Dihydrofolate Reductase from *Lactobacillus casei* 

X-RAY STRUCTURE OF THE ENZYME-METHOTREXATE-NADPH COMPLEX

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David A. Matthews, Richard A. Alden, Jeffrey T. Bolin, David J. Filman, Stephan T. Freer, Ronald Hamlin, Wim G. J. Hnl, Roy I. Kislik, Edward J. Pastore, Laurence T. Plante, Nguyen-huu Xuong, and Joseph Kraut

From the Department of Chemistry, University of California, San Diego, La Jolla, California 92037

The structure of a *Lactobacillus casei* dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADPH oxidoreductase, EC 1.5.1.3) ternary complex consisting of enzyme, NADPH, and methotrexate has been solved at 2.5 Å resolution. Backbone geometry for this complex is almost identical with that described previously (Matthews, D. A., Alden, R. A., Bolin, J. T., Freer, S. T., Hamlin, R., Xuong, N., Kraut, J., Poe, M., Williams, M., and Hoogsteen, K. (1977) *Science* 197, 452-455) for the binary *Escherichia coli* dihydrofolate reductase-methotrexate complex. Several small differences in backbone conformation between the two complexes are explained by coenzyme binding to the *L. casei* enzyme. The substrate and coenzyme binding functions of dihydrofolate reductase are carried out by overlapping portions of the amino acid sequence rather than by separate domains.

Methotrexate binds to *L. casei* dihydrofolate reductase in an open conformation which is very similar to that found in the binary complex with the *E. coli* enzyme. Some minor differences occur in the conformation of the benzoylglutamate portion owing to direct interactions with residues that are not conserved between these two bacterial enzymes.

NADPH assumes an open conformation when bound to dihydrofolate reductase. A distance of 17 Å (C=C) separates the adenine and nicotinamide rings which are almost perpendicular to one another. The nicotinamide mononucleotide ribose ring is puckered in a C3'endo conformation and the adenosine ribose is C2'endo. NADPH binds at the carboxyl end of a parallel β-sheet plane structure. However, the secondary structure composing the coenzyme binding domain in dihydrofolate reductase differs from the dinucleotide fold in the four NADPH-linked dehydrogenases of known structure.

The geometrical relationship between the nicotinamide ring of NADPH and the pteridine ring of methotrexate is consistent with the known A side reaction stereochemistry. It is proposed that Asp-26 serves as the proton source in the enzyme reaction.

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†Recipient of stipend from the Dutch Organization for the Advancement of Pure Research (ZWO). Present address, Department of Structural Chemistry, University of Groningen, Groningen, The Netherlands.

‡Present address, Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, Mass. 02111

1 D. A. Matthews *et al.* manuscript in preparation.
tion intensities were measured at 4°C using a multiwire area detector diffractometer (Xuong et al., 1978). At room temperature the crystals deteriorated rapidly in the x-ray beam, with intensity losses of 50% after only 20 h of exposure. However, stability is greatly enhanced at 4°C so that crystals could be irradiated for up to 4 days before average intensities had dropped by 15%.

There are 9,513 symmetry-independent and Friedel-independent reflections within the 2.5 Å sphere, i.e. the total number of reciprocal lattice points is 12 × 9,513. Intensity data were replicated about five times on the average for the parent crystal, and about four times for the heavy atom derivative. The number of individual intensity measurements was 46,450 on the parent and 77,190 on the derivative, with R_{sym} = \sum_n \sum_n |I(h) - I(h_1)| / \sum_n \sum_n |I(h)|, I(h_1), - 4.3% and 5.7%, respectively, where I(h) is the ith measurement of reflection h and I(h_1) is the mean value of the N equivalent reflections. Only one parent crystal and one derivative crystal were used for all intensity measurements. This data set contained phasing information for 8,527 independent reflections.

**PHASE CALCULATION AND REFINEMENT**

Heavy atom difference Patterson and anomalous difference Patterson syntheses showed two equally occupied sites of platinum substitution in the derivative crystal (see Table I). Phase calculations included contributions from anomalous scattering (Matthews, 1966). Statistics from the final cycle of phase refinement are shown in Table I. Notice that the figure of merit remains high in the outer shell of data between 3.0 and 2.5 Å. Also, the ratios \((E)/(F_N)\) and \((E^*)/(F_N^*)\) are low overall, 0.15 and 0.62, respectively, and remain low out to 2.5 Å.

**ELECTRON DENSITY MAP**

An electron density map was calculated on a 1 Å grid with phases obtained from the single isomorphous platinum derivative. For preliminary interpretation the map was first displayed as a “mini-map” on a scale of 0.25 cm/Å. Almost all features of the enzyme complex were exceptionally clear, and within a 20-h period the entire enzyme backbone was traced, the NADPH and methotrexate molecules were located, and \(\alpha\)-carbon positions for all 162 residues in the structure were identified. The course of the polypeptide backbone revealed that \(L.\) casei dihydrofolate reductase is structurally quite similar to \(E.\) coli dihydrofolate reductase although the extent of sequence homology is only about 28% (Bitar et al., 1977).

In the next stage of interpretation the same electron density map was displayed on an Evans & Sutherland Picture System for construction of the detailed molecular model. This was accomplished as follows.

A primary sequence alignment for the \(L.\) casei and \(E.\) coli dihydrofolate reductases was obtained by assuming that deletions and insertions will occur so as to minimize disruption of important secondary and tertiary structural features. The \(L.\) casei sequence (Fig. 1) is 3 residues longer (162 as compared with 159). From comparison of the two sequences alone it is clear that there must be about 9 insertions or deletions, or both relating the two. Our alignment turned out to differ only slightly from that proposed by Bitar et al. (1977).

