Photoaffinity Analogues of Methotrexate as Folate Antagonist Binding Probes. 1. Photoaffinity Labeling of Murine L1210 Dihydrofolate Reductase and Amino Acid Sequence of the Binding Region†

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ABSTRACT: N^{α}-(4-Amino-4-deoxy-10-methylpteroyl)-N^{ϵ}-(4-azido-5-[^{125}I]iodosalicylyl)-L-lysine, a photoaffinity analogue of methotrexate, is only 2-fold less potent than methotrexate in the inhibition of murine L1210 dihydrofolate reductase. Irradiation of the enzyme in the presence of an equimolar concentration of the ^{125}I-labeled analogue ultimately leads to an 8% incorporation of the photoprobe. A 100-fold molar excess of methotrexate essentially blocks this incorporation. Cyanogen bromide digestion of the labeled enzyme, followed by high-pressure liquid chromatography purification of the generated peptides, indicates that greater than 85% of the total radioactivity is incorporated into a single cyanogen bromide peptide. Sequence analysis revealed this peptide to be residues 53–111, with a majority of the radioactivity centered around residues 63–65 (Lys-Asn-Arg). These data demonstrate that the photoaffinity analogue specifically binds to dihydrofolate reductase and covalently modifies the enzyme following irradiation and is therefore a photolabeling agent useful for probing the inhibitor binding domain of the enzyme.

The chemotherapeutic action of methotrexate (MTX),[1] a 4-amino analogue of the vitamin folic acid, depends on the ability of the compound to bind and inhibit dihydrofolate reductase (DHFR).[1] A wealth of information has been generated dealing with the molecular basis of MTX binding. X-ray crystallographic data have been obtained in studies of inhibitor binding to DHFR from *Escherichia coli* (Matthews et al., 1977; Bolin et al., 1982), *Lactobacillus casei* (Matthews et al., 1978), and chicken liver (Volz et al., 1982; Matthews et al., 1985). These data indicate that the α-carboxylate moiety of MTX plays an important role in binding by a charge interaction between this group and an invariant arginine (Arg-70 in L1210) residue of the enzyme. Past studies with α- and γ-carboxyl-modified MTX derivatives further indicate the essential role of a free α-carboxylate in MTX binding to DHFR (Montgomery et al., 1979; Piper et al., 1982; Rosowsky et al., 1981). These studies also showed that modifications of the γ-carboxyl group of MTX did not significantly alter the drug's binding to DHFR. We have synthesized lysine and ornithine analogues of MTX that possess a free α-carboxyl group as well as a terminal, modifiable amino group (Kempton et al., 1982; Kumar et al., 1983a). Both compounds are potent inhibitors of DHFR in spite of the replacement of a carboxyl group by an amino group. Furthermore, fluorescent dansyl derivatives of the lysine and ornithine analogues have been synthesized (Kumar et al., 1983a, 1983b) and are also good inhibitors of DHFR despite the presence of the bulky dansyl group.

Although many binding interactions between MTX and DHFR have been elucidated by X-ray crystallography and studies with MTX derivatives, few attempts have been made to characterize the product of an affinity-labeled enzyme. Diaminopteridineglyoxal has been used to covalently modify bacterial DHFR via the guanido group of the invariant arginine (vide supra) (Johanson & Henkin, 1985). Identification of the modified residue(s) was unsuccessful due to the lability of the covalent adduct. Subsequently, a radioactive 4,6-diaminodihydrotriazine with a terminal sulfonyl fluoride moiety was used to label avian DHFR, and tyrosine-31 was unambiguously identified as the site of covalent attachment of the antifolate affinity label (Kumar et al., 1981).

Photogenerated affinity labels present a different approach to the study of biological systems. The use of photoaffinity analogues allows the study of reversible, noncovalent as well as irreversible, covalent interactions between a specific ligand and its receptor. The reactive nature of such compounds allows for the generation of a highly reactive nitrene species that will covalently modify otherwise unreactive amino acids [for a review, see Chowdhry and Westheimer (1979)]. A photoaffinity analogue of MTX, 2-azidoaminopterin, has been synthesized (Holmes et al., 1982) and used to label *E. coli* DHFR. However, the efficiency with which covalent incorporation occurred was only 0.5%. The present study describes the photoaffinity labeling of murine L1210 DHFR with N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-N^{ϵ}-(4-azido-5-[^{125}I]iodosalicylyl)-L-lysine and identification of the labeled residues. The structure of this compound is indicated in Figure 1. A

