

Crystallographic investigation of the cooperative interaction between trimethoprim, reduced cofactor and dihydrofolate reductase

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The structure of the complex between *E. coli* (RT500) form I dihydrofolate reductase, the antibacterial trimethoprim and NADPH has been determined by X-ray crystallography. The inhibitor and cofactor are in mutual contact. A flexible chain segment which includes Met 20 is in contact with the inhibitor in the presence of NADPH, but more distant in its absence. By contrast, the inhibitor conformation is little changed with NADPH present. We discuss these observations with regard to the mutually cooperative binding of these ligands to the protein, and to the associated enhancement of inhibitory selectivity shown by trimethoprim for bacterial as opposed to vertebrate enzyme.

Crystal structure Drug-enzyme interaction Dihydrofolate reductase Cooperativity

1. INTRODUCTION

Dihydrofolate reductase (EC 1.5.1.3) (DHFR) is an intracellular receptor for a number of drug molecules. Inhibition of the DHFR-catalysed reaction, the NADPH-dependent reduction of dihydrofolate, attenuates the biosynthesis of purines, thymidylate and certain amino acids [1]. Some drugs show selective inhibition of DHFR from different species; for example, trimethoprim (TMP) is used as an antibacterial and pyrimethamine as an antimalarial [2]. For TMP an approx. 3000-fold difference in K_i has been reported for mammalian compared with bacterial (*E. coli*) DHFR [3]. Cooperativity between inhibitor binding and cofactor binding has been observed for a variety of DHFR ligands [3–6] and it has been shown that a component of the selectivity depends upon the cooperative binding to DHFR of TMP and reduced cofactor, NADPH. This cooperativity is larger with *E. coli* than with mammalian enzyme [3]. It is not known whether cooperativity between cofactor binding and TMP binding arises through direct interaction between

the ligands at the active site or through conformational changes induced in the enzyme. Certainly, NMR studies have indicated the accessibility of more than one conformational state of the enzyme in some ternary complexes ([7–9]; also G.C.K. Roberts, personal communication).

We reported previously the crystal structure of the TMP-bound *E. coli* DHFR enzyme in the absence of NADPH [10,11]. More recently, this structure has been compared with the complex of TMP with NADPH and chicken DHFR [12]. This showed subtle differences in the binding sites for the drug, and these give rise to different drug conformations. Bolin et al. [13] have also compared a binary *E. coli* DHFR-methotrexate complex with a ternary *L. casei* DHFR-methotrexate-NADPH complex. We report here the structure of the ternary *E. coli* DHFR-TMP-NADPH complex, compare this with the binary *E. coli* DHFR-TMP complex and discuss possible structural contributions to cooperative binding. Attempts to diffuse NADPH into pre-grown *E. coli* DHFR-TMP binary complex (hexagonal) crystals resulted in loss of crystallinity. Co-crystallisation of the ter-

nary DHFR-TMP-NADPH complex gave rise to a new crystal form requiring de novo structure determination.

2. EXPERIMENTAL

2.1. Crystallisation and data collection

E. coli (RT500) form 1 DHFR [14] was crystallised from aqueous ethanol solutions at 4°C. Enzyme (20 mg/ml) was dialysed against 3 changes of 1 mM NADPH, 1 mM TMP, 3.6 mM CaCl_2 in 50 mM Hepes buffer (Na^+), pH 6.8, with 10% ethanol overnight. The solution was centrifuged at $20000 \times g$ for 30 min. 50- μl droplets were placed in a vapour diffusion apparatus and the reservoir contained variously 15, 17, 19 and 21% aqueous ethanol. Crystals grew as large trigonal bipyramids up to 2 mm long during a period of several weeks. The integrity of the co-crystallised NADPH over this time was demonstrated by spectral absorption at 340 nm after one such crystal had been dried and redissolved. The space group of the crystals was determined to be $P3_121$ or $P3_221$ with cell dimensions $a = 61.8 \text{ \AA}$ and $c = 105.8 \text{ \AA}$ having one enzyme molecule in the asymmetric unit. $\text{CuK}\alpha$ X-ray diffraction data to 3.0 \AA resolution were collected using an Arndt-Wonacott rotation camera [15]. Upon data reduction, the R factor ($R = \Sigma |F_o - F_c| / \Sigma F_o$) for data collected from two native crystals and subsequently merged was 0.113 (5007 unique data, each arising from typically 4 or 5 reflexions). Two heavy-atom derivative data sets were collected from crystals which had been soaked in ethylmercuriphosphate or *p*-chloromercuribenzoate and which gave rise to X-ray intensity differences.

