THE BINDING OF TRIMETHOPRIM TO BACTERIAL DIHYDROFOLATE REDUCTASE

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1. Introduction

Dihydrofolate reductase (DHFR, EC 1.5.1.3) catalyses the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate; further metabolites of tetrahydrofolate are involved in the incorporation of single-carbon units into purines, pyrimidines and amino acids [1,2]. The inhibition of DHFR can therefore lead to a deficiency of the components of nucleic acids and proteins, to eventual cessation of DNA synthesis and hence to cell death. DHFR inhibitors are used in the control of a number of disease processes including various tumours and bacterial infections. Understanding of inhibitor binding at the molecular level is of potential value in the design of drugs having greater inhibitory potential and improved selectivity towards the appropriate organism. Trimethoprim (TMP), a widely used antibacterial drug [3–6], is a potent inhibitor of bacterial DHFRs but a much weaker inhibitor of the vertebrate enzymes (e.g., IC_{50} values against *Escherichia coli* and human enzyme [7] are, respectively, 5×10^{-9} M and 3×10^{-4} M). To provide information on the action of this drug at the molecular level, we have determined the structure of the binary complex of *E. coli* (strain RT500) form I DHFR with TMP and compared it with that of the complex of DHFR with methotrexate (MTX), a drug which binds tightly to both bacterial and vertebrate DHFR. The structure of our TMP–enzyme complex differs from that in [8] of an MTX–enzyme complex from a different strain (MB1428) of *E. coli*. The amino acid sequences of the two enzymes are currently thought to differ at 3 positions [9].

2. Experimental

Escherichia coli (RT500) form I DHFR was prepared as in [10] and was crystallized from aqueous ethanol by vapour diffusion. Hexagonal bipyramidal crystals, space group $P6_1$, with axial length of up to 1.2 mm grew in ~1 month. The unit cell dimensions ($a = b = 93.6$ Å; $c = 73.9$ Å) were similar whether crystals were grown in the presence of TMP or MTX and resemble those reported for the MTX–enzyme complex in [8]. One isomorphous heavy atom derivative was prepared by soaking DHFR–TMP crystals in saturated solutions of sodium diuranate, another by growing crystals in the presence of dithiothreitol and transferring them to a solution containing ethylmercuriphosphate. X-ray data were collected using an automatic [11] rotation camera (Enraf Nonius) and a graphite monochromator (Stöe). Heavy atom sites were located by difference Patterson and difference Fourier functions. The parameters for 1 uranium and 7 mercury sites were refined by minimisation of lack of closure [12] and data to a minimum spacing of 2.8 Å were used in the calculation of electron density maps. Two protein molecules in the crystallographic asymmetric unit were located in maps plotted at a scale of 0.15 cm/Å and, with the aid of the amino acid sequence of the enzyme [13], the peptide chain was traced in maps at a scale of 0.75 cm/Å. Kendrew skelelal models were fitted to maps at a scale of 2.0 cm/Å using a modified design of the optical comparator devised in [14].

3. Results and discussion

The overall folding of the polypeptide backbone is substantially in accord with that in [8] for the *E. coli* DHFR–MTX complex. An 8-stranded β-sheet begins at the N-terminus and ends, with its only antiparallel strand, at the C-terminus. Viewed in the direction of

