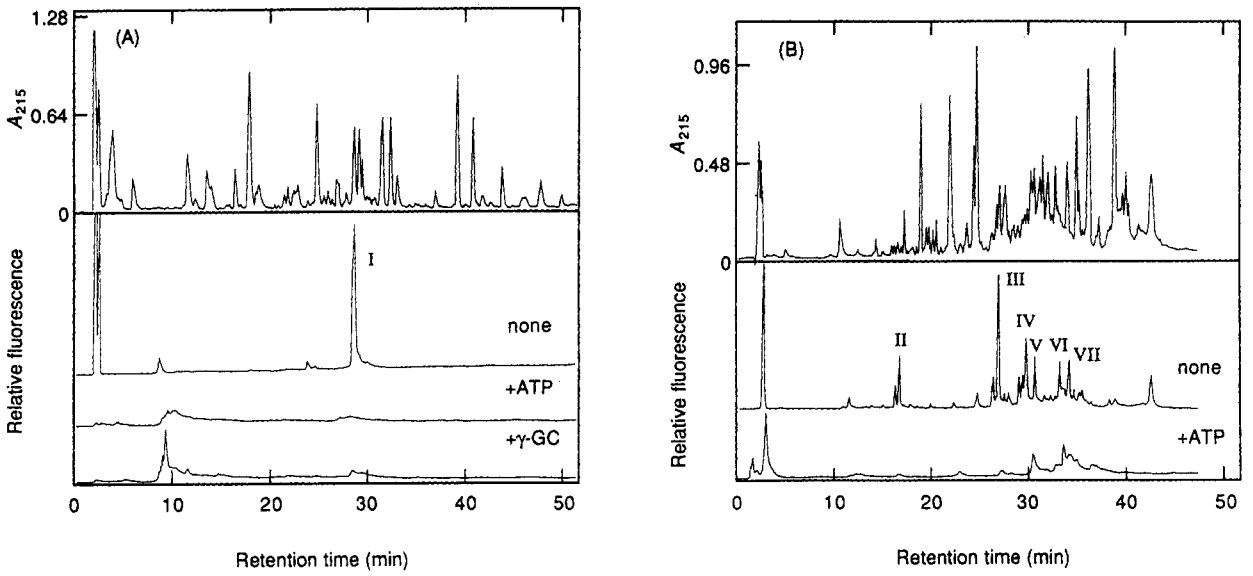

FIGURE 5: High-performance liquid chromatography of the AP_4-PL-labeled (A) and AP_3-PL-labeled (B) peptides of glutathione synthetase. The labeled enzyme was digested with lysyl endopeptidase (and also V8 protease for the AP_4-PL-labeled enzyme) and was applied to a C_{18} column, as described in Experimental Procedures. Absorbance at 215 nm (upper panels) and fluorescence (lower panels) were monitored. The single dominant peak of the AP_4-PL-labeled peptides and the six main peaks of the AP_3-PL-labeled peptides were numbered I and II–VII, respectively. Each of the peaks was further purified to homogeneity by HPLC. In the experiment shown in (A), the enzyme was labeled with 40 μM AP_4-PL in the absence (none) or presence of 10 mM MgATP (+ATP) or 10 mM γ-glutamylcysteine (+γ-GC). In the experiment shown in (B), the enzyme was labeled with 0.4 mM AP_3-PL in the absence (none) or presence (+ATP) of 10 mM MgATP.

Table I: Amino Acid Sequences of the AP_4-PL- and AP_3-PL-Labeled Peptides of Glutathione Synthetase

peptide	amino acid sequence[a]	labeled residue
I	XDSSFAMLLE	Lys-18
II	TRNXAQLK	Lys-144
III-1	XDSSFAMLL	Lys-18
III-2	AQLXAFWEK	Lys-148
IV	LGIVMDPIAN INIXK	Lys-17
V	not determined	–
VI	XDSSFAMLLE AQRRGY···	Lys-18
VII	LFTAWFSDLT PETLVTRNXA QLK	Lys-144

[a] The underlined "X" represents the labeled lysine. The sequence of the peak VI peptide was determined up to the 16th cycle.

(monomer), which suggests specific labeling of the enzyme by the reagent. In contrast, a similar experiment with AP_3-PL at 3–20 times higher concentrations than its K_d value (see above) gave about 1.5 mol of the incorporated label/mol of subunit, with more than 50% of the initial activity retained (data not shown).

