Affinity Labeling of Dihydrofolate Reductase with an Antifolate Glyoxal*

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Dihydrofolate reductases from different species contain several highly conserved arginines, some of which have been shown by x-ray crystallography to have their guanido groups near the p-aminobenzoyl glutamate moiety of enzyme-bound methotrexate. The orientation of one of these (Arg-52) appears to be completely reversed in comparing the crystal structures of Escherichia coli with Lactobacillus casei enzyme (Bolin, J. T., Filman, D. J., Matthews, D. A., Hamlin, R. C., and Kraut, J. (1982). J. Biol. Chem. 257, 13650–13662). We synthesized a novel antifolate containing a glyoxal group designed to react specifically with active-site guanido groups which are able to approach the p-aminobenzoyl carbonyl of methotrexate. The binding of this compound to the enzyme was competitive with dihydrofolate (DHF) in ordinary buffers. In borate buffer at pH 8.0 it inactivated dihydrofolate reductases from both E. coli and L. casei at similar maximum rates, while the chicken liver enzyme was more slowly inactivated. The inactivation was stoichiometric, paralleled the loss of the glyoxal chromophore, and showed saturation kinetics. Inhibitor binding and thus inactivation was enhanced by NADPH, while DHF protected the enzyme. This allowed calculation of the Kₐ for DHF which was found to be identical with its Kₐ. The stoichiometrically inactivated enzyme displayed the 340-nm chromophore characteristic of 4-aminopterideridines bound to dihydrofolate reductase confirming active-site labeling with normal orientation of the ligand. The ligand remained covalently bound to inactivated enzyme upon denaturation at low pH but dissociated at neutral pH. Computer graphic modeling of the crystal structures predicted reaction of Arg-31 but not Arg-52 in L. casei dihydrofolate reductase and of only Arg-52 in the E. coli enzyme. Purification of the CNBr fragments from the inactivated enzymes gave a single labeled peptide for each species. The particular peptide tagged in each case was unaffected by the presence of NADPH and was in excellent agreement with the crystallographic predictions.

Dihydrofolate reductase (EC 1.5.1.3) utilizes NADPH to recycle tetrahydrofolate which is required for many biosynthetic processes, including the formation of deoxythymidylate (Blakley, 1969; Friedkin, 1973). Aminopterin, the 4-amino analog of folate and its N²-methylated derivative, MTX,² are antifolates that bind very tightly to dihydrofolate reductases (Hitchings and Burchall, 1965; Chaykovsky et al., 1977). The importance of these and related compounds in chemotherapy directed against both microbes (Chaykovsky et al., 1974) and tumor cells (Goldin et al., 1959) has stimulated extensive physiochemical studies of the enzyme and its complexes (Gready, 1980; Kimber et al., 1977; Matthews et al., 1978).

X-ray crystallographic analysis of the bacterial enzyme in complex with MTX has elucidated many binding interactions between the ligand and the protein. Initial x-ray data showed that in both the Escherichia coli and Lactobacillus casei complexes arginine 57 is ion paired with the α-carboxylate of the ligand's glutamyl moiety (Matthews et al., 1978). The refined 1.7-Å resolution crystal structure of the E. coli binary complex, E-MTX (Bolin et al., 1982) revealed that the guanido group of Arg-52 is located in hydrogen bonding distance to the ligand's carboxamido group between its PABA and glutamyl moieties. By contrast, in the L. casei ternary complex (E-MTX-NADPH) crystal structure, the Arg-52 side chain appears pointed away from the ligand and hydrogen bonds to a protein backbone carbonyl group (Bolin et al., 1982). Avian and mammalian dihydrofolate reductases have conserved homologous arginines to Arg-52 and Arg-57 (Kumar et al., 1980; Smith et al., 1979) at positions 65 and 70. Lys-32, adjacent to Arg-33 of the E. coli enzyme, may correspond to Arg-31 in L. casei and to Arg-36 in avian and mammalian species and is thought to have hydrophobic interactions between its own methylene group and those of the ligand's glutamyl side chain (Bolin et al., 1982).

Essential arginines of the enzyme have been modified by the arginine-specific reagent phenylglyoxal (Vehe and Freisheim, 1976). The role of arginines in ligand binding (Cocco et al., 1978) has also been examined by ¹³C-NMR studies using ¹⁳C-Arg enriched enzyme. In the present affinity labeling study, a novel antifolate was used to deliver an arginine-specific glyoxal group near to the predicted position of the Arg-52 side chain in the E. coli enzyme. The observed time-dependent inactivation of several species of dihydrofolate reductase by the above compound provided an opportunity to test and refine the crystallographic results for this enzyme as they apply to its solution structure where additional conformational states may be accessible. It also provided a means to determine whether the altered crystal conformation of Arg-52 between E. coli and L. casei is due to a species difference or to the fact that the former crystal was of a binary

