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Letter to the Editor: NMR-based solution structure of the complex of *Lactobacillus casei* dihydrofolate reductase with trimethoprim and NADPH

Vladimir I. Polshakov[a,c], Eugeni G. Smirnov[c], Berry Birdsall[a], Geoff Kelly[b] & James Feeney[a,*]
[a]*Division of Molecular Structure and* [b]*MRC Biomedical NMR Centre, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.;* [c]*Center for Drug Chemistry, Moscow 119815, Russia*

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Abbreviations: C12E5, n-dodecyl-penta(ethylene glycol); DHFR, dihydrofolate reductase; DHPC, dihexanoyl phosphatidylcholine; DMPC, dimyristoyl phosphatidylcholine; TMP, trimethoprim.

Biological context

Dihydrofolate reductase (DHFR) catalyses the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate using NADPH as co-enzyme (Blakely, 1985). This enzyme has proved to be an excellent target for antifolate drugs such as methotrexate (anticancer), pyrimethamine (antimalarial) and trimethoprim (antibacterial). Such agents act by inhibiting the enzyme in parasitic or malignant cells (Blakely, 1985; Matthews et al., 1985). For example, the usefulness of the antibacterial agent trimethoprim (TMP) stems from the fact that it binds at least 3000 times more tightly to bacterial DHFR than it does to human DHFR as determined by enzyme inhibition (Dauber-Osguthorpe et al., 1988). Although X-ray crystal structures of TMP complexes with bacterial and vertebrate DHFR have been studied, no single hypothesis convincingly explains the molecular basis of TMP binding specificity (Baccanari and Kuyper, 1993). A large part of the decrease in binding to the human enzyme can be attributed to the loss of the co-operative binding involving TMP and NADPH as seen in the complex with the bacterial enzyme (Baccanari et al., 1982). We are undertaking a comparison of the solution structures of binary and ternary complexes of DHFR formed with TMP and NADPH in order to reveal differences which might be responsible for the co-operative binding effect in this ternary complex with bacterial DHFR. Here we report on the NMR assignments and structure of *L. casei* DHFR-TMP-NADPH, the first determination of a solution structure for a DHFR ternary complex.

*To whom correspondence should be addressed. E-mail: jfeeney@nimr.mrc.ac.uk

Methods and results

The preparation of ^{15}N-labelled *Lactobacillus casei* DHFR has been described earlier (Gargaro et al., 1998). Equimolar complexes of DHFR-TMP-NADPH (ligands from Sigma) were prepared as 1–4 mM concentration samples in either D_2O or 90% H_2O/10% D_2O and 50 mM potassium phosphate, 100 mM KCl, $pH^* = 6.5$.

2D and 3D NMR experiments were carried out on Varian UNITY and UNITY plus spectrometers operating at 600 and 500 MHz and the results were analysed to obtain the spectral assignments and the restraints required for structural analysis (see supplementary material provided in the electronic version of this paper). 2724 distance, 337 dihedral angle, 100 chemical shift, 76 ^{3}J coupling and 229 residual dipolar coupling constant (RDC) restraints were obtained. The CNS program (Brünger et al., 1998) with a simulated annealing protocol was used in the structure calculations. Initially only unambiguously assigned NOEs and torsion angle restraints were used in the calculations but the final refinement included all the experimental restraints and contained 10 ps of high