A set of 7 widely separated \(\alpha\)-carbon coordinates derived from the mini-map were fitted by least squares to their structurally equivalent \(E.\) coli dihydrofolate reductase \(\alpha\)-carbon coordinates. Then by applying the inverse coordinate transformation, the entire set of \(E.\) coli dihydrofolate reductase backbone atoms was positioned in the \(L.\) casei dihydrofolate reductase unit cell. Next, a computer program written by Hermans and McQueen (1974) was used to attach appropriate side chains, in arbitrary orientations, to the backbone model and to insert the three extra amino acids. The model and the electron density map were then displayed simultaneously on the Picture System and the fitting process was begun.

Backbone density was strong and continuous for the entire length of the main chain. The great majority of side chains could be positioned unambiguously and the electron densities for many of the valine, leucine, and isoleucine side chains were distinctly forked in a manner not usually seen in isomorphous replacement-phased maps at 2.5 Å resolution. The two tightly bound ligands were among the most prominent features in the map. The only area of ambiguity occurred at a sharp bend (residues 88 to 91) following three turns of \(\alpha\)-helix and the only portion of the structure for which no electron density was visible is the side chain of Asp-16, which was consequently omitted from the model.

As far as we know, this was only the second instance where a computer graphics system has been used to fit a model directly to the electron density map for a protein molecule. Recently, Tsermoglou et al. (1977) described a similar application of the graphics system at the University of North Carolina, Chapel Hill to construct a model of snake venom \(\alpha\)-neurotoxin. On the whole our experience confirms their favorable impression of this type of procedure.

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**Table I**

| Figure of merit | \(R_x = 0.035 = \sum |F_{\text{obs}} - F_{\text{calc}}|/\sum F_{\text{obs}}\) | \(R_c = 0.41 = \sum |F_{\text{obs}} + F_{\text{calc}}|/\sum F_{\text{obs}}\) |
|----------------|-------------------------------------------------|--------------------------------------------------|
| Overall        | 2810                                           | 2807                                             |
| Figure of merit| 0.96                                           | 0.81                                             |
| \(F_{\text{obs}}\) | 11.78                                          | 8.19                                             |
| \(F_{\text{calc}}\) | 1.02                                           | 0.71                                             |
| \((E)/\langle F_N\rangle\) | 1.67                                           | 1.51                                             |
| \((E^*)/\langle F_N^*\rangle\) | 0.51                                           | 0.57                                             |
Following completion of model building, the atomic coordinates were adjusted with the Hermans-McQueen constraint program (1974) in order to ensure the best possible agreement with idealized geometries of the various amino acids. These idealized coordinates were then further adjusted using one cycle of Diamond's real space refinement procedure (Diamond, 1971, 1974) to achieve better overlap with the electron density map. The program variables were chosen as in Huber et al. (1974). The crystallographic R factor calculated from the adjusted coordinates is 0.45.

At this point a brief comment should be made concerning the L. casei dihydrofolate reductases that are being studied by various workers. Dihydrofolate reductase has been independently isolated from methotrexate-resistant strains of L. casei at three different laboratories (Pastore et al., 1974a; Gundersen et al., 1972; Dann et al., 1976). The crystal structure work reported here was carried out using dihydrofolate reductase isolated and characterized by Pastore et al. (1974a). The electron density map for this enzyme was subsequently interpreted using the amino acid sequence for a dihydrofolate reductase from another independently isolated methotrexate-resistant strain of L. casei, the one first reported by Gundersen et al. (1972). It is possible that the reductases isolated from these two mutant strains may be slightly different although the fit of the primary sequence to the electron density map was very good in most places. Thus if any differences do exist they must be very minor. Sequence comparison between dihydrofolate reductases from two of the independently derived strains (those of Gundersen et al., 1972; and Dann et al., 1976) has shown that these enzymes are in fact identical (Batley and Morris, 1977) for at least the first 50 residues, with the possible exception of a substitution of Asn for Asp at residue 8.

**DESCRIPTION OF BACKBONE STRUCTURE AND COMPARISON OF DIHYDROFOLATE REDUCTASE FROM L. CASEI AND E. COLI**

The polypeptide backbone of the L. casei ternary complex is folded into an eight-stranded β-sheet with seven parallel strands and one antiparallel strand. The sheet begins at the NH₂ terminus and ends with a single antiparallel strand at the COOH terminus (Fig. 2). Four helices and six more or less extended loops provide connectivity within the sheet. Methotrexate is draped over helix αB, and resides in a deep cavity that cuts across one whole face of the reductase. The left side of the cavity provides a binding site for the nicotinamide ring of NADPH while the remainder of the coenzyme molecule occupies a shallow groove that winds back over β strands B, C, D, and E, appearing at the top in Fig. 2.

Unlike the four NAD⁺-linked dehydrogenases of known molecular structure, the substrate and coenzyme binding functions of dihydrofolate reductase are carried out by overlapping portions of the amino acid sequence rather than by separate domains. In fact well over half the methotrexate and coenzyme interactions involve the first 50 residues, and these residues are frequently conserved from species to species. Only 2 residues beyond Ile-102, namely Thr-116 and Thr-126, interact directly with either methotrexate or NADPH. In contrast, the last two strands of β-pleated sheet, βG and βH, and the long loop connecting βF and βG exhibit very little sequence homology among dihydrofolate reductases. The primary function of the last two β strands and the connecting loops may be merely to provide a structural framework for correctly positioning the more obviously important residues that are responsible for binding and catalysis. However, in view of the evidence that dihydrofolate reductase may be an autogenous repressor (Sheldon, 1977), one is tempted to entertain the possibility that this structural feature may have some role in such repressor activity.