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[1] Abbreviations: MTX, methotrexate [*N*-(4-amino-4-deoxy-10-methylpteroyl)glutamic acid]; DHFR, dihydrofolate reductase (EC 1.5.1.3); TFA, trifluoroacetic acid; APA-ASA-Lys, N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-N^{ϵ}-(4-azidosalicylyl)-L-lysine; APA-[^{125}I]ASA-Lys, N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-N^{ϵ}-(4-azido-5-[^{125}I]iodosalicylyl)-L-lysine; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; PTH, phenylthiohydantoin; ATZ, anilinothiazolinone; AUFS, absorbance units full scale.

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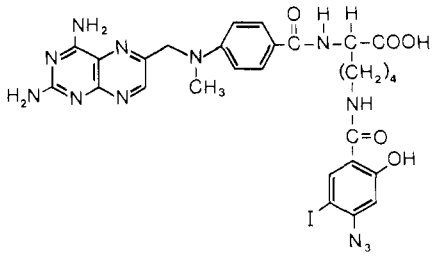

N^{α}-(4-AMINO-4-DEOXY-10 METHYLPTEROYL)-
N^{ϵ}-(4 -AZIDO-5 -IODO SALICYLYL)-L-LYSINE

APA-[I]ASA-LYS

FIGURE 1: Structure of APA-[^{125}I]ASA-Lys.

preliminary account of this work has been reported elsewhere (Price et al., 1986a).

Materials and Methods

Reagents. Cell culture products were purchased from KC Biologicals (Lenexa, KS). Sephadexes and AH-Sepharose 4B were obtained from Pharmacia (Piscataway, NJ), and Bio-Gel HTP (hydroxylapatite) was from Bio-Rad Laboratories (Richmond, CA). ^{125}I (as Na^{125}I) was obtained from Amersham (Arlington Heights, IL). Cyanogen bromide, Iodobeads, and sequanal grade TFA were purchased from Pierce Chemical Co. (Rockford, IL). HPLC-grade 1-propanol was purchased from Burdick and Jackson Laboratories (Muskegon, MI). Protein sequencer chemicals were supplied by Applied Biosystems (Foster City, CA). All other chemicals were of the highest quality commercially available.

Cells. MTX-resistant cells (L1210/R81) with a 35-fold elevation of DHFR were initially provided by Dr. R. C. Jackson (Warner-Lambert, Ann Arbor, MI) and were maintained in 10 μM MTX. Prior to harvesting, the cells were passed for three transfer generations (12 doublings) in MTX-free media in order to eliminate MTX bound to DHFR.

Enzyme Purification. DHFR was purified by MTX affinity chromatography as previously described (Price et al., 1986b). The purified enzyme migrated as a single species on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The enzyme was stored at −20 °C and was stable for several months.

Iodination. The following steps were performed in very subdued light. Approximately 200 μg of APA-ASA-Lys[1] was dissolved in 60 μL of dimethylformamide. The synthesis of APA-ASA-Lys was carried out as previously described (Price et al., 1986b). ^{125}I (1 mCi) (as 5 μL of Na^{125}I in dilute NaOH) was added, followed by the addition of two Iodobeads. After 2 min, the iodinated product was applied to a 4 × 20 cm analytical, fluorescent TLC sheet (silica gel) and developed in 3:1:1 *i*-PrOH/MeOH/NH_4OH. The product was visualized via autoradiography and UV fluorescence quenching and had a specific radioactivity of ca. 2 Ci/mmol. The iodinated compound was eluted from the silica gel with 0.1 M potassium phosphate, pH 7.5.

Photoaffinity Labeling of DHFR. L1210 DHFR (95 nmol) in 0.1 M potassium phosphate, pH 7.5, was incubated with an equimolar amount of APA-[^{125}I]ASA-Lys at 4 °C for 5 min in subdued light. The solution was then transferred to a quartz cuvette and irradiated with a long-wave UV lamp (Spectroline Model B-100, Spectronics Corp., Westbury, NY, 15 mW/cm^2) for 1 min. The solution was then made 5% in β-mercaptoethanol and 6 M in urea sequentially. After incubation for 1 h at 70 °C, the labeled, denatured enzyme was applied to a 1.4 × 38 cm Sephadex G-25 column, and elution was achieved with 50 mM NH_4HCO_3/6 M urea, pH 9.5. The single protein peak that coeluted with radioactivity was pooled, exhaustively dialyzed against several changes of H_2O, and lyophilized. A protection experiment was performed similarly to the above method, except that a 100-fold molar excess of MTX was preincubated with the enzyme for 5 min prior to the addition of the radiolabeled photoaffinity analogue.