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‡ The abbreviations used are: MTX, methotrexate; NADPH, nicotinamide adenine dinucleotide phosphate-reduced; DHF, dihydrofolate; PABA, p-aminobenzoyl; DAP, diaminopimelidene; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
complex and the latter of a ternary complex containing NADPH.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Reactivity of DAP-Glyoxal (II) with Arginine—The structures of the diaminopteridine DAP-acetal (I) and the DAP-glyoxal (II) antifolates synthesized as described under "Experimental Procedures" are shown on Fig. 1. The chemical activity of the DAP-glyoxal (II) is demonstrated in Fig. 2A, where loss of optical density shows that this compound reacts rapidly with polyarginine in borate buffer. Arginine also reacted with compound (II) but about 50-fold more slowly than did polyarginine ($k_{obs} = 1.7 \times 10^{-3}$ min$^{-1}$). The reaction with arginine followed pseudo first-order kinetics, required borate, and was six times more rapid at pH 8.0 than at pH 7.5 in 100 mM borate. DAP-acetal (I) which has nearly the same UV spectrum as (II) did not react with arginine or polyarginine under identical conditions. Reaction of (II) with either arginine or polyarginine led to substantial loss of the compound's major aminophenacyl (337 nm) chromophore and at completion gave a long wavelength UV peak only at 370 nm which is characteristic of 4-aminopteridines at neutral pH. The covalent nature of this reaction can be demonstrated by the fact that most of the 370-nm absorption remained bound to the polyarginine after 12-h dialysis against borate buffer. By assuming a millimolar extinction coefficient of 8.0 (Hood and Roberts, 1978) for the pteridine chromophore at 370 nm, the $A_{370}$ remaining after dialysis was used to quantitate the glyoxal and establish its purity.

Enzyme Inhibition—In the absence of borate buffer (e.g. in HEPES or phosphate), both of these compounds (DAP-acetal and DAP-glyoxal) were inhibitors of *E. coli* and *L. casei* dihydrofolate reductases and were competitive with the substrate, DHF, as determined by the common ordinate intercepts of double reciprocal plots with and without the added antifolates (data not shown). By measurement of the activity of *E. coli* enzyme at varying DHF concentrations with fixed inhibitor concentrations, the $K_i$ values for the acetal and the glyoxal were, respectively, found to be $1.3 \pm 0.5 \times 10^{-3}$ and $1.8 \pm 0.5 \times 10^{-3}$ M at pH 7.2 (50 mM HEPES, 75 mM KCl). In making these calculations we used the literature value (Poe et al., 1972) for the $K_a$ of DHF and the standard equation for competitive inhibition (Walter, 1965). Similar values of $K_i$ (Table I) were obtained for the *L. casei* enzyme. As competitive inhibitors, the two compounds (I and II) are nearly indistinguishable. As shown in Table I, replacement of the glyoxal moiety with an acetyl group (compound IV) has little effect on inhibition of the enzyme from either species while replacement with a methyl group (compound III) diminished binding about 5-fold but only for *E. coli* dihydrofolate reductase (Table I).

Enzyme Inactivation—In borate buffer dihydrofolate reductase underwent a similar although faster reaction to that of the polyarginine after 12-h dialysis against borate buffer. By measurement of the activity of the DAP-glyoxal (II) antifolate synthesized as described under "Experimental Procedures," spectrum within 5 s as described under "Experimental Procedures," spectrum within 10 s of addition of (II); ---, after 2 min; ---, after 10 min; ---, after 30 min. No change was observed in the absence of polyarginine. *E. coli* dihydrofolate reductase (1.5 nM) was present in each buffer in both sample and reference cuvettes. Compound (II) was added, and the first spectrum was obtained within 10 s after mixing (----) after 2 min (- - -), after 10 min (---), and after 30 or 60 min (-----).

---"Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-946, cite the authors, and include a check or money order for $24.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
In the presence of saturating concentrations of NADPH, a nearly identical maximum rate of *E. coli* enzyme inactivation and loss of chromophore was observed, but much lower concentrations of the DAP-glyoxal (II) were required, a result consistent with the known cooperative binding between NADPH and diaminopteridine antifolates (Birdsall et al., 1978). The concentration of NADPH giving half of the maximal rate of enzyme inactivation in the presence of a well below saturating concentration (32 nM) of DAP-glyoxal (II) was measured by varying DHF in a kinetic assay. Obtained in the presence of 1 μM NADPH by chromophoric change (Fig. 6).

sulted from some continued inactivation after dilution into the assay due to the slow off rate for the dissociation of DAP-glyoxal from the enzyme.

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Similar but even more dramatic evidence of cooperativity was observed for the *L. casei* enzyme where complete inactivation of 1 μM dihydrofolate reductase by a stoichiometric amount of DAP-glyoxal required many hours in the absence of NADPH but only a few minutes in its presence with a maximum rate similar to that found for *E. coli* enzyme (Table II). The ternary complex E-(II)-NADPH for chicken liver dihydrofolate reductase also underwent a time-dependent inactivation in borate buffer but at a rate 10–15 times slower than the bacterial enzymes examined. Reaction with the chicken enzyme was not observable over a 5-h period in the absence of NADPH suggesting substantial cooperativity of binding in this species as well.