2.2. Structure solution

Early attempts to solve reflexion phases by use of the isomorphous heavy-atom derivatives of these crystals failed, since the difference-Patterson maps eluded interpretation. It was therefore decided to use the procedure of 'molecular replacement' to discover the orientation and location of a known related structure, the protein part of the previously determined binary *E. coli* DHFR-TMP complex, in the ternary complex unit cell, and use the atomic coordinates to calculate phases. Crowther's method [16] was used to determine the

rotation of the molecule, and an R factor search [17] in 3 dimensions in each of the space groups $P3_221$ and $P3_121$ was used to determine its translation. The rotation procedure calculated a map with grid separation in spherical polar coordinates of 5°. A complete vector-space search was done ignoring the inherent symmetry within the unit cell. Thus it was possible to identify 6 peaks in the map that were related by point group 32 symmetry, and whose coordinates could be transformed by the appropriate symmetry operation to a common value. The process gave rise to an initial approximate solution, derived from the highest peaks in the rotation function. This orientation was applied to the known molecule which was then subjected to a translation search by structure factor calculation within the unit cell of the ternary complex crystal. For each space group it was necessary to scan half the unit cell volume at approx. 1.1 \AA intervals; thus nearly 1.4×10^4 translations required to be examined. For each translation, 1090 structure factors to 4 \AA resolution were calculated. The procedure gave an unequivocal minimum R factor for one particular grid point in space group $P3_221$.

At this point the orientation and translation were used to determine reflexion phases, and difference maps were calculated for each of the two mercury-containing derivatives. Two clear peaks emerged in the ethylmercuriphosphate map and one in that of *p*-chloromercuribenzoate. The peak common to each derivative appeared to be close to Cys 85 which supported the molecular replacement solution. As a further check, this solution was found to avoid interpenetration of molecules. The approximate solution was further refined using a selected shell of data between 6 and 4.5 \AA resolution, which reduced the R factor from 0.41 to 0.34 against a background R factor of 0.43 and above for incorrect solutions.

2.3. Model building

Several 'omit' electron density maps, one for each region requiring interpretation, were calculated from all observed X-ray data to 3.0 \AA , with phases determined from atoms of the structure (90–95%) outside the region under scrutiny. Detailed fitting to the electron density in the omit regions was performed at Oxford University on an Evans and Sutherland PS2 display linked to a PDP 11/70; later this work was transferred to an Evans

and Sutherland PS300 linked to an in-house VAX 11/750. In each case the molecule fitting was achieved through use of the program FRODO [18]. Adjustment was stopped with an *R* factor of 0.44 for all data to 3 Å resolution.

3. RESULTS

3.1. Trimethoprim

The principal torsion angles (τ_1 and τ_2) between the rings of TMP differ rather little between the binary and ternary complexes of TMP with *E. coli* DHFR. For the binary complex they are respectively [12] 177 and 76° and in the presently unrefined ternary complex model 192 and 56°. This slight difference of torsion angles leads to small changes in the contacts between TMP and those neighbouring residues which remain essentially unshifted in the two types of complex. One *m*-methoxy group is positioned in van der Waals contact with the side chains of Leu 28 and Phe 31, slightly shifted from its binary complex location close to Ile 50 and Leu 54 side chains; the other is repositioned midway between peptide 49/50 and the newly located Met 20 side chain (see below and figs 1 and 2).