When 142 out of the 159 α-carbon coordinates in E. coli dihydrofolate reductase (Matthews et al., 1977) are matched by least squares to structurally equivalent α-carbon coordinates for the L. casei enzyme, the root mean square deviation is 1.7 Å. Thus, despite the rather low sequence homology for these two bacterial reductases and despite the presence of bound NADPH in the L. casei enzyme complex but not in the E. coli enzyme complex, the backbone geometries for the two are almost identical. At first sight this might seem somewhat surprising since CD (Greenfield et al., 1972; Reddy et al., 1978) and NMR spectroscopic data (Pastore et al., 1974b; Kimber et al., 1977) on dihydrofolate reductases from several different sources suggest that the enzyme-methotrexate complex undergoes conformational changes upon binding a molecule of NADPH. Unfortunately, not only has it been impossible to ascribe these changes to particular parts of the struc-
ture, but it remains open to speculation as to whether they involve major movements of protein backbone, simply changes in side chain conformations, or both. In fact we do see that there are some definite differences in backbone conformation in at least three chain segments when comparing the L. casei ternary complex with the E. coli binary complex. However, care must be taken to distinguish, insofar as possible, effects due to coenzyme binding from intrinsic species differences between the two apoenzymes.

When the α-carbon backbones for the two enzyme complexes are superimposed on the Picture System it is apparent that there are at least three regions where differences in backbone conformation occur (compare Figs. 2 and 3). It is argued in a forthcoming paper that two of these conformational differences probably result from coenzyme binding, and that the remaining difference represents an inherent dissimilarity due to sequence variation between these two bacterial reductases. Both of the former are small changes, which strongly suggests that the two dihydrofolate reductase-methotrexate complexes would be nearly identical and that there are no large conformational changes in the backbone when NADPH binds. In contrast, when NAD⁺ binds to lactate dehydrogenase, backbone movements of up to 1 Å are observed (Holbrook et al., 1975).

We now briefly consider differences between the structure of the L. casei ternary complex and the E. coli binary complex. Two segments in which coenzyme binding appears to have induced conformational changes in the enzyme backbone are the flexible loop regions consisting of residues 12 to 21 and of residues 125 to 128. Both these parts of the backbone move by up to 3 Å in order to make several hydrogen bonds and van der Waals interactions with the coenzyme.

At one extremity of the large β-pleated sheet, the two longest strands, βG and βH, are connected by a short loop consisting of residues 147 to 150. In the L. casei ternary complex this loop is pushed out into solvent on the back side of the sheet as seen in Fig. 2. The same loop in the E. coli binary complex (residues 144 to 148) is on the front side of the β-pleated sheet and extends down toward the NH₂-terminal end of the helix aB. It must be cautioned, however, that the loop 144 to 148 is involved in a dimer interaction between the 2 molecules in an asymmetric unit. No such interaction occurs in crystals of the L. casei ternary complex, and therefore this structural difference may reflect a difference in molecular packing rather than an intrinsic species-specific effect.

Differences between the L. casei and E. coli enzyme complexes of up to 3 Å can also be seen in other parts of the long loop on the back side of the molecule (Fig. 2) that connects βF and βG, but this region was somewhat ambiguous in the original 2.5 Å isomorphous replacement-phased map for E. coli dihydrofolate reductase and a final description of possible differences must await refinement of both structures. Other differences arising solely from amino acid insertions and deletions will be discussed elsewhere.

**Methotrexate Bound to Dihydrofolate Reductase**

Methotrexate binds to L. casei dihydrofolate reductase in an open conformation with its pteridine ring nearly perpendicular to the aromatic ring of its p-aminobenzoyl group (Fig. 2). The overall conformation of methotrexate is very similar to that reported in the binary complex with E. coli dihydrofolate reductase (Matthews et al., 1977). Several minor differences will be discussed below where we consider specific enzyme-methotrexate interactions.

The structural formulas and conventional numbering systems for folate and for methotrexate are shown in Fig. 4, a and b, for convenient reference.

![Fig. 4. Covalent structure and atom numbering for (a) folate and (b) methotrexate. The substrate for dihydrofolate reductase is the 7,8-dihydro derivative of folate which is reduced to 5,6,7,8-tetrahydrofolate.](http://www.jbc.org/)}
Structure of Dihydrofolate Reductase·Methotrexate·NADPH Complex

Table II

Interactions between dihydrofolate reductase and methotrexate

Residue numbering for both species of dihydrofolate reductase is independent of proposed sequence homologies (Bitar et al., 1977). Thus the number 1 is simply assigned to the first residue in each case. Structurally equivalent residues are on the same line of the table.

| Methotrexate component | L. casei | E. coli | Type of interaction |
|------------------------|----------|---------|---------------------|
| Pteridine              | Leu-4    | Ile-5   | Hydrophobic         |
|                        | Carboxyl of Leu-4 | Carboxyl of Ile-5 | H bond to 4-amino |
|                        | Peptide 5-6 | Peptide 6-7 | Hydrophobic or pi-pi |
|                        | Ala-6    | Ala-7   | Hydrophobic         |
|                        | Leu-19   |         | Hydrophobic         |
|                        | Asp-26   | Asp-27  | Charge interaction and H bonds |
|                        | Leu-27   | Leu-28  | Hydrophobic         |
|                        | Phe-30   | Phe-31  | Hydrophobic         |
|                        | Carboxyl of Ala-97 | Ile-94 | van der Waals/hydrophobic |
|                        | Thr-116  | Thr-113 | H bond to 2-amino   |
|                        | Nicotinamide ring |         | Hydrophobic         |
|                        | N(10) methyl | Ser-48 | Hydrophobic         |
|                        | p-Aminobenzoyl | Leu-27 | Hydrophobic         |
|                        | Phe-49   | Ile-50  | Hydrophobic         |
|                        | Leu-54   | Leu-54  | Hydrophobic         |
|                        | Glutamate | His-38  | Hydrophobic         |
|                        | Arg-57   | Arg-57  | Charge interaction   |
|                        | Lys-32 or water |         |                     |

Table II lists those reductase residues which directly interact with methotrexate in the L. casei ternary complex. For comparison, the corresponding interactions between methotrexate and the E. coli enzyme are also listed. Residues that are homologous or that are nonhomologous but perform the same structural function in the methotrexate binding pocket are given on the same line in Table II. It is striking that every residue interacting with methotrexate in the E. coli binary complex with the exception of Leu-19 has its counterpart in the E. coli complex. Indeed in only 4 out of 13 interactions are the enzyme side chains involved merely analogous rather than identical.