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**Table I**

| Source of dihydrofolate reductase | *E. coli* | *L. casei* |
|----------------------------------|----------|-----------|
| DAP-acetal (I)                   | 1.3 ± 0.5 × 10⁻⁹ | 0.85 ± 0.1 × 10⁻⁹ |
| DAP-glyoxal (II)                 | 1.8 ± 0.5 × 10⁻⁹ | 0.85 ± 0.2 × 10⁻⁹ |
| 4-Methylamino anti-folate (III)  | 11.3 ± 3 × 10⁻⁹ | 0.95 ± 0.2 × 10⁻⁹ |
| 4-Acetylaminio anti-folate (IV)  | 2.3 ± 0.6 × 10⁻⁹ | 0.93 ± 0.2 × 10⁻⁹ |

**Table II**

| Kinetic constants for dihydrofolate reductase obtained by inactivation with (II) | *E. coli* | *L. casei* |
|---------------------------------------------------------------------------------|----------|-----------|
| **DAP-glyoxal (II)**                                                            |          |           |
| *Kₐ* (inactivation)                                                             | 1.0 ± 0.4 × 10⁻⁸ M | 0.9 ± 0.2 min⁻¹ |
| *kᵢ* (loss of chromophore)                                                      | 1.8 ± 0.3 min⁻¹ | 0.7 ± 0.1 min⁻¹ |
| *K₉* (by protection)                                                            | 1.4 ± 0.3 min⁻¹ |           |
| **Dihydrofolate**                                                               |          |           |
| *K₉*                                                                             | 1.1 ± 0.3 × 10⁻⁹ |           |
| *K₉*                                                                             | 1.1 ± 0.2 × 10⁻⁹ M |           |
| **NADPH**                                                                        |          |           |
| *K₉* (inactivation)                                                             | 1.2 ± 0.2 × 10⁻⁷ M |           |
| **Methotrexate**                                                                | 1.2 ± 0.3 × 10⁻¹¹ M |           |

* Measurements for *L. casei* dihydrofolate reductase were carried out at pH 8.0 in 100 mM potassium borate, 50 mM KCl. Those for *E. coli* dihydrofolate reductase were carried out in 100 mM potassium borate, 50 mM potassium phosphate, 50 mM KCl, 1.0 mM EDTA, pH 8.0.

* *Kₐ* is the concentration of NADPH giving half of the maximal rate of enzyme inactivation in the presence of a well below saturating concentration (32 nM) of DAP-glyoxal (II). *K₉* was measured by varying DHF in a kinetic assay.

* Obtained in the presence of 1 μM NADPH by chromophoric change (Fig. 6).
The substrate, DHF, protected *E. coli* enzyme from inactivation by the glyoxal (II). Using a fixed concentration of (II) at 0.33 μM to carry out inactivation of 0.072 μM enzyme in the absence of NADPH, we included varying concentrations of DHF ranging from 0 to 1.5 μM DHF. As DHF was increased, the t½ for inactivation increased. A plot of the reciprocal of the apparent kᵢ against DHF concentration gave a straight line. The data were analyzed by treating the DAP-glyoxal (II) as the substrate of an enzyme reaction for which V/E is kᵢ in the absence of DHF. DHF was treated as a competitive inhibitor of the reaction and its inhibition constant obtained by standard analysis (Walter, 1965) of the slope of the above line taking Kᵣ for (II) to be its previously determined Kᵢ of 1.0 × 10⁻³ M. This yielded an inhibition constant for DHF at pH 8.0 of 1.1 × 10⁻⁴ M, which we assume to be equivalent to its binary dissociation constant since NADPH was absent. Interestingly, we also obtained (Table II) a Kᵣ value for DHF of 1.1 × 10⁻⁶ M under the same conditions by kinetic analysis of the normal enzymic reaction. Thus, at pH 8.0, Kᵣ and Kᵣ for DHF are equal.

**Inactivation Stoichiometry**—In order to establish the stoichiometry of the inactivation reaction, enzyme and the DAP-glyoxal (II) at varying ratios were incubated overnight in borate buffer. Fig. 5 shows the correspondence between moles of the DAP-glyoxal added to *E. coli* enzyme and the amount of enzyme inactivated. The loss of enzyme activity was stoichiometric with added inhibitor up to approximately 70% inactivation. At higher inhibitor levels, less than stoichiometric inactivation was observed if the enzyme assay was prolonged. Under these conditions, upon dilution into the assay mix, enzyme activity was initially very low (<2%) and consistent with stoichiometric inactivation but gradually increased over several minutes during the assay to a partially reactivated level. The rate and final extent of inactivation was enhanced by increased borate concentration ranging from 20 to 300 mM. Less reactivation was observed with the *L. casei* enzyme than with *E. coli* dihydrofolate reductase.