Cyanogen Bromide Digestion of Labeled DHFR. A 2-fold (w/w) excess of cyanogen bromide was added to the lyophilized, labeled protein as a 3-mL aliquot of 70% formic acid. After 12 h, the solution was diluted 10-fold with ice-cold H_2O and lyophilized several times. The cyanogen bromide generated peptides were chromatographed on a reversed-phase C3 HPLC column by use of a Waters Model 720 controller and Model 510 pumps, a Rheodyne injector, and a linear gradient of 1-propanol/0.1% TFA. The peak that contained radioactivity was pooled and lyophilized. This material was designated CB-3.

Peptide Sequencing. Automated Edman degradations were performed with an Applied Biosystems Model 470A gas-phase protein sequencer and a Beckman 890C spinning cup sequencer. For the gas-phase sequence analysis, the radiolabeled cyanogen bromide fragment (CB-3) was sequenced according to the standard program 02NVAC from the manufacturer. The phenylthiohydantoin amino acid derivatives recovered from the sequencer were dried under vacuum in a Savant Speed-Vac and redissolved in 40 μL of acetonitrile/methanol (1:1). Appropriate aliquots were taken for HPLC injections and radioactivity determinations. Phenylthiohydantoin amino acid derivative separation was accomplished according to the protocol devised by Touchstone (1983). The system consists of a Waters gradient HPLC station comprised of two Model 510 pumps, a Model 710B WISP, a Waters temperature control module, a Model 440 dual wavelength detector, and a Waters 840 data and chromatography station for system control, data storage, and integration. Most of the phenylthiohydantoin amino acid derivatives were identified by their absorbance at 254 nm. PTH-δ-Ser and PTH-δ-Thr were detected at 313 nm.

For the spinning cup analysis of CB-3, the ATZ-amino acids were manually collected as was each cycle wash that would have normally been delivered to waste.

Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10%) was performed according to Laemmli (1970).

Computer Modeling. The molecular model of the interaction between APA-[^{125}I]ASA-Lys and chicken liver DHFR was constructed by utilizing the coordinates of the 2.2-Å resolution crystal structures of the chicken liver DHFR–3-$NHCOCH_3$-triazine holoenzyme complex as determined by Matthews et al. (1985). Placement of the inhibitor was based on MTX and the superimposition of the 2,4-diaminopyrimidine ring of the inhibitor onto the corresponding atoms of the triazine (Blaney et al., 1984). APA-[^{125}I]ASA-Lys was built on the basis of the X-ray structure of MTX and by use of standard bond angles and lengths. The model was then fit and optimized into the DHFR active site according to the program MIDAS on an Evans and Sutherland picture system 2.

Results

Covalent Incorporation of APA-[^{125}I]ASA-Lys into Murine L1210 Dihydrofolate Reductase. An equimolar concentration of APA-[^{125}I]ASA-Lys was used to covalently modify purified L1210 DHFR. As indicated in Figure 2, covalent incorporation of APA-[^{125}I]ASA-Lys occurred only when the en-

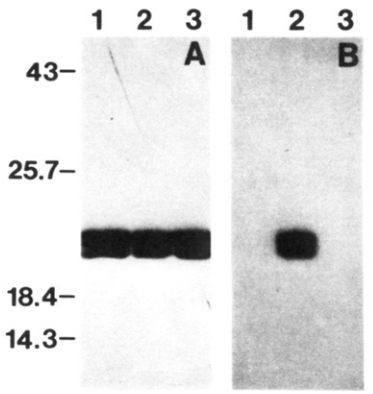

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel (10%) electrophoresis of the DHFR–APA-[^{125}I]ASA-Lys complex with and without irradiation. (A) Coomassie-stained gel: lane 1, 15 μg of L1210 DHFR; lane 2, DHFR plus an equimolar amount of APA-[^{125}I]-ASA-Lys, with irradiation; lane 3, DHFR plus an equimolar amount of APA-[^{125}I]ASA-Lys, without irradiation. Molecular weight standards (Bio-Rad) are myosin H chain (200K), phosphorylase *b* (97.4K), bovine serum albumin (68K), ovalbumin (43K), α-chymotrypsinogen (25.7K), and β-lactoglobulin (18.4K). (B) Autoradiogram of (A). Details of the irradiation procedure are given under Materials and Methods.