The pteridine binding pocket is lined by side chains from Leu-4, Ala-6, Leu-19, Leu-27, Phe-30, and the carbonyl of Ala-97. The backbone a-carbon of Trp-5 and the peptide bond between Trp-5 and Ala-6 closely approach the pteridine ring at N(1), C(6), its attached amino group, and N(3). The 2- and 4-amino groups hydrogen bond with Oy of Thr-116 and the backbone carbonyl of Leu-4, respectively. The side chain of Leu-19 is tucked up under the pyrazine portion of the bound pteridine ring in close proximity to the nicotinamide ring of NADPH. The corresponding residue in the E. coli binary complex is Met-20, but the latter is over 7 Å away from any part of the pteridine ring. Three bacterial and three vertebrate dihydrofolate reductases that have been partially or fully sequenced2 3 (Bennett, 1974; Stone et al., 1977; Gleisner et al., 1974, Bitar et al., 1977, Stone and Phillips, 1977) all have leucine at this position, except for the E. coli enzyme. This residue is near the end of a loop connecting 6A and 6B which appears to be flexible in the nucleotide-free E. coli enzyme. NADPH appears to be flexible in the nucleotide-free E. coli enzyme undergoes the same movement on binding of the coenzyme. This side chain movement could provide a sensitive means for positioning the dihydropteridine ring of the substrate in order to facilitate catalytic reduction of dihydrofolate at N(5)-C(6). It could also partially explain enhanced binding of methotrexate to dihydrofolate reductase in the presence of NADPH.

Moreover, hydrophobic interactions between the pteridine ring of methotrexate and the nicotinamide ring of NADPH must also contribute to stabilizing the ternary complex.

The side chain carboxyl of Asp-26 closely approaches N(1) of bound methotrexate. The same interaction was observed in the E. coli reductase·methotrexate binary complex. However, this feature was attributed to a charge interaction resulting from partial or complete donation of a proton on the aspartate side chain to N(1) of methotrexate. The affinity of methotrexate for most dihydrofolate reductases is 10 5 10 4 greater than that of folate, even though the only difference between methotrexate and folate that significantly affects binding to the enzyme is substitution of a 4-oxo group in folate by a 4-amino group in the inhibitor. It was first suggested by Baker (1959) that enhanced binding of methotrexate in comparison with folate is a result of increased basicity of N(1) due to the 4-amino substituent. In our earlier paper we pointed out that this hypothesis is supported by the existence of a strong interaction between Asp-26 and N(1). The possible significance of this interaction for the mechanism of dihydrofolate reductase is further discussed below.

The aromatic ring of the p-aminobenzoyl portion of methotrexate resides in a second hydrophobic pocket formed by the side chains of Leu-27, Phe-49, and Leu-54. The a-carbon of Ser-48 is in van der Waals contact with the methyl group on N(10) in methotrexate. The aromatic side chain of Phe-49 is nearly parallel to and in van der Waals contact with the benzene ring in the inhibitor and this interaction could account for a 10 to 20° difference between the dihedral angle C(9)-N(10)-C(1)-C(4) observed when methotrexate is compared in the E. coli binary and in the L. casei ternary complex. The analogous hydrophobic interaction in the E. coli binary complex is provided by Ile-50. Such a stacking interaction between the side chain of Phe-49 and the p-aminobenzoyl groups would contribute to the stability of the inhibitor. It was first suggested by Baker (1959) that enhanced binding of methotrexate in comparison with folate is a result of increased basicity of N(1) due to the 4-amino substituent. In our earlier paper we pointed out that this hypothesis is supported by the existence of a strong interaction between Asp-26 and N(1). The possible significance of this interaction for the mechanism of dihydrofolate reductase is further discussed below.

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The glutamate portion of the inhibitor is bound at the enzyme surface with the side chain of Arg 57 hydrogen bonded to the a-carboxyl group. An identical interaction occurs in the E. coli reductase binary complex. The γ-carboxyl group hydrogen bonds with a side chain Nε2 of His-28. The structurally equivalent residue in the E. coli enzyme is Ala-29, which of

2 J. Freisheim (1977), private communication.
3 R. L. Blakley (1977), private communication.
course has no side chain hydrogen bonding capabilities. However, progressing one turn further along helix αB, Lys-32 in E. coli dihydrofolate reductase interacts with the γ-carboxyl group of methotrexate. Thus the side chain of the glutamate moiety of methotrexate is held in a different conformation in these two bacterial enzymes.

Before concluding this discussion of methotrexate binding, we should further consider the possible role of hydrogen bonding at the 4-amino group. In our earlier report we noted that the 4-amino group of methotrexate appears to donate a hydrogen bond to the backbone carbonyl oxygen of Ile-5, but that on the other hand, a 4-oxo group, as in folate, might be able to accept a hydrogen bond from the backbone NH of Gly-95. We therefore concluded that no net enhancement of methotrexate binding could be expected from differences in hydrogen bonding at the 4-substituent of the pteridine ring.

The L. casei dihydrofolate reductase structure presents a somewhat different picture with respect to the environment of the 4-substituent on the pteridine ring. Although a 4-amino group can hydrogen bond to the backbone carbonyl oxygen of Leu-4 (equivalent to Ile-5 in the E. coli enzyme), because of a small conformational difference at Ala-97 and Gly-98 (equivalent to Ile-94 and Gly-95 in the E. coli enzyme), there is no hydrogen bond donor close to the 4-substituent of methotrexate that might interact with an oxo group at the folate molecule. Thus direct hydrogen bonding with the 4-amino group of methotrexate may, after all, contribute to enhanced binding of the latter. Moreover, examination of the E. coli dihydrofolate reductase electron density map now reveals that the backbone amide of Gly-95 is also close to the backbone carbonyl of Ile-5. If the peptide plane of Gly-95-Gly-96 is in reality rotated by only 30° from its present orientation in our model, probably within the limits of the model’s accuracy, we would conclude that a normal parallel β-sheet hydrogen bond exists between these two backbone groups. Thus, until high resolution refinement of this structure is completed, we are unable to determine whether the backbone NH of Gly-95 could, after all, really form a hydrogen bond with the 4-oxo group of folate, and whether it is therefore possible that hydrogen bonding differences at the 4-substituent in methotrexate and in folate could contribute to the enhanced affinity that methotrexate exhibits for most dihydrofolate reductases.