When equimolar (1 μM) amounts of *L. casei* dihydrofolate reductase and the DAP-glyoxal (II) were combined at pH 8.0 in borate, the 337-nm peak of (II) rapidly disappeared (Fig. 6, top). If 1 eq of MTX was also included, the final extent of chromophore decomposition was reduced roughly 50%, while the apparent first-order rate of chromophoric loss was reduced by 85-fold as shown on Fig. 6 (bottom). Kinetic constants obtained from the above measurements are summarized in Table II.

**Covalent Nature of Enzyme Inactivation**—The addition compound formed between phenylglyoxal and arginine side chains is known to be stabilized at low pH in the absence of borate as well as at neutral pH in the presence of borate (Takahashi, 1966). Therefore, in order to establish the covalent nature of the reaction of *E. coli* enzyme with the DAP-glyoxal, an inactivation of 0.05 μmol of enzyme was carried out at pH 8.0 in 100 mM borate buffer using a stoichiometric amount of the DAP-glyoxal (II). When 75% permanent enzyme inactivation was achieved, the pH was rapidly brought to 1.5-2.0 by addition of concentrated HCl. Solid urea was then added to the solution to give a final concentration of 8 M. This was then dialyzed overnight against 500 volumes of 8 M urea in 10 mM HCl. The spectrum of the dialyzed protein contained a UV absorption peak at 345 nm (Fig. 7) corresponding to 0.7-0.8 eq of protonated aminopteridine/mole of protein. Upon reneutralization to pH 8 with concentrated borate buffer in 8 M urea this peak shifted to 370 nm (Fig. 7). The latter chromophoric also was partly removed by further overnight dialysis against borate in 8 M urea. These results indicate covalent binding of the DAP-glyoxal to the enzyme but with some instability of the addition compound at neutral pH. Essentially the same results were obtained when 6 mM guanidine HCl was substituted for urea.

**Labeled Peptide of *L. casei* Enzyme**—The site of labeling of dihydrofolate reductase from *L. casei* was investigated after inactivation of 0.05 μmol of the enzyme in ternary complex with NADPH and the DAP-glyoxal (II). Enzyme and (II) each at a final concentration of 10 μM and NADPH at 12 μM were incubated for 1 h at 25 °C in 0.1 M potassium borate, pH...
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Fig. 6. Reaction of L. casei enzyme with the DAP-glyoxal (II) and effects of MTX. Enzyme (1.0 |m|) and NADPH (2.0 |m|) were present in both sample and reference cells in 100 mM borate buffer, pH 8.0. Top, DAP-glyoxal was added to a final concentration of 0.9 |m|, and spectra were taken at high speed as described under “Experimental Procedures” within 10 s after mixing (—), after 30 s (— — —), after 50 s (— — —), after 90 s (— — —), and after 184 min (— — —). Bottom, same as above but with 0.9 |m| MTX present in both sample and reference cells before the addition of compound II. Spectra were taken within 10 s after mixing (—), after 36 min (— — —), after 106 min (— — —), after 161 min (— — —), and 184 min (— — — (lower)).

8.0, containing 50 mM KCl. Reaction of (II) with enzyme was followed spectrophotometrically. The addition compound formed upon inactivation was then stabilized by bringing the pH to 1–2 with concentrated HCl. Enough solid guanidine HCl to give a final concentration of 6 M was immediately dissolved in the acidic reaction mixture. It was then dialyzed for 12 h against 4 liters of 10 mM HCl and, further, against 2 liters of 2% formic acid. The dialyzed protein solution contained pteridine (340 nm) chromophore and was concentrated by lyophilization. The sample was then treated with 15 mg/ml CNBr in 70% formic acid under argon in the dark for 20 h, which is known to yield three peptide fragments (Freisheim et al., 1978). The largest peptide (Val-40 to homoserine lactone 128) was separated from the two smaller ones (N-terminal Thr to homoserine 39 and Ile-129 to C-terminal Ala-162) by gel filtration through Bio-Gel P-30 equilibrated with 10% formic acid, essentially as described by Freisheim et al., (1977). The pteridine chromophore co-chromatographed only with material that exhibited a 280-nm absorption peak of the unresolved smaller peptides. This material was concentrated by lyophilization and dissolved in 0.25 ml of 10 mM HCl.

Separation was accomplished by high pressure liquid chromatography with a reverse-phase (Vydac 214TP54) C4 column. Development was with a linear gradient (flow rate, 1.5 ml/min) from 8 to 50% acetonitrile in water over a 15-min interval. All solvents contained 0.1% trifluoroacetic acid (freshly distilled). Two major 280-nm absorption peaks were found with retention times of 8.5 and 10.1 min, respectively. Only the later eluting material contained 340-nm pteridine absorption. The early eluting material was concentrated, subjected to sequenator analysis as described under “Experimental Procedures,” and sequentially yielded Ile-Pro-Leu . . . precisely as expected for the C-terminal peptide. The other substance, containing the pteridine chromophore of the affinity label, gave Thr-Ala-Phe-Leu . . . and was thus identified as the N-terminal peptide which contains Arg residues only at positions 9 and 31 (Freisheim et al., 1978).