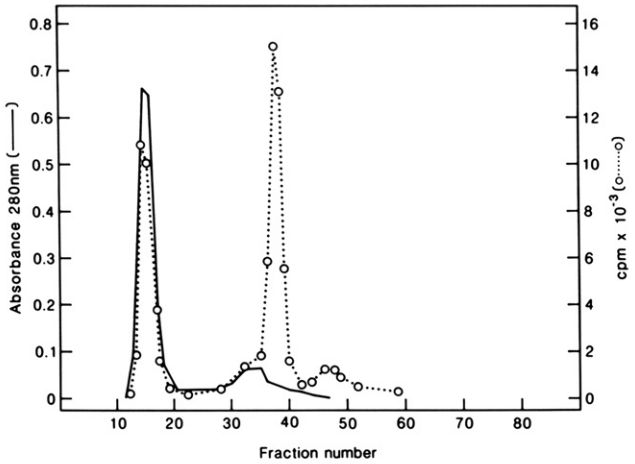

FIGURE 3: Sephadex G-25 gel filtration of labeled, denatured L1210 DHFR. The labeling experiment was performed a number of different times, under a variety of conditions. Shown are the results of one experiment, whose chromatographic profile is typical of all such chromatograms obtained. DHFR (62 nmol) was labeled as described under Materials and Methods with a 2-fold excess of photoprobe (1.57 $\times$ 10^7 total cpm). After denaturation, the labeled protein, in a 2.5-mL volume, was applied to a 1.4 $\times$ 38 cm Sephadex G-25 column, and elution was achieved with 50 mM NH_4HCO_3/6 M urea, pH 9.5. Fractions (1.4 mL) were collected at a flow rate of 18 mL/h. Aliquots (50 μL) from each fraction were analyzed for radioactivity. A total of 2.7 $\times$ 10^6 cpm was recovered in the protein fraction, corresponding to the labeled, denatured enzyme.

zyme–probe complex was irradiated. In the absence of irradiation, no covalent modification occurs. Gel filtration of the labeled DHFR, denatured in 6 M urea, was used to separate the reversibly bound probe from the covalently modified enzyme. This procedure will effectively separate [^{3}H]MTX from DHFR (data not shown), which binds tightly, but noncovalently, to the enzyme. As shown in Figure 3, two peaks absorbing at 280 nm and coeluting with radioactivity are obtained. The first peak, which elutes essentially at the void volume of the column, was associated with nondialyzable radioactivity. The second peak elutes at the bed volume and represents unbound photoproduct(s) of APA-[^{125}I]ASA-Lys. The identification of the first and second peaks as DHFR and unbound probe, respectively, was determined from separate gel filtration studies of denatured DHFR and free APA-

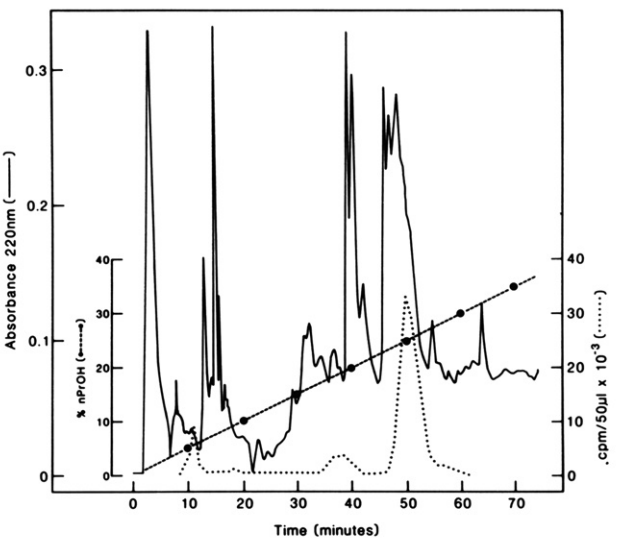

FIGURE 4: HPLC of cyanogen bromide digested, radiolabeled L1210 DHFR. A total of 22 nmol of the digest (see Materials and Methods for digestion conditions) in 250 μL of 35% formic acid was injected onto a C3 reversed-phase HPLC column, and elution was achieved on a linear gradient of 1-PrOH/0.1% TFA at a flow rate of 1 mL/min. The effluent was monitored at 220 nm at a sensitivity of 0.4 AUFS. One-milliliter fractions were collected, and 50 μL of each was analyzed for radioactivity. A total of 5.28 $\times$ 10^6 cpm was injected onto the column, and the recovery was approximately 75%. The material eluting at 50 min, CB-3, contained 3.4 $\times$ 10^6 cpm, which corresponds to 85% of the recovered radioactivity.