3.2. Cofactor

The binding of reduced cofactor to *E. coli* DHFR had not previously been determined by X-

ray crystallography, although other studies have shown how NADPH binds to *L. casei* [19], chicken [20,21] and mouse L1210 [22] enzymes. The location and conformation of the cofactor appear to be similar in all cases, including *E. coli* enzyme, and the rms deviations between molecules of DHFR-bound cofactor superimposed one on the other are 0.9 Å for *E. coli* DHFR compared with *L. casei* DHFR, and 1.2 Å compared with chicken DHFR. The corresponding value for *L. casei* enzyme vs chicken enzyme is 1.0 Å. (See table 1 for details of the superposition method.) As indicated by Filman et al. [19] the residues and contacts involved in cofactor binding are highly conserved. In common with the other ternary complexes determined, the reduced end of the cofactor is close to the pyrimidine ring of the inhibitor and the amide is probably hydrogen-bonded to the main chain of Ala 7, as reported for *L. casei* enzyme [19]. The nicotinamide group is also close to the bridging methylene of TMP and one edge of the benzene ring as well as to one *m*-methoxy group.

3.3. Protein

Over the majority of the protein structure the chain folding differs little from that observed for the *E. coli* binary complex. A marked difference is observed however in the vicinity of residues 15–21, and as can be seen from fig.1 the change in confor-

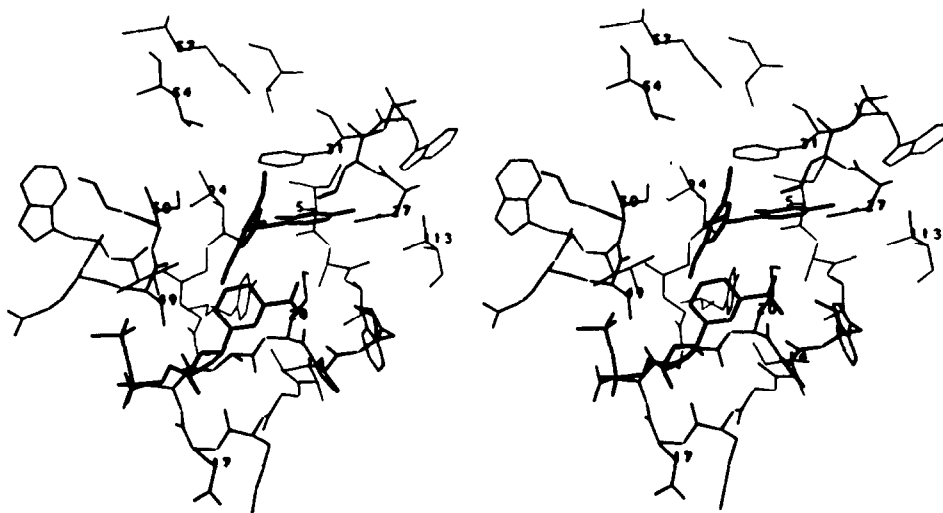


Fig.1. Stereo skeletal representation of part of the active site of *E. coli* DHFR in ternary complex with TMP and cofactor NADPH. The two ligand molecules are drawn with bold lines.