At this juncture it must be emphasized that throughout the above discussion we are assuming the pteridine ring of folate, when bound to dihydrofolate reductase in the productive mode, has the same orientation as the pteridine ring of bound methotrexate. This point will be further considered below.

CONFORMATION OF NADPH BINDING TO DIHYDROFOLATE REDUCTASE

X-ray crystallographic investigations of four NAD⁺-dependent dehydrogenases have provided detailed information concerning the conformation of NAD⁺ when bound to this family of enzymes (Rossman et al., 1975). In the case of lactate dehydrogenase a complete description of the hydrogen bonding, van der Waals, and charge interactions between NAD⁺ and the enzyme has been given (Holbrook et al., 1975). The work reported here is the first high resolution crystallographic study of the closely related cofactor NADPH bound to an enzyme. Zappe et al. (1977) have recently reported a 6 Å study of human erythrocyte glutathione reductase, a dimeric NADPH-dependent flavoenzyme with molecular weight 10⁶ daltons. Only a brief description of cofactor binding to dihydrofolate reductase is presented here since it will be discussed more fully in a future paper.¹

The NADPH molecule assumes an open conformation when bound to dihydrofolate reductase. In contrast, nucleotide coenzymes in solution are thought to exist primarily in a folded conformation, with their two bases stacked parallel to one another (Weber, 1957; Velick, 1961). A distance of 17 Å separates the adenine and nicotinamide bases (C6-C2a) in the reductase methotrexate-NADPH complex, with the rings oriented almost perpendicular to one another. The nicotinamide mononucleotide ribose ring is puckered in a C2′, endo conformation, and the adenosine ribose is C3′, endo.

A noteworthy feature of the NADPH conformation when bound to dihydrofolate reductase is that the torsion angle about the C5′-O5′ bond in the adenine portion, θa = 122°, differs by almost 60° from the value of 181° ± 2° usually found for 5′-nucleotides in mono- and dinucleotide crystal structures (Arnott and Hukins, 1969). Also the conformation with both θa and θr near 180° is found in the lactate dehydrogenase and malate dehydrogenase enzyme–NAD⁺ complexes (Chandrasekhar et al., 1973; Webb et al., 1973) and for NADPH in solution (Sarma and Mynott, 1973). Our results are consistent with recent 31P NMR experiments reported by Feoney et al. (1975) showing that either θa or θr differs by at least 55° from this usual 180° value.

COMPARISON OF COENZYME BINDING TO DIHYDROFOLATE REDUCTASE AND TO LACTATE DEHYDROGENASE

We have previously noted (Matthews et al., 1977) the close geometrical similarity between the dinucleotide binding domain in NAD⁺-linked dehydrogenases and a portion of the dihydrofolate reductase structure comprising the innermost four strands of β-sheet and two bridging helices. Furthermore, a glycine residue is conserved at geometrically equivalent positions on these (βαβ)3 folds in the dehydrogenases and at the corresponding location in the known dihydrofolate reductase sequences. In addition, an invariant aspartate in the dehydrogenases makes a hydrogen bond with the 2′-hydroxyl of the adenosine ribose in NAD⁺. This aspartate, which occurs at the COOH terminus of one β strand in the (βαβ)3 fold, is replaced by an arginine at the corresponding geometrical location in E. coli dihydrofolate reductase and is conserved in the other dihydrofolate reductase sequences. In Matthews et al. (1977), it was argued that the arginine would interact with the 2′-phosphate of NADPH and thus that the (βαβ)3 fold in dihydrofolate reductase is probably the binding site for NADPH. To some degree, the validity of the above arguments have been substantiated by the present crystallographic studies of coenzyme binding to L. casei dihydrofolate reductase. However, in several respects NADPH binding to dihydrofolate reductase differs significantly from what was expected on the basis of the structural homology with lactate dehydrogenase.

NADPH binding in relation to the α-carbon backbone of L. casei dihydrofolate reductase is shown in Fig. 2, and Fig. 5 compares NADPH binding to dihydrofolate reductase with the analogous NAD⁺ binding to the dinucleotide fold in lactate dehydrogenase. In both dihydrofolate reductase and the dehydrogenases the respective coenzymes are bound at the carboxyl end of a parallel β-pleated sheet structure. In all these enzymes, including dihydrofolate reductase, the adenine mononucleotide portion of the dinucleotide is situated on the back side of the β-sheet as it appears in Fig. 2. The pyrophosphate moiety then leads around and over the end of the β-sheet to the nicotinamide binding site on the front side. Surprisingly, NADPH binds to L. casei dihydrofolate reductase in a position that is shifted by one β strand (toward the top of Fig. 2) from the location expected on the basis of structural comparison between lactate dehydrogenase and the E. coli dihydrofolate reductase–methotrexate complex. Even so, Arg-43 does bind to the 2′-phosphate of NADPH as we
had anticipated, but only because the arginine side chain, when fully extended, is about 5 Å longer than an aspartate side chain.

On the basis of structural comparison with lactate dehydrogenase, conservation of Gly-99 (structurally equivalent to Gly-96 in E. coli dihydrofolate reductase) was thought to result from its close proximity to the adenosine ribose of bound NADPH, as mentioned above. However, the present work reveals that translation of the coenzyme across the β-sheet by one strand relative to its anticipated location causes this invariant glycine to make van der Waals contact with the pyrophosphate group rather than with the adenosine ribose. Although both NADPH and NAD⁺ are bound in an open conformation to dihydrofolate reductase and lactate dehydrogenase, respectively, NADPH is more extended by about 3 Å primarily because the 5'-oxygen atoms bonded to the NMN ribose and to the adenosine ribose rings, respectively, are trans to one another rather than gauche.