Labeled Peptide of E. coli Enzyme—E. coli dihydrofolate reductase was inactivated under identical conditions except that NADPH was initially omitted since the x-ray structure for comparison in this species is only available for the binary complex (E-MTX). Initial workup of the tagged enzyme was the same as that for L. casei through P-30 gel filtration. The E. coli enzyme containing methionine at its N terminus is expected to give only 4 CNBr peptides of molecular weight greater than 500 g/mol (Bennett et al., 1978), including two smaller peptides (Ile-2 to homoserine lactone 16, and Pro-21 to homoserine lactone 42) and two larger ones (Gly-43 to homoserine lactone-92, and Val-93 to C-terminal Arg-159).

From Bio-Gel P-30 gel filtration three peaks of 280-nm absorption were evident. For the two early eluting materials, 340-nm pteridine absorption paralleled that at 280 nm. The third substance contained no pteridine chromophore. Sequenator analysis of this substance gave Ile, Trp; Pro, Leu; Ser, Asn as expected for a mixture of the two smaller peptides. Both the first and second substances were shown, by sequenc- ing, to contain the two larger peptides with the first presumably containing them in an associated form.

Separation of the components of the second substance was carried out by HPLC in an acidic system as described above but with a slightly different linear gradient (20 to 60% acetonitrile over 20 min). Two major materials were separated with retention times, respectively, of 8.1 and 11.3 min. The early eluting material contained the pteridine chromophore and yielded Gly-Arg-His . . . upon sequencing as expected for peptide 45–92. The latter eluting substance had no pteridine absorption.
Absorption and was shown to be the C-terminal peptide containing mainly Val-Ile-Gly... by sequenator analysis.

Thus, affinity labeling of the E. coli enzyme was shown to occur only in the peptide Gly-43 to Met-92 which contains Arg residues at positions 44, 52, 57, and 71. No labeling could have occurred at Arg-33. In the case of L. casei, by contrast, only the peptide containing Arg-9 and Arg-31 was labeled while the middle peptide containing Arg at residues 43, 44, 52, 57, and 118 was not.

Labeling of Ternary Versus Binary Complexes—In order to determine whether the site of labeling was affected by the presence of NADPH, a set of the above experiments was carried out in the reverse sense, in which the L. casei binary (rather than ternary) complex or where the E. coli ternary (rather than binary) complex was labeled. These were treated as described above and gave precisely the same chromatographic patterns with respect to the protein and affinity label chromophores. Thus, the CNBr peptides tagged were identical for binary or ternary complexes of the same species.

Instability of the Covalent Adduct to Further Analysis—Efforts were also made to further narrow and identify the tagged arginine residues. These were, however, not successful. For example, it was attempted to cleave further the pteridine-bearing peptides at Trp residues by exposure to CNBr and heptafluorobutyric acid (Ozols and Gerard, 1977), but the pteridine chromophore did not remain bound to protein after this treatment. Hydrolysis of the labeled peptides with pepsin at pH 2.2 did not yield any high pressure liquid chromatography-isolable unique subfragments. Finally, we employed the strategy of blocking all other (nontagged) Arg residues of the affinity-labeled enzyme with malondialdehyde in 10 N HCl as described by King (1966) followed by lysine acetylation. We anticipated that this would generate a single trypsin cleavage site to yield a new N terminus adjacent to the site of labeling. This procedure failed to yield a new N terminus, and analysis by disc gel electrophoresis showed that this was because most of the protein had been cross-linked to a trypsin-resistant high molecular weight species by treatment with malondialdehyde.

DISCUSSION

Previous affinity-labeling studies of dihydrofolate reductase by other workers complemented and refined those from x-ray analysis. For example, Kumar et al. (1981) labeled chicken liver dihydrofolate reductase at a specific (Tyr-31) active-site residue by means of a diaminotriazine antifolate bearing a terminal sulfonyl fluoride. These results demonstrated that the location of the Tyr-31 side chain in solution was similar to its assigned crystallographic position. It was concluded that Tyr-31 had a role of hydrophobic interaction with the PABA moiety similar to that of Leu-27 in the homologous structure of the L. casei enzyme. Rosowsky et al. (1982) affinity labeled the L. casei enzyme employing an N'-idoacetyl-lysylmethotrexate analog. The inactivation rate displayed a t$_{1/2}$ of 1–2 h at pH 7.4, and the pH rate profile was consistent with alkylation of a histidine residue. These workers suggested that this may be His-28 which has been implicated by x-ray data (Matthews et al., 1978) in a charge interaction with the 3-glutamyl carboxyl of MTX.

Dihydrofolate reductase from a wide range of species contain several highly conserved arginine residues. The DAP-glyoxal reagent (II) described here was designed to have inherent affinity for the active site and thereby react specifically with the guanido side chains of the enzyme which are able to closely approach the enzyme-bound PABA carbonyl group.