[^{125}I]ASA-Lys. The amount of radioactivity associated with the first peak (3.7 $\times$ 10^7 cpm) was ascertained after exhaustive dialysis (Figure 3). From this measurement, a labeling efficiency of ca. 8% was calculated (on the basis of the application of 4.3 $\times$ 10^8 cpm to the column).

The labeling specificity of the photoprobe for DHFR was examined by using an excess of MTX prior to the addition of APA-[^{125}I]ASA-Lys. DHFR (8.8 nmol) was photoaffinity labeled with radiolabeled reagent as described under Materials and Methods in the presence and absence of a 100-fold molar excess of MTX. In the absence of MTX 8520 cpm was covalently incorporated into DHFR. In the presence of MTX 790 cpm was incorporated into the enzyme, approximately a 90% reduction.

Separation of the Cyanogen Bromide Digest of Radiolabeled DHFR. The digest was subjected to chromatography on a C3 reversed-phase HPLC column by employing a linear gradient of 0–50% 1-propanol/0.1% TFA in 100 min (Figure 4). Under these conditions, undigested DHFR elutes at 56 min (28% 1-propanol) and free APA-[^{125}I]ASA-Lys elutes at 16 min (8% 1-propanol). Determination of the radioactivity in each fraction revealed that the material (CB-3) eluting at 50 min (25% 1-propanol) contained ca. 85% of the total radioactivity associated with the protein.

Sequence Analysis of CB-3. Two types of instruments were utilized in order to determine the sequence of the isolated peptide as well as to elucidate the actual residues labeled with the photoprobe. One method (Table I) used a gas-phase protein sequencer into which 2 nmol of material was loaded. Fifteen automated Edman cycles were performed, and a single phenylthiohydantoin amino acid derivative was recovered in each of these steps. This established both the purity and identity of the labeled peptide. Comparison of the sequence of CB-3 with the established sequence of L1210 DHFR (Stone et al., 1979) unequivocally identifies the peptide as residues 53–111 of the enzyme. As indicated in Table I, there is a decrease in the yield of PTH-amino acids between cycles 9

Table I: Sequence Analysis of Radiolabeled CB-3

cycle	PTH-amino acid found[a]	sequence[b]	pmol recovered
1	Gly	Gly	524
2	Arg	Arg	243
3	Lys	Lys	301
4	Thr	Thr	83
5	Trp	Trp	85
6	Phe	Phe	349
7	Ser	Ser	16
8	Ile	Ile	223
9	Pro	Pro	76
10	Glu	Glu	82
11	Lys	Lys	67
12	Asn	Asn	67
13	Arg	Arg	0
14	Pro	Pro	70
15	Leu	Leu	181

[a] A total of 2 nmol of material was applied to a gas-phase sequencer and sequenced as described under Materials and Methods. The initial yield was about 25%. [b] From Stone et al. (1979). The sequence of CB-3 begins with Gly-53.

and 14, after which the yield increases. This decrease in PTH-amino acid yield corresponds to a region of radioactivity observed in a sequence analysis that utilized 8 nmol of CB-3 and in which the entire amount of PTH-amino acid released in each cycle was analyzed for radioactivity (Figure 5). A peak of radioactivity was centered around cycles 11–13 (Lys-Asn-Arg) in a gas-phase sequencer experiment (data not shown). However, summation of the radioactivity recovered in each cycle (1–59) of the gas-phase analysis as well as the radioactivity remaining on the sample filter indicated that only 21% of the applied radioactivity was accounted for. When the material in the waste receptacle from the sequencer was analyzed for radioactivity, it was found to contain the remainder of the radioactivity. Therefore, approximately 80% of the total applied radioactivity was found in the waste from the sequencer run. Accordingly, with the spinning cup sequencer, 8 nmol of CB-3 was analyzed by collecting both the anilinothiazolinone amino acid derivative and the waste from each individual cycle and determining the radioactivity present. The results of these analyses are indicated in Figure 5. A region of radioactivity was observed corresponding to Edman cycles 11–16, with a preponderance of radioactivity centered around Lys-Asn-Arg (residues 63–65). Although 56 cycles were analyzed in this manner, the total yield of radioactivity recovered was only 18%. Essentially no radioactivity was observed in two blank cycles performed prior to the actual sequence run, indicating that no free probe was present in the spinning cup.