**Fig. 5.** Upper, schematic diagram showing how individual strands are connected to form the central β-pleated sheet in dihydrofolate reductase (DHFR) and how NADPH binds in relation to this sheet. Each strand is represented as either V or A depending on whether the carboxyl or amino end is nearer the viewer. Helices with their axes nearly parallel to adjacent β-sheet are indicated by circles, while the one helix (aC) which is almost perpendicular to adjacent β-sheet is depicted as a two-turn helix. Connections occurring at the end of the sheet nearest the viewer are drawn with thick lines. Hexagons labeled N and AD represent the nicotinamide and adenine mononucleotide portions of NADPH, respectively. Lower, schematic diagram for the NAD⁺ binding region in lactate dehydrogenase (LDH).

**Fig. 6.** Stereo drawing showing the relative orientations of Asp-26, the reduced nicotinamide (NIC) portion of NADPH, and the pteridine portion of methotrexate (MTX). Nitrogen and oxygen atoms are indicated by blackening and shading, respectively.

**COENZYME-METHOTREXATE INTERACTION AT THE ACTIVE SITE**

Enzyme-mediated reduction of dihydrofolate occurs across the N(3')-C(6) bond of the dihydropteridine ring (Pastore et al., 1963; Pastore, 1967). Stereospecificity for the A side of NADPH has been demonstrated for dihydrofolate reductase from chicken liver, mouse leukemic cells (Pastore and Friedkin, 1962), Strep-tococcus faecalis (Blakley et al., 1963), and E. coli (Pastore and Williamson, 1968). It is also known (Pastore et al., 1963) that reduction of the substrate results in transfer of a hydride ion from NADPH to C(6) and incorporation of a proton at N(3). As shown in Fig. 6, in the L. casei dihydrofolate reductase:methotrexate:NADPH structure the pteridine ring of methotrexate and the nicotinamide ring of NADPH are close together in the active site of the enzyme, with the C(6) carbon of the nicotinamide ring close to the N(3')-C(6) bond of the inhibitor. The pteridine ring is inclined at about 45° to the plane of the nicotinamide ring and C(6) of methotrexate is about 4.5 Å directly above C(6) of the nicotinamide. C(6) is also close (3.5 to 4 Å) to N(3), C(4), and the 4-amino group of methotrexate. Examination of Fig. 6 confirms that the transferable hydride ion at C(4) must come from the A side of the nicotinamide ring; the B side points down and away from the pyrazine ring.

Perhaps it is worth noting here that availability of the kind of detailed structural information represented in Fig. 6 provides an opportunity for rational design of a new class of dihydrofolate reductase inhibitors that would incorporate elements of both substrate and cofactor in a single molecule. In the case of several dehydrogenases, analogous compounds already exist. Thus, a reduced adduct of NADPH and pyruvate has been synthesized and was shown to bind strongly and specifically to lactate dehydrogenase (Everse et al., 1972).

**RELEVANCE TO ENZYME MECHANISM**

A growing body of spectroscopic evidence suggests that methotrexate, folate, and dihydrofolate are each bound somewhat differently in the active site of dihydrofolate reductase (Erickson and Mathews, 1972; D'Souza and Freisheim, 1972; Greenfield et al., 1972; Erickson and Mathews, 1973; Pastore et al., 1974b; Roberts et al., 1974; Poe et al., 1975), but the extent of these differences is unclear since relative movements of certain chemical groups by a few tenths of an angstrom could dramatically perturb NMR, ultraviolet, circular dichroism, and fluorescence spectra of the complexes. We must thus address the question of whether the binding geometry reported here for the dihydrofolate reductase: methotrexate-NADPH complex corresponds to the productive binding geometry for substrate and coenzyme.

Huennekens et al. (1970) have reported fluorescence titration results that indicate methotrexate and dihydrofolate compete for the same binding site. More recently a number of kinetic investigations have shown methotrexate to be a competitive inhibitor of dihydrofolate reductase (see for example...
Williams et al., 1973). Furthermore, Pastore et al. (1976) report that addition of methotrexate to the \(^{13}C\) (benzoylcarbonyl)-labeled enzyme-folate-NADP\(^+\) complex causes folate to be displaced, as evidenced by the appearance of a single sharp resonance in the \(^{13}C\) NMR spectrum at 170.3 ppm corresponding to the expected chemical shift for free folate in solution.

The present work strongly points to the conclusion that the geometry seen here in the ternary complex must at least closely resemble productive substrate binding. One reason for this conclusion is that out of 21 strictly conserved residues in the five known reductase sequences, 15 interact directly with either methotrexate or NADPH, while conservation of the remaining 6 are readily explained by intramolecular interactions required to maintain the correct chain folding. A second reason is that the geometrical relationship between coenzyme and the pteridine ring of methotrexate is, as described above, consistent with the known reaction stereochemistry.

A question remains, however, concerning the orientation of the pteridine ring within its binding site. Model building experiments show that it may be possible to turn the pteridine ring over with respect to the rest of the inhibitor molecule while retaining a reasonable fit within the substrate binding crevice. This can be accomplished by rotations of 180° about the C\(_{15}\)-C\(_{39}\) bond, and a 30° rotation about the C\(_{27}\)-N\(_{12\ast}\) bond, and results in a pteridine ring oriented with N\(_{1}\) and N\(_{8}\) at the top in Fig. 1, and N\(_{3}\) at the bottom. Significantly, such a rearrangement does not much alter the close approach between C\(_{49}\) of the NADPH nicotinamide ring and C\(_{6}\) of the pteridine ring. Consequently, in view of the evidence alluded to above that folate and dihydrofolate are bound in a somewhat different manner from methotrexate, we must entertain the possibility that substrate does in fact bind with its pteridine ring turned over in this way. It is likely that this problem will eventually be resolved directly by difference-Fourier studies on the enzyme-substrate complex.