The parent compound (I), similar in structure but unable to react with guanido groups, was used as a control. Both DAP-acetal and DAP-glyoxal were competitive inhibitors of both E. coli and L. casei enzymes in ordinary buffers and were shown by kinetic analysis of competition against DHF to have essentially identical inhibition constants ($K_i$, Table I). The UV spectra of both compounds is dominated by an intense absorption at 340 nm due to the p-aminophenacyl group. The chromophore in the DAP-acetal (I) is unaffected by the presence of arginine, polyarginine, or the enzyme in borate buffer attesting to its inertness.

The DAP-glyoxal (II) was reacted with 10$^{-7}$–10$^{-4}$ M enzyme from E. coli or L. casei in borate buffer leading to enzyme inactivation and concomitant loss of the ligand's p-aminophenacyl chromophore, the reaction being very rapid ($t_{1/2}$ - 20–40 s) at pH 8.0. A free arginine concentration near 1 M would be required to give the same rate of reaction with (II). Borate dependence and acid stability of the addition product indicate that the reaction of the glyoxal moiety of (II) with an Arg side chain was the inactivating event. The rate of enzyme inactivation approached a maximum value with increasing inhibitor concentration. Such saturation behavior is an essential criterion of affinity labeling at the active site (Molecio, 1967). Considerable other evidence for active-site-directed inactivation was also accumulated. These included the enhancing effect of NADPH, the second substrate, protection by substrate (DHF), protection against reaction by another competitive inhibitor (MTX), and the 1:1 stoichiometry of inactivation with maintenance of the characteristic spectrum of an enzyme-bound pteridine.

Effects of Substrates—Saturation of the inactivation rate was reached at much lower concentrations of DAP-glyoxal (II) if NADPH was present. This is a consequence of the cooperative binding between NADPH and antifolates which is well established for dihydrofolate reductase (Birdsall et al., 1978). The concentration of NADPH giving half-maximal enhancement of the rate of inactivation of E. coli enzyme by a low concentration (0.03 X Kd) (II) at pH 8 was about 1 X 10$^{-7}$ M. This value can be taken as the dissociation constant for NADPH in ternary complex with enzyme, and (II) and is 200-fold lower than the $K_m$ value for NADPH at pH 8.0. The effect of NADPH on the inactivation of L. casei enzyme by sub saturating DAP-glyoxal was even more pronounced. In fact, it was impractical to accurately measure a $K_i$ for DAP-glyoxal in the absence of NADPH. This can be accounted for only by a several hundred-fold cooperativity in binding between NADPH and (II). A 675-fold mutual enhancement of binding between NADPH and MTX has been estimated for the L. casei enzyme by Birdsall et al. (1980).

The substrate, DHF, protected E. coli enzyme from inactivation by DAP-glyoxal (II), a further indication of true active-site labeling. Accurate values for the $K_a$ of binding of DHF to dihydrofolate reductase is difficult to obtain due to the instability of DHF over the time course of equilibrium measurements. Here the competition by DHF against the rapid inactivator, DAP-glyoxal (II), allowed calculation of $K_a$ for DHF with the use of our measured $K_i$ for (II). $K_a$ was found to be 1.1 X 10$^{-4}$ M which was identical with the $K_a$ determined for DHF in borate at pH 8.0 (Table II). Since $K_a$ for DHF was measured in the presence of NADPH while $K_a$ was not, their equivalence shows that NADPH yields no apparent cooperativity for the binding of DHF. This is in distinct contrast with its cooperative effect on antifolate binding. The noncooperativity of apparent binding thus demonstrated does not necessarily mean that the binding of these substrates has no mutual effect on each other's interaction with the enzyme.
If they do, however, this must be transformed mainly into catalytic power rather than into enhanced affinity.

Reversibility of Enzyme Adduct Formation—Just as the inactivation reaction was slowed by substrate, the corresponding reaction of enzyme-bound DAP-glyoxal as observed through loss of its chromophore was substantially slowed by the active-site ligand, MTX (Fig. 6). Under these conditions, the $K_i$ of (II) for inactivation can be assumed to be similar to the value obtained by simple kinetic inhibition, $K_i$ (Table I), since NADPH was present. Slowing of the rate of reaction of enzyme with (II) by MTX (Fig. 6) allows calculation of the $K_i$ for MTX assuming competitive behavior. The $K_i$ value of $1.2 \times 10^{-11}$ M for MTX obtained for $L$. casei enzyme (Table II) agrees well with values estimated for this enzyme at lower pH (Birdsell et al., 1980). The fact that MTX not only reduced the rate of reaction of (II) with $E$. NADPH but also reduced its final extent (Fig. 6) strongly suggests that the covalent complex formed between dihydrofolate reductase, DAP-glyoxal, and borate is not formed irreversibly. Thus an equilibrium can be reached as expressed by the following equation.

$$
E^* \text{NADPH} \overset{\text{MTX}}{\rightleftharpoons} E^* \text{NADPH} \overset{\text{borate}}{\rightleftharpoons} E^* \text{NADPH} \overset{\text{borate}}{\rightleftharpoons} (\text{II}) \text{borate}
$$

Since the extent of reaction as estimated by the loss of 337-nm chromophore was about 50% of the control where MTX was omitted, it is reasonable to conclude that formation of the covalent complex in 100 mM borate, pH 8.0, transformed the DAP-glyoxal (II) from an inhibitor which was roughly 100 times more poorly bound than MTX to one which was bound with similar affinity to that of MTX when equilibrium was attained.