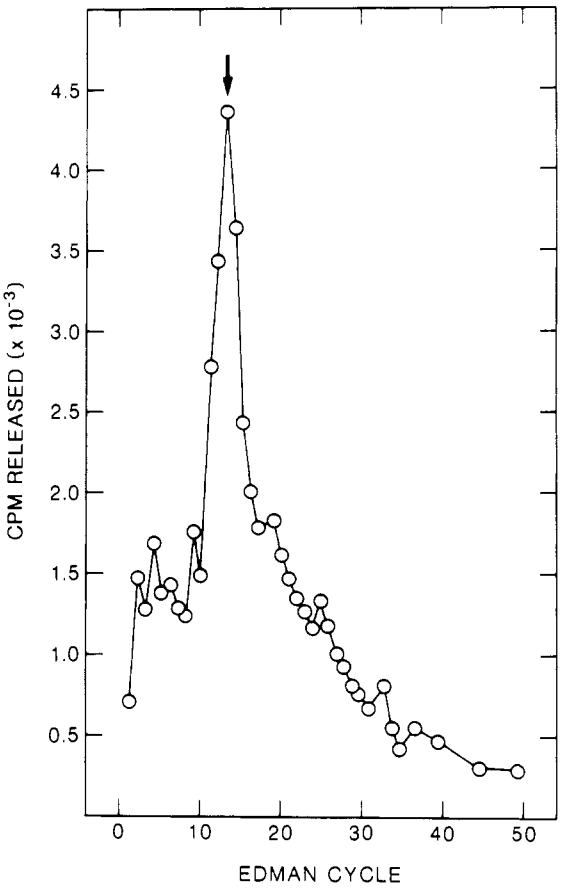

FIGURE 5: Analysis of radiolabeled CB-3 using a Beckman spinning cup sequencer (890C). Approximately 8 nmol of material, obtained from the HPLC run shown in Figure 3, was loaded onto the sequencer. Aliquots were analyzed as described under Materials and Methods. The results of this analysis are indicated as radioactivity released from each cycle by counting both the anilinothiazolinone amino acid derivative recovered at that step and the wash/waste fraction recovered from the same cycle. The indicated cycle (arrow) corresponds to Arg-65 of the native enzyme.

Discussion

It was recently shown that APA-ASA-Lys and APA-[^{127}I]ASA-Lys are both potent inhibitors of murine DHFR (Price et al., 1986b). For this reason the radioiodinated compound was chosen as an agent that would covalently and specifically modify the enzyme in a UV irradiation dependent manner. First, initial experiments involving a 3-min irradiation of the reductase following a 20-min incubation with a 2-fold excess of APA-[^{125}I]ASA-Lys resulted in a very high (35%) covalent incorporation of the probe (Price et al., 1986b). Subsequent gel filtration of the cyanogen bromide generated peptides derived from the labeled protein revealed a wide distribution of radioactivity throughout the chromatogram (data not shown), which is indicative of nonspecific random labeling. However, an excess of MTX will effectively block ca. 90% of the incorporation of the photoprobe, leading to the conclusion that the initial binding reaction, at least, is specific. Since the azidosalicylyl moiety of the analogue is located at the ϵ-amino group of lysine, there is the potential for flexibility of the reactive species (nitrene) via the four methylene groups of the lysine residue. This could favor the modification of a number of residues in the peptide backbone of the enzyme. Second, immediately after irradiation and prior to gel filtration the labeled enzyme is denatured in urea. In the absence of agents that will scavenge any unreacted nitrene groups, it is possible that the photoprobe, once dissociated from the active site of the enzyme by denaturation, will modify a number of different regions of the enzyme. These considerations may explain why a 35% labeling efficiency was originally observed (Price et al., 1986b).