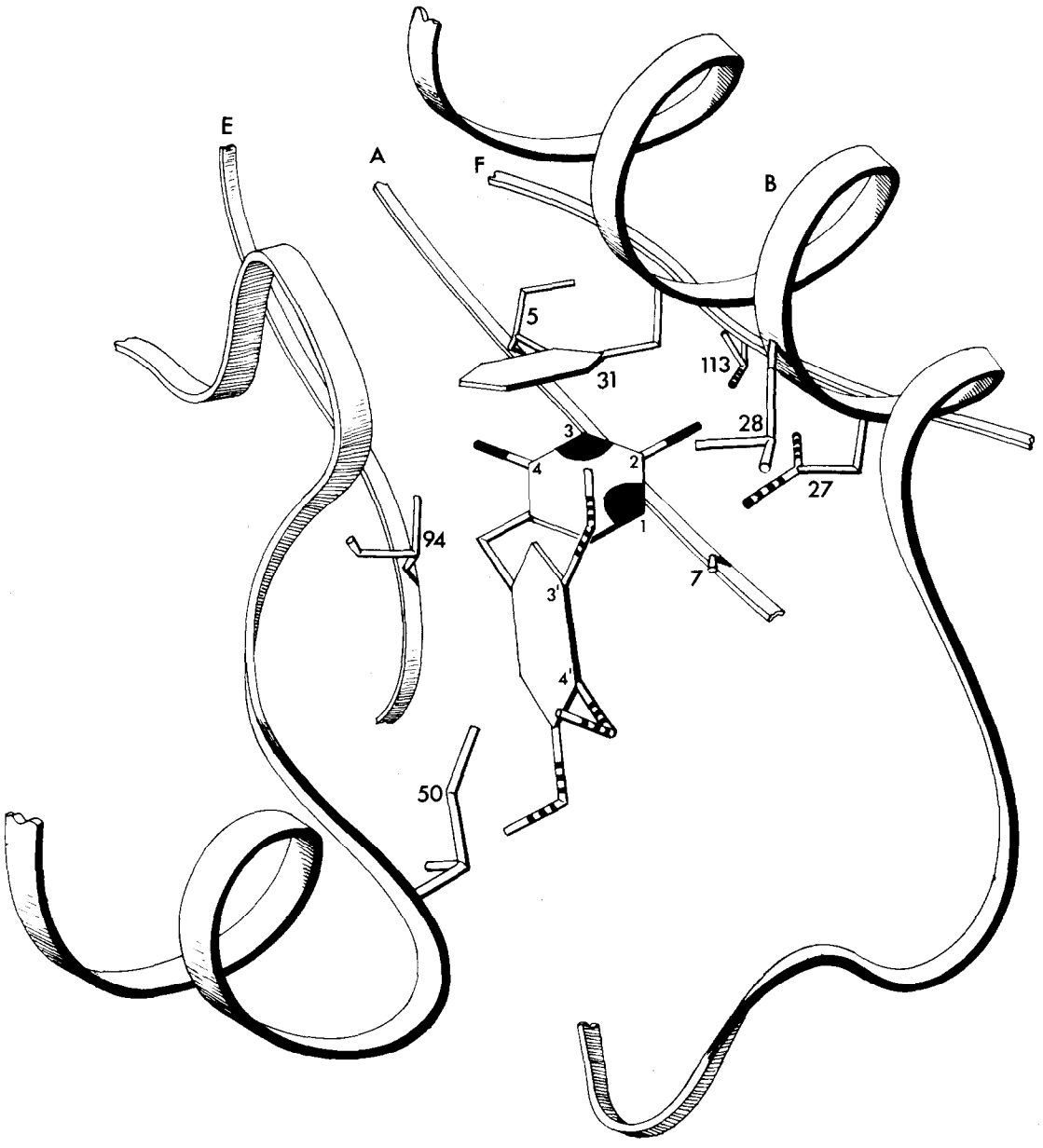

Fig.1. Schematic illustration of the binding site for TMP in *E. coli* DHFR. Nitrogen atoms are shown black, oxygen striped. Only those side chains which might interact with TMP are shown. Three segments of peptide chain form the rear of the binding cleft and these are adjacent strands in the β-sheet (E, A and F in the nomenclature of [8]). The left-hand side of the cleft is formed by irregular peptide chain whilst the right-hand side contains an irregular region and contiguous helix (B). The diaminopyrimidine ring is shown surrounded by Asp 27, Phe 31, Ile 94, Thr 113, and the protein chain between Ile 5 and Ala 7. Tyr 100 is omitted for clarity but would appear below and well behind the ring. The trimethoxybenzyl moiety of TMP is partially enclosed by the Phe 31 above, Ile 50 on the left and Leu 28 on the right, the latter two groups being situated at the entrance to the cleft.

the strands, the sheet shows the usual right-handed twist [15].

