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Dihydrofolate reductase (EC 1.5.1.3) catalyses the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. The product, together with other reduced folates, serves as a cofactor in a variety of 1-carbon transfers in the biosynthesis of purines, pyrimidines and amino acids (Blakley, 1969). The enzyme is considered to be the major cellular receptor for several important drugs including methotrexate (2,4-diamino-N,N-dimethylpteroylglutamate) (Hitchings & Burchall, 1965) and inhibition of the enzyme leads to inhibition of DNA synthesis and cell death. Certain inhibitors in particular trimethoprim (2,4-diamino-5-(3',4',5'-trimethoxyphenyl)pyrimidiné) and pyrimethamine (2,4-diamino-5-p-chlorophenyl-6-ethylpyrimidine) are more potent inhibitors of bacterial dihydrofolate reductases than of the mammalian enzymes and, consequently, these compounds have been employed as antibacterial agents. In an attempt to understand the underlying molecular basis for the differential susceptibilities of dihydrofolate reductases to inhibition, a considerable effort has been devoted to the determination of the structures of these enzymes from a variety of bacterial and vertebrate sources.

The complete amino acid sequences of the bacterial enzymes from Streptococcus faecium (Gleisner et al., 1974), Escherichia coli RT500 (Stone et al., 1977), E. coli MB1428 (Bennett et al., 1978), Lactobacillus casei (Bitar et al., 1977) and the enzyme specified in E. coli by the R-plasmid R67 (Stone & Smith, 1979) have been determined. In addition, the amino acid sequences of the enzymes from the following vertebrate sources have also been determined; mouse lymphoma L1210 (Stone et al., 1979), porcine liver (Smith et al., 1979), chicken liver (Kumar et al., 1980) and bovine liver (Lai et al., 1979). The primary sequences are compared in Fig. 1., and in the following discussion all residue numbers refer to this Figure. With the exception of the R-67-plasmid-specified enzyme, which does not appear to be related to the other dihydrofolate reductases, the bacterial enzymes generally contain 159–167 amino acids, whereas the vertebrate enzymes all contain between 186 and 189.

The vertebrate enzymes are closely homologous, possessing an overall identity of 72–89%, whereas the degree of identity observed between bacterial enzymes and between bacterial and vertebrate enzymes is of the order of 30%. The constant and variable residues are distributed throughout the molecules, suggesting that there is probably overall structural similarity, although it is clear that certain restricted regions of the molecule show more extensive homology than is observed in other regions. In particular the regions between residues 16–29, 49–76, 111–125 and 135–152 are highly conserved, and it is interesting to consider these areas in terms of the X-ray-crystallographic studies of enzyme–ligand complexes that have been published to date.

The structures of the E. coli-enzyme–methotrexate binary complex (Matthews et al., 1977) and the L. casei-enzyme–NADPH–methotrexate ternary complex (Matthews et al., 1978) have both been solved at 2.5nm (25 Å) resolution. Although the two proteins have substantially different amino acid sequences (29% identity), the protein backbone and inhibitor conformations in the two structures are very similar, and it seems likely, therefore, that all homologous dihydrofolate reductases will possess the same general structure.

The overall folding of the polypeptide chain is dominated by an eight-stranded b-sheet composed of seven parallel strands and a single antiparallel strand at the C-terminus. The sheet shows the usual right-handed twist. The molecule contains three helical regions. A cleft some 1.5 nm (15 Å) wide cuts across one face of the molecule and gives the structure a bi-lobed appearance. The pteridine ring of methotrexate, and, by analogy, the substrate dihydrofolate, occupies a hydrophobic area within this cleft and the nicotinamide ring of the cofactor is located in the other portion of the cleft. It is perhaps not surprising that all of the four highly conserved areas of the molecule described above contribute residues to the surfaces of this cleft, and thus, on the basis of the amino acid sequence homology, it seems likely that the active sites of mammalian and bacterial enzymes are structurally similar. This hypothesis is further strengthened by a consideration of the specific residues involved in binding methotrexate. According to the X-ray-crystallographic studies, the pteridine ring is bound in the active-site cleft by hydrogen bonds formed between the 2-amino, 4-amino, N(15) and residues threonine-136, isoleucine-7 and aspartic acid-30 respectively and by hydrophobic interactions with isoleucine-7, alanine-9, leucine-31, phenylalanine-34 and isoleucine-114. These residues are all either conserved or conservatively replaced in the dihydrofolate reductase sequences that have been determined. Similarly residues leucine-31, isoleucine-60, leucine-67 and isoleucine-114, all of which interact with the p-aminobenzoate portion of the methotrexate molecule are either conserved or conservatively replaced. The cofactor is bound in an extended conformation with its adenine portion on one side of the b-sheet and the nicotinamide portion on the other side. The residues that interact with the cofactor, in particular the basic residue at position 54, which interacts with the 2'-phosphate, are well conserved, and this observation provides further support for the hypothesis that the enzymes possess very similar structures.

The crystallographically observed structure of the ternary complex predicts that, assuming the pteridine ring of dihydrofolate is bound in the same orientation as that of methotrexate, the absolute configuration of C-6 of the product would be R according to the Cahn-Ingold-Prelog conversion (Cahn et al.,
Residue numbers are based on the chicken-liver-enzyme sequence. Gaps have been introduced to maximize the homology. A, *Escherichia coli* RT500 (Stone et al., 1977); B, *Streptococcus faecium* (Petersen et al., 1975); C, *Lactobacillus casei* (Lai et al., 1979); D, porcine liver (Smith et al., 1979); F, bovine liver (Lai et al., 1979); G, chicken liver (Kumar et al., 1979); H, R67-plasmid-specified.