In this paper we describe the specific photoaffinity labeling of murine L1210 DHFR. This was achieved by shortening the incubation and irradiation times to 5 and 1 min, respectively. In addition, after irradiation and prior to denaturation of the labeled enzyme, β-mercaptoethanol was added to the irradiated complex. This agent will compete for any remaining nitrene moieties that have not reacted with the enzyme. Although the labeling efficiency dropped to ca. 8%, essentially only one cyanogen bromide generated peptide was photolabeled (Figure 4), which indicated a specific reaction had occurred. When this peptide was subjected to sequence analysis, a preponderance of radioactivity was found in the Edman cycles 11–13. Previous experience in our laboratory indicates that peptides that have been covalently modified by affinity or

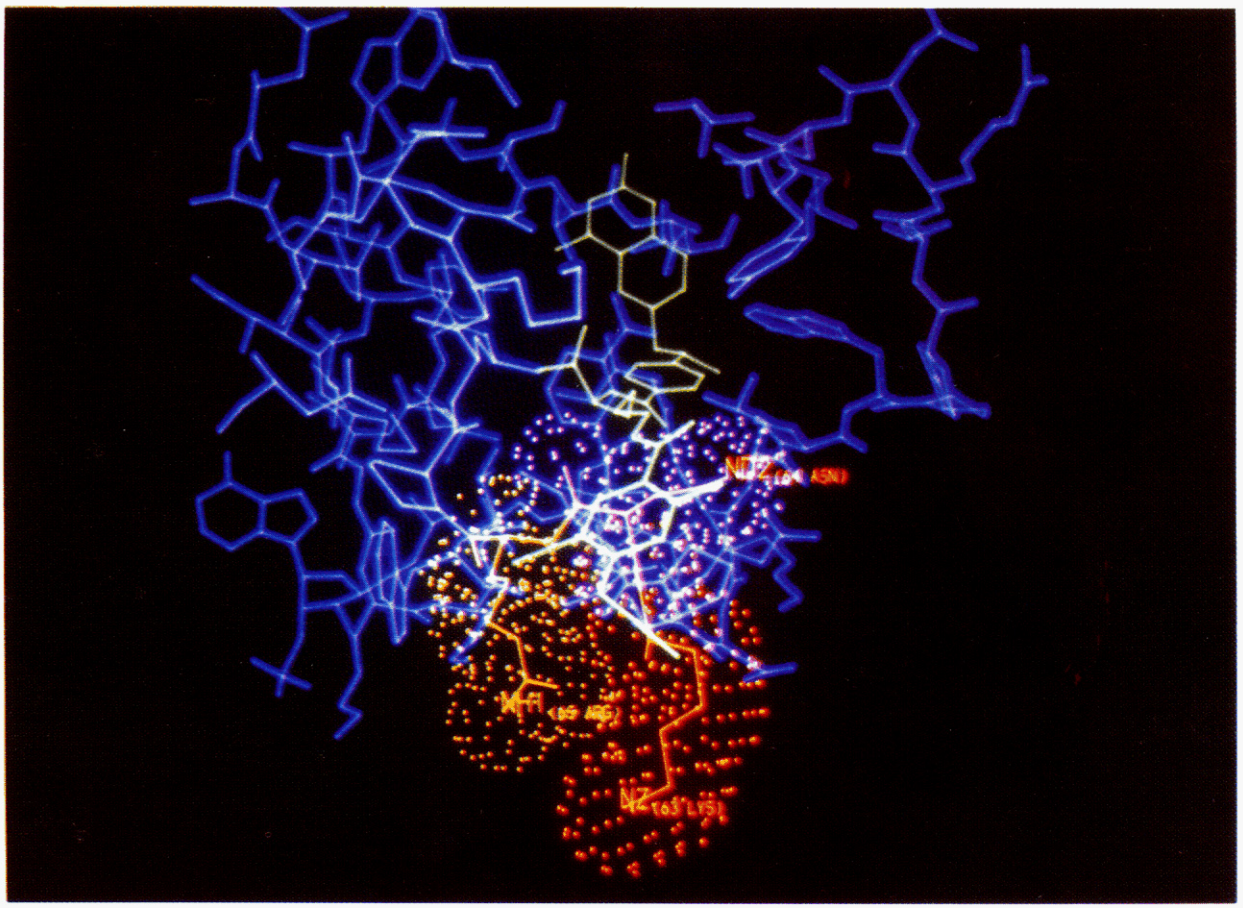

FIGURE 6: Computer placement of APA-[^{125}I]ASA-Lys in the MTX binding site of avian DHFR. Shown is the carbon backbone (blue) of the complex with the inhibitor (green) at the active site of the enzyme. The *p*-azidosalicylyl moiety is the green structure in the center of the figure, and the side chains of residues 63–65 are shown. In this figure the van der Waals surfaces for Lys-63 (red), Asn-64 (magenta), and Arg-65 (yellow) are shown. Modeling was performed as described in the text.