In the meantime, it is relevant to consider what group within the enzyme might be available to transfer a proton to N\(_{3}\) during the course of the reaction. The NAD\(^+\)-dependent dehydrogenases of known structure all appear to utilize a histidine side chain (although indirectly, in the case of alcohol dehydrogenase) as the required acid-base catalyst (Dalziel, 1975), and speculation has also focused on histidine as the proton source in the dihydrofolate reductase-catalyzed reduction of dihydrofolate (Baker and Ho, 1964). However, we find no histidine residue anywhere near the pteridine binding pocket in either E. coli or E. coli dihydrofolate reductase. In fact the only potential proton donor groups in this neighborhood are Asp-26 (Asp-27 in the E. coli enzyme) and Thr-116 (Thr-113). Both are invariant in all known dihydrofolate reductase sequences.

Matthews et al. (1977) found that the side chain of Asp-27 in the E. coli reductase interacts closely with N\(_{1}\) of methotrexate in the binary complex and proposed that Asp-27 is involved in the exceptionally strong binding of methotrexate. Exactly the same close interaction is seen between N\(_{1}\) of methotrexate and Asp-26 in the L. casei ternary complex described here. Additionally, the hydrophobic crevice in which Asp-26 resides is now completely blocked off from access to surrounding solvent by the nicotinamide ring in the ternary complex, and consequently the pH of this side chain in the complex must be considerably higher than that of a solvent-exposed aspartic acid. It seems likely in view of the foregoing that Asp-26 is the required proton source in the enzymic reaction. But herein lies the slight apparent difficulty which, indeed, is the source of our question concerning the "productive" orientation of the substrate's pteridine ring. The proton is ultimately delivered to N\(_{3}\) in the enzymic reduction but the carboxylate group of the Asp-26 side chain interacts most closely with N\(_{1}\), and is also close to N\(_{3}\) and the 2-amino group. However, if the pteridine ring in the substrate were to be turned over, the carboxylate group of Asp-26 could instead be close to N\(_{3}\). In fact there may be no problem at all, for it is not unreasonable to invoke a proton jump within the pteridine ring or a proton rearrangement mediated by water, either during the enzymic reaction or after release of products (Dreyfus et al., 1976). Thus at present, all we can say is that during the productive binding of substrate the pteridine ring may be turned over from the orientation of the pteridine ring observed in bound methotrexate, but the evidence does not require it.

In this connection, it should be pointed out that the model represented in Fig. 6 makes a prediction about the absolute configuration of the asymmetric center of C\(_{6}\) in enzymically reduced tetrahydrofolate. If productive binding of dihydrofolate has the geometry of Fig. 6, hydride attack from C\(_{4\ast}\) of the reduced nicotinamide ring will occur from underneath C\(_{6}\) of the dihydropteridine ring, producing the enantiomer with absolute configuration designated R according to the Cahn-Ingold-Prelog convention (1966). If, on the other hand, productive binding takes place with the pteridine ring turned over from the way it is depicted in Fig. 6, the opposite enantiomer will be produced. Obviously it would be interesting to determine directly, by independent x-ray crystallographic methods, the absolute configuration of natural tetrahydrofolate.

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REFERENCES
Arnott, S., and Hukins, D. W. L. (1969) Nature 224, 866-868
Baker, B. R. (1959) Cancer Chemother. Rept. 4, 1-10
Baker, B. R., and Ho, B. T. (1964) J. Pharmacol. Sci. 53, 1457-1466
Bailey, K. E., and Morris, H. R. (1977) Biochem. Biophys. Res. Commun. 75, 1010-1014
Bennett, C. D. (1974) Nature 248, 67-68
Bittar, K. C., Blankenship, D. T., Walals, K. A., Dunlap, R. B., Reddy, A. V., and Freisheim, J. H. (1977) FEBS Lett. 80, 119-122
Blakley, R. L. (1969) The Biochemistry of Folic Acid and Related Pteridines, John Wiley and Sons Inc., New York
Blakley, R. L., Ramassar, B. V., and McDougall, B. M. (1963) J. Biol. Chem. 238, 3075-3079
Calu, R. S., Ingold, C. K., and Freelig, V. (1969) Angew. Chem. Int. Ed. Engl. 8, 385-394
Chabner, B. A., and Johns, D. G. (1976) in Cancer (Becker, F. F., ed) pp. 363-377, Plenum Press, New York
Chandrasekhar, K., McPherson, A., Jr., Adams, M. J., and Rossmann, M. G. (1973) J. Mol. Biol. 78, 503-518
Crusberg, T. C., Leary, K., and Kasiuk, H. L. (1970) J. Biol. Chem. 245, 5292-5296
Dalziel, K. (1975) in The Enzymes (Boyer, P. D., ed) Ed 3, pp. 1-60, Academic Press, New York
Dann, J. G., Ostler, G., Bjur, R. A., King, R. W., Scudder, P., Turner, P. C., Roberts, G. C. K., Burgener, A. S. V., and Harding, N. G. L. (1976) Biochem. J. 157, 559-571
Diamond, R. (1971) Acta Crystallogr. A27, 436-462
Diamond, R. (1974) J. Mol. Biol. 82, 371-391
Drees, M., Dodin, C., Benzsaude, O., and Dubois, J. E. (1976) J. Am. Chem. Soc. 97, 2589-2596
D’Souza, L., and Freisheim, J. H. (1972) Biochemistry 11, 3770-3774
Erickson, J. S., and Mathews, C. K. (1972) J. Biol. Chem. 247, 5661-5667
Erickson, J. S., and Mathews, C. K. (1973) J. Biol. Chem. 248, 379-380
Everse, J., Berger, R. L., and Kaplan, N. O. (1972) in Structure and Function of Oxidation-Reduction Enzymes (Akesson, A., and Ehrenberg, A., eds) pp. 691-708, Pergamon Press, New York
Structure of Dihydrofolate Reductase·Methotrexate·NADPH Complex