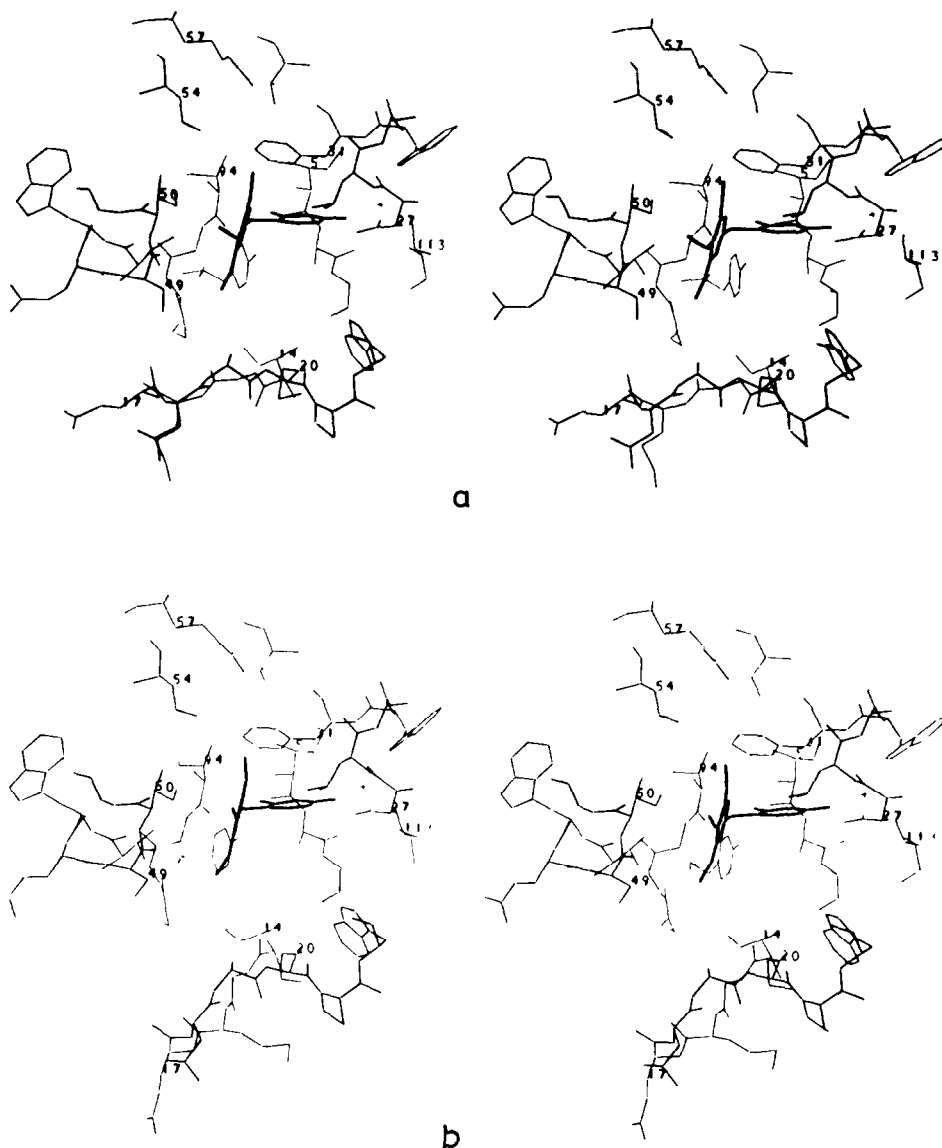


Fig.2. Stereo skeletal representation of part of the active site of the binary *E. coli* DHFR-TMP complex. Two crystallographically independent molecules are observed, (a) molecule I and (b) molecule II [12]. The TMP skeletons are shown in bold lines.

mation of the peptide chain in this region brings Met 20 into contact with the inhibitor and with the nicotinamide moiety of the cofactor. Residues 15–21 are not directly involved in intermolecular packing as they are in the binary complex. In section 4 the segment of chain containing these residues, and its counterpart in other DHFRs, will be termed the 'flexible loop'.

4. DISCUSSION

Table 1 compares flexible loop regions of various DHFR complexes, including those of *E. coli*. The smallest rms difference (0.7 Å) occurs between the two ternary complexes of highest resolution; however, rms differences not much greater occur between either of these and the

Table 1

The rms differences in α -carbon positions (\AA) of residues in the sequence 13–23 (*E. coli* numbering) between DHFR molecules superimposed from different crystal structures

DHFR complex (resolution)	<i>E. coli</i> DHFR molecules in binary complex with TMP (2.3 \AA resolution) [12]		DHFR molecules in ternary complex	
	Molecule I	Molecule II	<i>E. coli</i> / TMP/NADPH	<i>L. casei</i> / MTX/NADPH
<i>E. coli</i> binary molecule II (2.3 \AA)	1.8			
<i>E. coli</i> ternary (3.0 \AA)	2.4	1.8		
<i>L. casei</i> ternary (1.7 \AA) [13]	2.7	2.2	1.0	
Chicken ternary (2.2 \AA) [12]; 10 residues only	2.8	2.2	1.1	0.7

Each value was obtained after superposition of a pair of complexes so that the discrepancy between each whole set of α -carbons was minimised, followed by a further translation to achieve closer coincidence of the particular region of interest. The residual rms value then provides a comparison of conformations (The numbering of the distinct *E. coli* binary molecules follows the convention of Matthews et al. [12].)