group-specific agents are often difficult to analyze via Edman degradation. When a peptide that had been previously modified by an affinity analogue of trimethoprim was subjected to sequence analysis, it was found that the anilinothiazolinone amino acid derivative of the modified amino acid was insoluble in the usual extraction solvent (butyl chloride) and required the use of a more polar solvent. In the present case, inefficient extraction can possibly be attributed to two factors. First, the mass of the photoprobe (M_r 740) would greatly increase the size of the labeled phenylthiohydantoin amino acid derivative. Second, since both the pteridine ring and salicylate substituent of the photoprobe are reasonably hydrophilic, the solubility of such a modified PTH-amino acid in butyl chloride would be reduced. This is supported by the fact that of the 80% of the applied radioactivity that was found in the waste, 92% was in the aqueous phase (gas-phase sequencer; data not shown). Similar difficulties have been described in work involving the identification of amino acids that had been modified with the photoaffinity reagent 8-azido-ATP (Knight & McEntee, 1985; Walker et al., 1986). It was found that the polar ATZ derivatives of the 8-azido-ATP-derivatized amino acids, due to their insolubility in butyl chloride, remained in the reaction vessel of either the spinning cup or gas-phase sequencer. Other potential problems involved in sequencing the labeled peptide include steric hindrance of the amino-terminal residue by the adduct, thereby preventing phenyl isothiocyanate derivatization, and an inefficient cleavage of the phenyl isothiocyanate derivatized amino acid from the remainder of the peptide. Considering these possibilities, the identification of residues 63–65 (Lys-Asn-Arg) as the major region of covalent modification, albeit in low yield, is not unreasonable, on the basis of these problems of derivatization, cleavage, and extraction.

Assuming that L1210 DHFR exhibits MTX binding characteristics similar to those displayed by chicken liver DHFR, whose three-dimensional structure has been elucidated (Volz et al., 1982; Matthews et al., 1985), the azide moiety of the analogue, when bound by the enzyme, can be aligned with a region of the peptide backbone that encompasses residues 63–65. This is depicted graphically in Figure 6. The peptide backbone in the region of amino acid residues 63–65 is a loop that connects helix αC to β-strand βC and is part of the boundary of the active site (Volz et al., 1982). Computer modeling indicates that, when bound in the MTX binding site of the enzyme, the *p*-azidosalicylyl moiety can theoretically fit into two possible channels. When it is fit into the first channel, the *p*-azidosalicylyl group is near amino acid residues 68–70. When it is fit into the second channel, amino acid residues 63–65 are in close proximity to the *p*-azidosalicylyl moiety. Proline-66, which is between the two channels, cannot react with the photolabel, stereochemically, since it is not orientated in the proper spatial position, being pointed away from the binding pocket. As shown in Figure 6, the *p*-azidosalicylyl group is positioned atop the ridge that borders the active-site cavity and is itself somewhat outside of the MTX binding site. Since the compound is a potent DHFR inhibitor, it is reasonable to assume that it binds tightly to the enzyme (Price et al., 1986b). Assuming that the 2,4-diamino-10-methylpteroyl portion of the inhibitor remains firmly bound by the enzyme, it is highly unlikely that residues outside

of this region (63–65) are within the distance required for contact with the azido group. Placement of APA-[^{125}I]-ASA-Lys in the active site of avian DHFR by the computer modeling program MIDAS (molecular interactive display and simulation) shows that the *p*-azidosalicylyl moiety is indeed juxtaposed with the region that includes Lys-63, Asn-64, and Arg-65. The amino acid sequences of both avian and L1210 DHFR in this region are identical.

These results indicate the usefulness of APA-[^{125}I]ASA-Lys in probing the MTX binding site of DHFR. The results include a reasonable efficiency of covalent incorporation, specificity for the folate/MTX binding site, and covalent modification only after long-wave UV irradiation. For these reasons the photoprobe should prove useful in studying other folate-binding proteins, especially membrane-derived transport proteins. In the following paper the successful use of this photoprobe to label and identify the MTX/reduced folate carrier protein from murine L1210 cells is described (Price & Freisheim, 1987).

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