Identification of Labeled Sites. To identify the lysyl residue or residues labeled by AP_3-PL or AP_4-PL, the enzyme was modified with 40 μM AP_4-PL or 0.4 mM AP_3-PL (a concentration much higher than its K_d value was used to achieve extensive labeling), then carboxymethylated, and cleaved with lysyl endopeptidase (and then with V8 protease for AP_4-PL-labeled enzyme). The digests were separated on a reverse-phase column, as described above under Experimental Procedures. For the AP_4-PL-labeled enzyme, a single dominant fluorescence peak (peak I) was eluted from the reverse-phase column (Figure 5A). The peak I peptide was further purified, and the amino acid sequence was determined with an automated protein sequencer (Table I). Since the phenylthiohydantoin derivative of an AP_n-PL-modified lysine is undetectable in the sequence analysis (Hountondji et al., 1990; Tagaya et al., 1987), we concluded that the residue of cycle 1 was the labeled lysine. The following sequence of the peak I peptide corresponds to the sequence from Asp-19 to Glu-27 in the complete sequence of *E. coli* glutathione synthetase (Gushima et al., 1984). Thus, Lys-18 was identified as the AP_4-PL-labeled amino acid residue. In an HPLC analysis of the enzyme that had been labeled with AP_4-PL in the presence of 10 mM ATP, no fluorescence was detected for the digests (Figure 5A), which confirms that AP_4-PL specifically labeled Lys-18 and that this labeling was prevented by ATP. The addition of 10 mM γ-glutamylcysteine also prevented considerably the incorporation of the label into Lys-18 (Figure 5A), in agreement with its protection of the enzyme from inactivation by AP_4-PL as described above.

In contrast, there were multiple fluorescence peaks in the chromatogram of the AP_3-PL-labeled and digested enzyme (Figure 5B). Six major peaks were designated II–VII, in order of elution. After each of these peaks was purified by repeated HPLC on a reverse-phase column as described under Experimental Procedures, their sequences were determined as summarized in Table I, except for peak V. The peak V material proved to be a mixture of labeled peptides, but the amounts were insufficient for sequence determination. Rechromatography revealed that the peak III material contained two different peaks, designated III-1 and III-2. Peak II and III-1 peptides were a part of peak VII and VI peptides, respectively, and were probably derived from aberrant digestion by a nonspecific protease contaminating the commercial lysyl endopeptidase. The residues labeled with AP_3-PL were identified as Lys-17, Lys-144, and Lys-148 in addition to Lys-18 that was specifically labeled with AP_4-PL. Note that none of the isolated peptides contained doubly labeled residues at Lys-17 and Lys-18, or at Lys-144 and Lys-148 (Table I).

Although the relative amounts of the labels in these four residues are unknown (because aberrant digestion produced different peptides containing the same labeled residue, and because of incomplete separation in the first HPLC), the sum of their labels appeared to account for more than 70% of the

FIGURE 6: A stereoview of the location and orientation of AP_4-PL bound to the enzyme crystal. The $|F_{complex}| - |F_{native}|$ difference Fourier map was calculated with multiple isomorphous replacement phases of the native enzyme (Yamaguchi et al., in press). Positive electron densities contoured at 3σ are superimposed on the Cα backbone structure of a subunit of the enzyme. The side chain of Lys-18 (thick line) and the γ-glutamylaminobutyrate-binding site (star mark) are also indicated.

total label in the enzyme. Other minor fluorescence peaks, which accounted for about 25% of the total label, remained unidentified. In accord with the considerable amount of AP_3-PL incorporated into the enzyme with a high activity remaining (see above), these results suggest nonspecific labeling by AP_3-PL of multiple lysyl residues in the enzyme. The multiple residues might have been modified because the concentration of AP_3-PL used to identify the labeled sites (0.4 mM) was higher than that of AP_4-PL (40 μM). However, there were multiple fluorescence peaks even with 40 μM AP_3-PL (data not shown). In addition, all the peaks were similarly lower in the chromatogram of the enzyme labeled with 0.4 mM AP_3-PL in the presence of 10 mM ATP (Figure 5B), which suggests that AP_3-PL also has an affinity for the ATP-binding site.