1966). In fact, however, the X-ray-crystallographic study of the absolute configuration of the natural diastereoisomer of 5,10-methenyltetrahydrofolic acid (Fonntecilla-Camps & Bugg, 1979) has shown that the configuration of C-6 of tetrahydrofolate is S. Thus the orientation of the dihydropteridine ring in the productive enzyme–substrate complex must differ from that
observed in enzyme–methotrexate complexes. The orientation that appears to be required for catalysis can be readily achieved by rotations of the pteridine of 180° around the C-6–C-9 bond and 30° about the C-9–N-10 bond. In this orientation, C-4 of the NADPH nicotinamide ring closely approaches C-6 of the substrate, and N-5 of the substrate is adjacent to aspartic acid-30 of the enzyme. It has been suggested that in this orientation either aspartic acid-30 or threonine-136 may provide the proton that is transferred to N-5 (Matthews et al., 1978).

Comparative studies on the structures and kinetic properties of two isoenzymes (forms 1 and 2) of dihydrofolate reductase from E. coli RT500 (Baccanari et al., 1981) provide further information regarding the mechanism of action of these enzymes. Although the amino acid sequences of these two isoenzymes differ only at position-31, which is arginine in form 2 and leucine in form 1, they do exhibit considerably different inhibitor-binding and kinetic properties. From equilibrium-dialysis studies it has been shown that the inhibitors methotrexate and trimethoprim bind more tightly to form 1 than to form 2. The isoenzymes also differ in their catalytic properties, form 1 having a 10-fold higher catalytic-centre activity (turnover number) at pH 7 than form 2. In the presence of Ba++, which is a powerful inhibitor of the form-1 isoenzyme but a much weaker inhibitor of the form-2 isoenzyme, the properties of form 1 resemble those of form 2. It has been suggested that the interaction of arginine-31 and other residues in the active-site region could account for the characteristic properties of the form-2 enzyme and that a similar interaction with Ba++ can alter the properties of the form 1 enzyme to those resembling form 2.


**Studies on the active site of pig kidney aldehyde reductase**

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Pig kidney aldehyde reductase is a monomeric NADPH-dependent oxidoreductase that catalyses the reduction of a variety of aromatic, aliphatic and sugar aldehydes to their corresponding alcohols (Bosron & Prairie, 1972; Flynn et al., 1975). The enzyme is one of a group of widely distributed aldo–keto reductases (Bosron & Prairie, 1973; Bachur, 1976; Davidson et al., 1978), all of which have similar, but not identical, characteristics. The most thoroughly characterized aldehyde reductases to date are those from mammalian sources, namely pig kidney (Flynn et al., 1975; Davidson and Flynn, 1979a; Morpeth & Dickinson, 1980), pig liver (Brantlan & Biellman, 1980), human liver (Wermuth et al., 1977) and rat liver (Felsted et al., 1974). However, the enzyme from these tissues is almost certainly identical with L-gulonate:NADP+ oxidoreductase (EC 1.1.1.19) (also previously known as ‘TPN-linked hexose dehydrogenase’: Bosron & Prairie (1972)), but it is currently referred to by workers in the field as NADPH-dependent aldehyde reductase from a specific organ and species.

Aldehyde reductase is intrinsically interesting enzymologically. Its single-subunit nature makes it a model for the study of nicotinamide-nucleotide–coenzyme–enzyme interactions without the complications of additional subunits. The enzymes are also interesting from an evolutionary point of view.

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Compositional studies have shown aldehyde reductases to be the most rapidly evolving family of oxidoreductases (Davidson & Flynn, 1979b), and it has been speculated that monomeric oxidoreductases such as aldehyde reductase and octopine dehydrogenase may be representative of a link between ancestral dehydrogenases and present-day existing oligomeric dehydrogenases (Flynn et al., 1975; Wermuth et al., 1977). Further evidence on this aspect will come from amino-acid-sequence studies on pig kidney aldehyde reductase (T. G. Flynn, unpublished work).

Aldehyde reductase also provides a model for examining the mechanism of action of an oxidoreductase at the single-subunit level. The kinetic mechanism of pig kidney aldehyde reductase is ordered BiBi, with NADPH adding first (Davidson et al., 1979), although a more complex mechanism has been suggested for the human liver enzyme (Wermuth & von Wartburg, 1979). In relation to these studies, and to form a base for determining the chemical reaction mechanism, we and others (Wermuth et al., 1977; Morpeth & Dickinson, 1980) have begun examining essential residues at the active site.

**Chemical-modification studies**

Arginine. This amino acid has been located at the nicotinamide-coenzyme-binding site of many oligomeric dehydrogenases (see, for example, Borders & Riordan, 1975; Lange et al., 1975; Ehrlich & Colman, 1978). It has been suggested that a major biological function of arginine may be interaction with phosphorylated substrates and coenzymes (Riordan et al., 1977). There are several apparently selective chemical reagents for the modification of arginine residues, and Davidson & Flynn (1979a) took advantage of butane-2,3-dione for the modification of pig kidney aldehyde reductase. The enzyme was rapidly inactivated by the reagent, and it was