The binding of TMP is similar in each of the two molecules in the asymmetric unit; it lies in a prominent cleft having irregular and helical peptide chain on one side and irregular chain on the other (fig.1). The face of the β-sheet closes the cleft at the rear. N1 and the 2-amino group of TMP are ~3 Å from the carboxyl oxygens of Asp 27. The side chain of Phe 31 is close to the pyrimidine and benzyl methylene of TMP while Ile 94 is also close to this methylene and to the 4-amino group. Thr 113 is near the 2-amino group and may accept a hydrogen bond from it. The 4-amino group, C2 and N3 are all close to the first β-strand, in the region of residues 5–7. The 4-amino group is in the vicinity of the hydroxyl substituent of Tyr 100 but it is not clear whether the oxygen and nitrogen atoms are close enough to form a hydrogen bond. The phenyl ring of TMP is directed outwards from the enzyme cleft with the ring plane roughly aligned with the cleft. The sidechain of Ile 50 is close to the 4′-methoxy group. The 3′-methoxy group approaches Leu 28 on the other side and also the edge of the ring of Phe 31.

Both TMP and MTX contain the diaminopyrimidine moiety. To establish whether there are similarities in the binding of this part of the 2 drugs we have also grown and observed DHFR–MTX crystals and have calculated the difference Fourier electron density between the MTX and TMP complexes. The difference features near the diaminopyrimidine rings are not identical for the 2 molecules in the asymmetric unit. In molecule 1 only there are indications that the pyrimidine ring of MTX may be slightly tilted relative to that in TMP away from the Phe 31. The carboxyl group of Asp 27 may also be slightly displaced away from Phe 31. There are also suggestions of small shifts in Phe 31 and Ile 50. No such differences have been detected in molecule 2.

Without further refinement of the crystal structure it would be premature to offer explanations for such small differences. The essential similarity in the TMP binding to the 2 protein molecules suggests that the binding we observe is not substantially affected by crystal packing forces, despite the fact that the clefts of the 2 protein molecules are in different environments within the crystal and are therefore subject to non-equivalent perturbations. In [8] there was a difference in hydrogen bonding between the protein and the glutamate moiety of MTX in the 2 molecules of their complex, and comparable small differences may become apparent when the current TMP complexes are refined.

The dissociation constant for TMP in the binary complex with *E. coli* enzyme has been estimated [16] as 15 nM and the observed structure of the complex between DHFR and TMP provides some insight into the source of the binding energy for this drug. The diaminopyrimidine ring interacts with the protein both ionically and through hydrogen bonding and there are many Van der Waal's contacts between protein and drug. The observed conformation of TMP in the crystalline binary complex is compatible with that deduced by solution NMR studies [17] and similar to that observed in the crystal structure of TMP–HBr itself [18,19]. The binding of the diaminopyrimidine moiety to an acidic group in the protein is consistent with an analysis [20] of UV difference spectra which indicated an increase in pK_a of TMP upon binding to DHFR.

The TMP geometry which we have observed in the DHFR–TMP binary complex is similar to that used to explain inhibition of the enzyme in the ternary complex by analogues of TMP [19,21]. Some structural differences may exist between the binary and ternary complexes which could explain the observation [10] that the dissociation constant of TMP from the ternary complex is 0.1 nM and therefore ~150-fold smaller than that for the binary complex. The possibility that these differences are small and localised is suggested by the observation that the protein backbone and the inhibitor conformation in the ternary *L. casei* DHFR–MTX–NADPH complex is similar to that in the binary *E. coli* DHFR–MTX complex [22] despite the substantially different amino acid sequences and the presence of cofactor in one of the complexes. To ascertain the extent of any such differences, we have grown and are studying crystals of the *E. coli* DHFR–TMP–NADPH complex. (These differ in form from the crystals of the *E. coli* DHFR–TMP complex.)

We also wish to understand why MTX, unlike TMP, inhibits mammalian DHFR much more effectively than bacterial DHFR. The structural features of MTX which give rise to this difference in binding energy would probably include: the pteridine group (in contrast to the pyrimidine of TMP), a glutamate moiety, and a differently located benzene ring (due to its linkage with pteridine). It would be premature to hypothesize on these points at present, so to study this further

we have crystallised the mammalian enzyme and are now determining that structure.

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