Difference Fourier Analysis of AP_4-PL-Binding Site. To identify more directly the binding site for AP_4-PL, we used the difference Fourier method to analyze the location and orientation of AP_4-PL bound to the enzyme and confirmed the binding site in the ligand-free enzyme structure determined recently to 2.0-Å resolution (Yamaguchi et al., in press). Crystals of the native enzyme were immersed in buffered ammonium sulfate solution (40% saturation) containing 5 mM AP_4-PL and were studied by X-ray diffraction. Diffraction data were collected to 2.7-Å resolution and merged with an R_{merge} of 4.78% for 10 989 independent reflections. The soaked crystal was isomorphous to the crystal of AP_4-PL-free enzyme, with unit-cell dimensions of $a = b = 88.3$ Å and $c = 164.0$ Å. The difference Fourier map was calculated with the native protein phases derived from multiple isomorphous replacement techniques (Yamaguchi et al., in press). The resulting map (2.7-Å resolution) showed a large lobe of electron density, between two β-sheets in the putative active-site cleft (Figure 6). The location and the shape of this lobe correspond to those of the adenosine moiety of ATP, as determined by a similar analysis with the crystals soaked with ATP (Yamaguchi et al., in press). Therefore, we conclude that the adenosine moiety of AP_4-PL binds to the nucleotide-binding site of the enzyme. In contrast, the location of the pyridoxyl moiety of the reagent is not known; the electron density lobe was expected to appear around Lys-18. This is probably because the AP_4-PL–enzyme complex was not reduced with sodium borohydride, and excess ammonium sulfate in the soaking buffer might have prevented the formation of the Schiff base between the reagent and Lys-18 in the crystal. However, the distance between the ϵ-amino group of Lys-18 and the adenosine moiety of the bound AP_4-PL (about 13 Å) is almost equal to the chain length of the tetraphosphoryl group in the reagent.

DISCUSSION

Affinity labeling studies with a series of nucleotidyl derivatives of PLP have revealed the presence of a highly reactive lysyl residue at the nucleotide-binding site in some proteins, including rabbit muscle adenylate kinase (Tagaya et al., 1987; Yagami et al., 1988), the F_1 of *E. coli* H^+-ATPase (Noumi et al., 1987), and the Ha-*ras* oncogene product p21 (Ohmi et al., 1988). The lysyl residue uniquely labeled by the reagents is contained in a glycine-rich sequence, Gly-X-X-X-X-Gly-Lys-(Thr/Ser) (where X represents an unspecified residue), which is conserved in various ATP- and GTP-binding proteins (Walker et al., 1982). Since the glycine-rich sequence has a loop structure in these proteins and plays a crucial role in binding of adenine and guanine nucleotides, it is also called a "phosphate-binding loop (P-loop)" (Saraste et al., 1990). Although glutathione synthetase is a member of the protein group requiring ATP as a substrate, no sequence similar to the P-loop has been detected in the primary structure. Moreover, the disordered loop (residues 226–241) contains two arginine residues instead of lysine, though it is also glycine-rich and is indispensable for catalytic functions of the enzyme (Tanaka et al., 1992). These structural features of glutathione synthetase led us to examine whether or not the enzyme is inactivated by AP_n-PLs and, if so, which lysyl residue is labeled by the reagents.

The present results show that AP_4-PL has a very high affinity for the enzyme (K_d = 23 μM), rapidly inactivates it in the presence of Mg^{2+}, and labels Lys-18 specifically. The degree of inactivation by AP_4-PL is stoichiometrically correlated with the amount of label incorporated into the enzyme protein. Both the inactivation of the enzyme and the labeling of Lys-18 by AP_4-PL can be prevented by either ATP or γ-glutamylcysteine. These results show that AP_4-PL is indeed an affinity labeling reagent for *E. coli* B glutathione synthetase.

In marked contrast to AP_4-PL, AP_3-PL inactivates the enzyme less efficiently, while multiple lysyl residues, including Lys-18, are labeled. Such a difference between the actions of two reagents with different numbers of phosphoryl groups has been observed rather infrequently in previous affinity labeling studies. For example, adenylate kinase is inactivated equally by all AP_n-PLs with concomitant labeling of the same lysyl residue, Lys-21 (Yagami et al., 1988). With H^+-ATPase, both AP_3-PL and AP_4-PL bind to and inactivate the enzyme, but AP_2-PL does so only slightly (Noumi et al., 1987). However, for efficient binding and inactivation of glutathione synthetase, the tetraphosphoryl linkage in AP_4-PL is necessary.