Feeney, J., Birdsall, B., Roberts, G. C. K., and Burgen, A. S. V. (1975) Nature 257, 564-566

Friedkin, M. (1973) Acta Cryst. 38, 235-292

Gleazer, J. M., Peterson, D. L., and Blakely, R. L. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 3001-3005

Greene, N. J., Williams, M. N., Poe, M., and Hoogsteen, K. (1972) Biochemistry 11, 4706-4711

Gundersen, L. E., Dunlap, R. B., Harding, N. G. L., Freisheim, J. H., Otting, F., and Huennekens, F. M. (1972) Biochemistry 11, 1018-1023

Hartman, S. C., and Buchanan, J. M. (1959) J. Biol. Chem. 234, 1527-1510

Hermans, J., and McQueen, J. E. (1974) Acta Crystallogr. A30, 730-739

Hitchings, G. H. (1973) in Trimethoprim-Sulfamethoxazole (Finland, M., and Kass, E. H., eds) pp. 1-4, University of Chicago Press, Chicago

Holbrook, J. J., Liljas, A., Steindel, S. J., and Rossmann, M. G. (1975) in The Enzymes (Boyer, P. D., ed) Ed 3, pp. 191-292, Academic Press, New York

Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J., and Steigemann, W. (1974) J. Mol. Biol. 89, 73-101

Huennekens, F. M., Moll, G. P., Harding, N. C. L., Gundersen, L. E., and Freisheim, J. H. (1970) in Chemistry and Biology of Pteridines (Iwai, K., Akino, M., Gato, M., and Iwanami, Y., eds) pp. 329-350, Internatinal Printing Co., Ltd. Tokyo, Japan

Kimber, B. J., Griffiths, D. V., Birdsall, B., King, R. W., Scudder, P., Feeney, J., Roberts, G. C. K., and Burgen, A. S. V. (1977) Biochemistry 16, 3492-3500

Matthews, B. W. (1966) Acta Crystallogr. 20, 82-86

Matthews, B. W. (1978) in The Proteins (Neurath, H., and Hill, R. L., eds) Ed 3, Academic Press, New York, in press

Matthews, D. A., Alden, R. A., Bolin, J. T., Freer, S. T., Hamlin, R., Xuong, N. H., Kraut, J., Poe, M., Williams, M., and Hoogsteen, K. (1977) Science 197, 452-455

Pastore, E. J., and Friedkin, M. J. (1962) J. Biol. Chem. 237, 3802-3810

Pastore, E. J., Friedkin, M., and Jardetsky, O. (1963) J. Am. Chem. Soc. 85, 3058-3059

Pastore, E. (1967) Abstracts of 15th Meeting of American Chemical Society, 107

Pastore, E. J., and Williamon, K. L. (1968) Fed. Proc. 27, 754

Pastore, E. J., Plante, L. T., and Kisiislu, R. L. (1974a) Methods Enzymol. 34, 281-288

Pastore, E. J., Kisiislu, R. L., Plante, L. T., Wright, J. M., and Kaplan, N. O. (1974b) Proc. Natl. Acad. Sci. U. S. A. 71, 3849-3853

Pastore, E. J., Plante, L. T., Wright, J. M., Kisiislu, R. L., and Kaplan, N. O. (1976) Biochem. Biophys. Res. Commun. 68, 471-475

Poe, M., Williams, M. N., Greenfield, N. J., and Hoogsteen, K. (1975) Biochem. Biophys. Res. Commun. 67, 240-247

Paston, J. M., and Stadtman, T. C. (1975) in Cobalamin (Babior, B. M. ed) pp. 112-140, John Wiley and Sons, Inc., New York

Reed, A. V., Behinke, W. D., and Freisheim, J. H. (1978) Biochim. Biophys. Acta 533, 419-427

Roberts, G. C. K., Feeney, J., Burgen, A. S. V., Yuferov, V., Dann, J. G., and Bjur, R. (1974) Biochemistry 13, 5351-5357

Rosemann, M. G., Liljas, A., Branden, C. I., and Banazak, L. J. (1975) in The Enzymes (Boyer, P. D. ed) Ed 3, pp. 61-102, Academic Press, New York

Sarma, R. H., and Mynott, R. J. (1973) in Conformation of Biological Molecules and Polymers (Bergmann, E. D., and Pullmann, B., eds) pp. 591-624, Israel Academy of Sciences and Humanities, Jerusalem

Sheldon, R. (1977) Mol. & Gen. Genet. 15, 215-219

Stone, D., and Phillips, A. W. (1977) FEBS Lett. 74, 85-87

Stone, D., Phillips, A. W., and Burchall, J. J. (1977) Eur. J. Biochem. 72, 613-624

Tsernoglou, D., Petsko, G. A., McQueen, J. E., Jr., and Herman, J. (1977) Science 197, 1378-1381

Velick, S. F. (1961) in Light and Life (McElroy, W. D., and Glass, B., eds) pp. 109-143, Johns Hopkins Press, Baltimore

Weber, G. (1957) Nature 180, 1499

Williams, M. N., Poe, M., Greenfield, N. J., Hirschfield, J. M., and Hoogsteen, K. (1973) J. Biol. Chem. 248, 6375-6379

Xuong, N. H., Freer, S. T., Hamlin, R., Nielsen, C., and Vernon, W. (1978) Acta Crystallogr. A34, 289-296

Zappe, H. A., Krohn-Ehrich, G., and Schulz, G. E. (1977) J. Mol. Biol. 113, 141-152
Dihydrofolate reductase from Lactobacillus casei. X-ray structure of the enzyme methotrexate.NADPH complex.
D A Matthews, R A Alden, J T Bolin, D J Filman, S T Freer, R Hamlin, W G Hol, R L Kisliuk, E J Pastore, L T Plante, N Xuong and J Kraut

J. Biol. Chem. 1978, 253:6946-6954.

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