If the above equilibrium can exist, presumably high concentrations of DHF could take the place of MTX in the above equation and then lead to product formation. This hypothesis is consistent with observations made here in attempts to measure the stoichiometry of enzyme inactivation (Fig. 5). Initial rate measurements indicated a stoichiometric inactivation, but during the assay some activity was always slowly restored. This was particularly noticeable at high extents of inactivation where little activity was initially observed. The amount of restored activity could be reduced by raising the concentration of borate. This is compatible with the reversible formation of an addition compound, which was stabilized by borate. The high DHF concentrations as are normally present in the assay appeared to compete with the formation of this complex. This complex was apparently more stable under assay conditions in the case of the $L$. casei enzyme than in that of $E$. coli.

Takahashi (1968) has shown that addition compounds between arginine and phenylglyoxal are stable in acid and only moderately stable in neutral borate buffer. For this reason $E$. coli enzyme inactivated by DAP-glyoxal (II) was rapidly acidified and dialyzed against acid under denaturing conditions in the absence of borate (Fig. 7). A protonated diaminopteridine chromophore remained undialyzed, consistent with covalent reaction of an arginine residue with the inhibitor. The instability of the covalent complex with denatured enzyme at neutral pH was demonstrated by the finding that the diaminopteridine chromophore was eventually lost during dialysis against borate buffer. The p-amino group may further stabilize addition compounds of (II) with arginine compared with those of a simple reagent such as phenylglyoxal.

In addition to meeting the usual criteria for affinity labeling, one particularly important observation was made concerning the reaction of dihydrofolate reductases with DAP-glyoxal. For most reactive affinity compounds it is difficult to rule out the possibility that enzyme labeling occurs in an unusual orientation of the ligand in or near the active site. In the present case this can be ruled out by the observation that the stoichiometrically reacted enzyme-inhibitor complex displayed the characteristic UV spectrum of an enzyme-bound rather than a free 2,4-diaminopteridine (Fig. 2). This requires that the pteridine moiety remained bound in its normal mode which, in turn, means that the aminoephnylglyoxal group was anchored with little freedom to attain unusual orientations. Thus, it can be concluded that the affinity label reacted only with arginines that normally closely approach the PABA carbonyl group of an enzyme-bound antifolate.

Computer Graphic Modeling—By molecular modeling of both bacterial dihydrofolate reductases as described under "Experimental Procedures" we examined the ability of all arginines and especially of the conserved $E$. coli Arg-33 (Arg-31 or Lys-32 in $L$. casei), Arg-52, and Arg-57 side chains of these complexes to closely approach the phenylcarbonyl group of the bound DAP-glyoxal antifolate (II). Main chain and side chain rotations were carried out to estimate closest distances observable for the corresponding carbonyl carbon atom of a bound truncated (glutamate and methyl removed) MTX molecule and the guanido nitrogen atoms of nearby Arg residues (Fig. 8). It was found that in the $E$. coli binary complex only Arg-52 guanido nitrogens can approach to within binding distance (<2 Å) of the p-aminobenzoyl carbonyl carbon of MTX. Arg-57 guanido nitrogens, however, cannot approach any closer than 5 Å. Arg-33 is part of a helical segment oriented away from the ligand. All other arginines were much too distant to be considered. The relative orientations of arginines for the $E$. coli complex with DAP-glyoxal (II) are shown in Fig. 8A. In the $L$. casei ternary complex (Fig. 8B) structure a different situation was found. By facile bond rotation Arg-31 can be brought close to the ligand carbonyl while Arg-52 is directed away from the ligand, as pointed out by Bolin et al. (1982) and also cannot be made to approach the ligand carbonyl by rotation because of severe steric inter-
ference from other side chains. Arg-57 of the L. casei enzyme is positioned similarly to Arg-57 in E. coli and cannot be brought closer than 4–5 Å. Other arginines of L. casei dihydrofolate reductase (e.g. Arg-9) were very distant from the ligand. Thus, the crystal structures (Bolin et al., 1982) indicated that Arg-31 and Arg-52 would, respectively, be the targets of DAP-glyoxal in the L. casei and E. coli enzymes. Tagging of Arg-52 in the L. casei ternary complex or of Arg-33 in the E. coli binary complex might occur only if the solution structures varied from or were much more flexible than those in the crystal. Our separation and identification of the CNBr fragments from the affinity-labeled enzymes showed that the pteridine co-migrated with an unique peptide in each case. This was the N-terminal peptide containing only Arg-9 and Arg-31 for E. coli and was a middle peptide containing arginine at positions 44, 52, 57, and 71 for E. coli. The results clearly show that homologous arginines did not react in these two species and in particular that Arg-52 did not react in L. casei. Computer modeling complemented these results and indicated that Arg-52 was the actual target in the labeled E. coli peptide since Arg-44 and Arg-71 could be ruled out by their great distance from the ligand. Arg-57 could not be brought closer than 4–5 Å and is thus highly unlikely to be tagged. Similarly for L. casei, Arg-9 could be ruled out as extremely distant, leaving only Arg-31 as the modified residue.