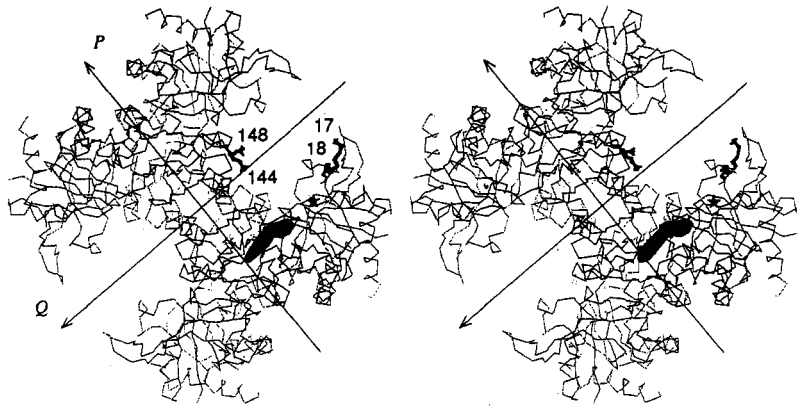

FIGURE 7: Schematic drawing of a tetrameric structure of the glutathione synthetase. There are two large inner-solvent regions between the tightly associated dimers, and the putative active site is at the bottom of these regions. The side chains of Lys-17, Lys-18, Lys-144, and Lys-148 (thick lines) are indicated around the mouth of one of the inner-solvent regions. The bound ATP (dark area) and the γ-glutamylaminobutyrate-binding site (star mark) are also indicated.

Furthermore, all the sequences around the lysyl residues labeled by AP_3-PL and AP_4-PL are not homologous with that of the P-loop (see Table I). Therefore, the structure of the ATP-binding site of glutathione synthetase probably differs from those of other nucleotide-binding proteins containing the P-loop.

Results of X-ray crystallographic analysis of *E. coli* B glutathione synthetase in complex with ATP have suggested that the nucleotide-binding site is located in the crevice between the central and C-terminal domains (Yamaguchi et al., in press). In the difference Fourier map between the free and AP_4-PL-soaked enzymes (Figure 6), there is a large lobe of electron density in this crevice. This lobe can be attributed to the adenosine moiety of the bound AP_4-PL, and it occupies the same place as the bound ATP. Thus, we conclude that the adenosine moiety of AP_4-PL binds at the nucleotide-binding site. In contrast, γ-glutamylcysteine appears to bind in the crevice between the N-terminal and central domains opening to the nucleotide-binding crevice, as indicated by the difference Fourier analysis of the enzyme crystals soaked with γ-L-glutamyl-α-L-aminobutyrate, a substrate analog (Yamaguchi et al., in press). According to the crystallographic structure of the unliganded enzyme, the Lys-18 that is labeled specifically by AP_4-PL is in a loop connecting the end of the first β-strand to the first α-helix in the N-terminal domain. This loop adjacent to the central domain is part of the putative γ-glutamylcysteine-binding crevice mentioned above. Therefore, Lys-18 is probably located at or near the γ-glutamylcysteine-binding site rather than at the nucleotide-binding site. This is supported by our preliminary finding that replacement of Lys-18 by Ala increases the Michaelis constants for amino acid substrates more significantly than it increases the constant for ATP (T. Hibi, H. Kato, T. Nishioka, and J. Oda, unpublished results). Consequently, AP_4-PL binds bivalently to the enzyme. Its adenosine moiety binds to the nucleotide-binding site, and the pyridoxyl moiety binds to the γ-glutamylcysteine-binding site, forming a Schiff base with the ε-amino group of Lys-18. The distance between the amino group of Lys-18 and the electron density lobe, which is caused by the adenosine moiety of the bound AP_4-PL, is about 13 Å, which coincides with the chain length of the tetraphosphoryl group in the reagent. Because of this binding, either ATP or γ-glutamylcysteine can protect the enzyme from inactivation by AP_4-PL.

The K_d value (81 μM) of the less efficient AP_3-PL determined from the inactivation process is not very much larger than that of AP_4-PL (23 μM) and is considerably smaller than the apparent K_m value for ATP (240 μM) (Tanaka et al., 1992). However, modification of the enzyme by AP_3-PL resulted in labeling of multiple lysyl residues: Lys-17, Lys-144, and Lys-148, in addition to Lys-18. Since the enzyme that was labeled with more than 1 mol of AP_3-PL/mol of subunit retained more than 50% of its initial activity, the labeling of Lys-17, Lys-144, and Lys-148 probably does not lead to inactivation. However, the addition of ATP almost completely retarded the incorporation of label into all of these lysyl residues (Figure 5B), suggesting that the AP_3-PL-labeled lysyl residues other than Lys-18 are also situated in the vicinity of the ATP-binding site. The labeling of multiple lysyl residues by AP_3-PL is probably due to their locations as described below and the less favored equilibrium ($K = 1.4$) of the Schiff base formation between AP_3-PL and the amino group of Lys-18.