unrefined, lower-resolution, *E. coli* ternary structure, for which errors in atomic positions will be somewhat larger. The tabulated differences point to a common conformation in complexes that contain NADPH and inhibitor, a conformation different from those observed in the *E. coli* binary complex which lacks NADPH. Moreover, the two crystallographically distinct molecules in the binary complex show conformations in this region substantially different from each other. This gives rise to the idea of a mobile region which can adopt different conformations in the absence of cofactor but which folds in a distinct fashion in a ternary complex. It happens that the flexible loops of molecules I and II of the binary complex are involved in intermolecular packing whereas this loop in the ternary complex is remote from adjacent molecules. Certainly, our attempts to prepare the ternary complex crystals by diffusion of NADPH into hexagonal crystals of the binary complex resulted in disorder, presumably because a cofactor-induced conformational change in the flexible loop disrupted important intermolecular interactions. Conversely, no trigonal crystals were observed during crystallisation of the binary complex, despite the use of similar conditions of growth, and it may be reasonable to suppose that the ternary conformer of the flexible loop is not favoured in the absence of cofactor.

To what extent might such conformational flexibility within the enzyme contribute to cooperativity between cofactor binding and inhibitor binding? Some published values for cooperativity are given in table 2. If we compare the interactions between TMP and the enzyme in the ternary conformation with those observed for TMP with the binary conformer we note that the overall interaction is

Table 2

Some cooperative interactions between trimethoprim, NADPH and DHFR

DHFR type	Cooperativity ^a	Reference
<i>E. coli</i> form 1 ^d	41 ^b	[3]
<i>E. coli</i> form 2 ^d	230 ^c 123 ^b	[3]
<i>L. casei</i>	100 ^b 135 ^{ba}	[3] [4]
Mouse	4.7 ^c 2.8 ^b	[3]

^a Binary complex is with inhibitor except for *L. casei* [4] where it is with NADPH

^b Ratio of binary and ternary dissociation rate constants

^c Ratio of dissociation constants

^d Isozyme forms 1 and 2 of *E. coli* (RT500) DHFR differ by a single amino acid substitution [14]

enhanced by the additional contacts between ligand and enzyme provided by the ternary conformer (notably between the substituted trimethoxybenzene and the side chain of Met 20). Furthermore, one would predict that NADPH binding in the ternary complex is enhanced by interactions additional to those that would be made with the binary conformer (notably between the ribose and residues 17 and 18 of the flexible loop, and between the nicotinamide and side chain of Met 20). Progressive binding of ligands, by favouring the ordered conformer, would increase the proportion of this tighter-binding species, and a positive cooperativity would result. Observations on a variety of DHFR-NADPH-inhibitor complexes [12,19–22] show that the nicotinamide of cofactor interacts with some part of the inhibitor, and no doubt a component of cooperativity arises from direct interaction between both types of bound ligand. The negative cooperativity observed for the binding of certain substrate analogues [6] might be explained by direct steric interference between inhibitor and cofactor, or a preference of the inhibitor for the flexible conformer.

With TMP binding, smaller positive values for cooperativity are obtained for mouse than for *E. coli* enzyme (table 2). In the chicken enzyme ternary complex, as with mouse, the conformation of TMP is substantially different from that in the *E. coli* enzyme complex [12]. This gives rise to a different position and orientation for the trimethoxybenzyl group. Moreover, the pyrimidine is somewhat differently located. This TMP conformer is readily accommodated in a cleft significantly larger than that of *E. coli* DHFR. However, this is at the expense of certain interactions with the cofactor and with the flexible loop. Leu 22 (homologous to Met 20 of *E. coli* enzyme), whilst able to interact with the nicotinamide of NADPH, is somewhat distant from TMP (4 Å from the bridging methylene). Thus, the lower cooperativity exhibited in vertebrate enzyme ligand binding might be due to a decrease in the number of contacts between inhibitor, cofactor and enzyme compared with the analogous *E. coli* complex, although possible contributions may also arise from intrinsic energy differences for the conformational transition.

Further understanding of the cooperativity could result from determination of the structures

of the *E. coli* apoenzyme and of the corresponding DHFR-NADPH binary complex.

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