Unambiguous chemical identification of the exact arginines tagged resisted considerable efforts because of the instability of the adduct compound with denatured enzyme under non-acidic conditions. It might be possible to accomplish this through the exhaustive pepsin digestion of enzyme labeled with radioactive DAP-glyoxal. Synthesis of the latter would require considerable effort and, if successful, might definitely rule out reaction of Arg-57 in E. coli. However, this would only slightly enhance the firm conclusion that homologous arginines did not react. The results strongly support predictions of the crystal structure in solution. This was particularly important to demonstrate since Arg-52, which is highly conserved in evolution, has such completely different orientations between the two species. It is surprising that the apparent functional role of this arginine is not also conserved.

Since the L. casei crystal contained NADPH but the E. coli enzyme did not, it might have been argued that the differential orientations of L. casei Arg-31 and E. coli Arg-33 and of Arg-52 in both species relative to the ligand could be due to a long range effect of NADPH on the enzyme conformation. Such an effect on the orientation of Arg-52 has recently been suggested (Clore et al., 1984) as one possible explanation for the observation, via 19F NMR measurements, that NADPH changed the tumbling rate of the PABA ring in 3',5'-difluoromethotrexate bound to L. casei dihydrofolate reductase. The results here make this hypothesis highly unlikely, since it was shown that the peptide which was tagged was independent of the presence of NADPH. Our results corroborated the crystal structures through chemical behavior in solution and also ruled out differences in the crystal structures which were ambiguous since the binary complex x-ray data were not available for L. casei nor the ternary complex data for E. coli. The compound employed here will probably also prove useful in examining arginines in the enzymes from other species such as chicken and pig where Arg-65 and Arg-70 appear to correspond with the bacterial Arg-52 and Arg-57. This will be important since no crystallographic data are yet available for eukaryotic dihydrofolate reductases in complex with MTX. It will be particularly interesting to ascertain whether Arg-65 can react in these species.

That there is an actual contribution in the E. coli enzyme of Arg-52 to ligand binding is suggested by our finding (Table I) that compound (III) was a superior inhibitor by about 5-fold to compound (IV) in this species. The former has a phenacyl group which could hydrogen bond to Arg-52, while the latter has only a phenylmethyl group. This effect is not a dramatically favorable interaction but might be enhanced in a ligand like MTX where the simultaneous α-COO- binding to Arg-57 might help to cooperatively orient the ligand’s PABA carboxyl with respect to Arg-52. In the L. casei enzyme (Table I) compounds (III) and (IV) are not noticeably different as inhibitors, again in agreement with the lack of hydrogen binding to arginine. Arg-31, of L. casei which could approach the carbonyl group of DAP-glyoxal, is thought rather to interact hydrophobically (Matthews et al., 1978; Bolin et al., 1982) with the glutamate chain of MTX and may only fortuitously reach the carboxyl group of our affinity label where this glutamyl chain is missing.

The results described here are highly indicative of rapid affinity labeling of arginines in E. coli and L. casei dihydrofolate reductases by a tightly bound antifolate glyoxal in borate buffer. The enzymes from other species appear to have arginine side chains poised near the same region of the folate binding site as potential targets of alkylation by affinity labels such as compound (II). For these, kinetic analysis of the effects of substrates and inhibitors on affinity labeling can be useful in directly measuring binding constants and estimating substrate cooperativity as demonstrated here. If sufficient specificity for compounds related to (II) exists and if these compounds do not require borate buffer for reaction, they may be of therapeutic importance. Borate-independent arginine-specific functional groups are known (Gilbert and O’Leary, 1975), and our observation that (II) reacted much more slowly with chicken dihydrofolate reductase, which closely resembles the mammalian enzyme, than with the bacterial enzymes should encourage future examination of other dihydrofolate reductases with DAP-glyoxal and closely related analogs.

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Continued on next page.
Supplementary material to:

**Affinity Labeling of Dihydrofolate Reductase with an Antibody-Catalyzed Soybean Isoflavone**: by H. A. Johnson and J. R. Small

**EXPERIMENTAL PROCEDURES**

**BRE-cell cultures** were purchased from the American Type Culture Collection, Rockville, MD. Determination of specific activity was performed by inhibitor titration and was based on the method of Ho et al. (1977). **Affinity purification** was performed by agarose- and solid-phase chromatography. The affinity-purified enzyme was used as the source for the enzyme used in the following experiments.

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