In the crystal structure of the enzyme consisting of four identical subunits arranged around three orthogonal dyad axes (*P*, *Q*, *R*), two subunits are in contact with each other and form a tightly associated dimer, and two of the dimers facing on the *QR* plane form a tetrameric molecule (Yamaguchi et al., in press). Two large solvent regions are formed between them only in the tetrameric structure (Figure 7). The inner-solvent regions are open to the surface of the molecule on only one side of each region, and the nucleotide-binding crevice is at the bottom of the region. Since Lys-17, Lys-144, and Lys-148 labeled by AP_3-PL are all located in the mouth of this inner-solvent region (Figure 7), it is likely that AP_3-PL is trapped by them when it enters and leaves the active site through the inner-solvent region. Because the Schiff base equilibrium constant of AP_3-PL is close to unity, AP_3-PL may frequently pass through this region at equilibrium. In other words, the multiple positive charges provided by Lys-17, Lys-144, and Lys-148 may play a role in attracting the negatively charged ATP and AP_n-PLs to the active site.

REFERENCES

Bergmeyer, H. U. (1983) in *Methods of Enzymatic Analysis* (Bergmeyer, J., & Grassl, M., Ed.) Vol. 1, pp 114–142, Verlag Chemie GmbH, Weinheim, Germany.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Colman, R. F. (1990) in *The Enzymes* (Boyer, P. D., Ed.) Vol. 17, pp 283–321, Academic Press, New York.

Fayat, G., Hountondji, C., & Blanquet, S. (1979) *Eur. J. Biochem. 96*, 87–92.

Furukawa, K., Tagaya, M., Inouye, M., Preiss, J., & Fukui, T. (1990) *J. Biol. Chem. 265*, 2086–2090.

Gushima, H., Miya, T., Murata, K., & Kimura, A. (1983) *J. Appl. Biochem. 5*, 210–218.
Gushima, H., Yasuda, S., Soeda, E., Yokota, M., Kondo, M., & Kimura, A. (1984) *Nucleic Acids Res. 12*, 9299–9307.
Hamilton, W. C., Rollet, J. S., & Sparks, R. A. (1965) *Acta Crystallogr. 18*, 129–130.
Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 199–203.
Hountondji, C., Schmitter, J.-M., Fukui, T., Tagaya, M., & Blanquet, S. (1990) *Biochemistry 29*, 11266–11273.
Kato, H., Chihara, M., Nishioka, T., Murata, K., Kimura, A., & Oda, J. (1987) *J. Biochem. (Tokyo) 101*, 207–215.
Kato, H., Tanaka, T., Nishioka, T., Kimura, A., & Oda, J. (1988) *J. Biol. Chem. 263*, 11646–11651.
Kato, H., Yamaguchi, H., Hata, Y., Nishioka, T., Katsube, Y., & Oda, J. (1989) *J. Mol. Biol. 209*, 503–504.
Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431–441.
Meister, A. (1974) in *The Enzymes* (Boyer, P. D., Ed.) Vol. 10, pp 671–697, Academic Press, New York.
Meister, A. (1985) *Methods Enzymol. 113*, 393–399.
Noumi, T., Tagaya, M., Miki-Takeda, K., Maeda, M., Fukui, T., & Futai, M. (1987) *J. Biol. Chem. 262*, 7686–7692.
Ohmi, N., Hoshino, M., Tagaya, M., Fukui, T., Kawakita, M., & Hattori, S. (1988) *J. Biol. Chem. 263*, 14261–14266.
Oppenheimer, L., Wellner, V. P., Griffith, O. W., & Meister, A. (1979) *J. Biol. Chem. 254*, 5184–5190.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
Saraste, M., Sibbald, P. R., & Wittinghofer, A. (1990) *Trends Biochem. Sci. 15*, 430–434.
Tagaya, M., & Fukui, T. (1986) *Biochemistry 25*, 2958–2964.
Tagaya, M., Yagami, T., & Fukui, T. (1987) *J. Biol. Chem. 262*, 8257–8261.
Tanaka, T., Kato, H., Nishioka, T., & Oda, J. (1992) *Biochemistry 31*, 2259–2265.
Walker, J. E., Saraste, M., Runswick, M. J., & Gay, N. J. (1982) *EMBO J. 1*, 945–951.
Yagami, T., Tagaya, M., & Fukui, T. (1988) *FEBS Lett. 229*, 261–264.
Yamaguchi, H., Kato, H., Hata, Y., Nishioka, T., Katsube, Y., Oda, J., & Kimura, A. (1990) *Acta Crystallogr. A46*, C132.
Yamaguchi, H., Kato, H., Hata, Y., Nishioka, T., Kimura, A., Oda, J., & Katsube, Y. (1993) *J. Mol. Biol.* (in press).