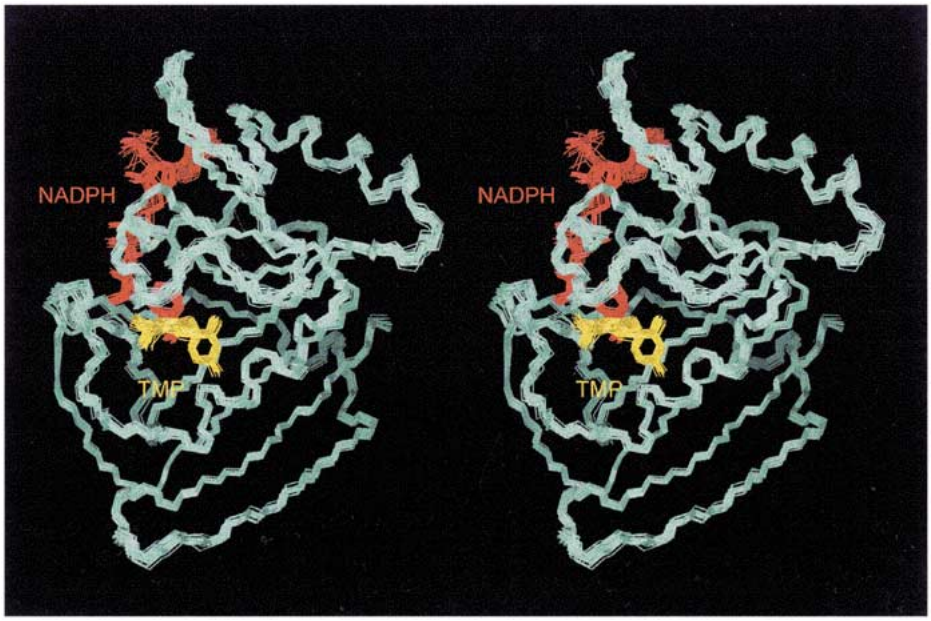

Figure 1. Stereoview of a superposition over the backbone atoms (N, Cα and C) of residues 1–162 of the final 24 structures of the DHFR-TMP-NADPH complex. The ligands TMP and NADPH are coloured yellow and red respectively. The superposition was made onto the backbone atoms of the representative structure, S_{rep}.

temperature (1000 K) restrained molecular dynamics followed by a 40 ps slow cooling stage and 1000 steps of energy minimisation. The final ensemble contained 24 structures (quality defined in Table 1 and Figures 2S to 8S in the Supplementary materials). The overall structure of the protein complex (see Figure 1) contains the well established eight stranded β-sheet structure with four α-helixes arranged two on either side of the β-sheet. TMP lies in a cleft while NADPH binds in an extended conformation over the surface of the protein. Details of the interacting residues in the binding site regions are shown in Figures 2a and 2b.

The spectral assignments and the structure ensemble for the DHFR-TMP-NADPH complex have been submitted to the BioMagRes Bank (accession # 5396) and the PDB (accession # 1LUD) respectively.

Discussion and conclusions

Crystal and solution structures of several complexes of DHFR have been reported (Bolin et al., 1982; Matthews et al., 1985; Sawaya and Kraut, 1997; Gargaro et al., 1998; Martorell et al., 1994; Polshakov et al., 1999; Groom et al., 1991; Li et al., 2000). Crystal structures of complexes with NADPH can suffer from stability problems because NADPH is unstable at pH values below 7 and several authors have mentioned or alluded to this problem (Li et al., 2000; Groom et al., 1991; Champness et al., 1994; Stammers et al., 1993). Fortunately, in our solution structural studies the integrity of the NADPH can be monitored continuously by NMR.

Conformation of bound TMP and its binding site

The structure of the bound TMP is very well defined in the family of structures (see Figure 1) with an RMSD value of 0.66 ± 0.25. The values of the torsion angles τ_1 and τ_2 are 195.57° ± 7.72 and 73.99° ± 7.51, respectively (where τ_1 is defined as C4-C5-C7-C11 and τ_2 as C5-C7-C11-C12). These are in excellent agreement with values calculated earlier from ring current chemical shift calculations (Birdsall et al., 1984).

The environment of the 2,4-diaminopyrimidine ring is very similar to the corresponding moiety in TMP, methotrexate and trimetrexate, in binary complexes with DHFR. The protonated N1 atom of TMP lies within hydrogen bonding distance of the OD1 atom of D26 (2.04 ± 0.05 Å for the N-O distance), in full agreement with previous findings (Roberts et al., 1981) that showed that TMP is protonated at N1 and involved in electrostatic interactions with the protein. The hydrogen bonding interactions formed by the 2-

amino and 4-amino protons with groups on the protein (Figure 2a) agree closely with similar interactions seen for the 2,4-diaminopyrimidine moiety of bound TMP, methotrexate and trimetrexate in their binary complexes (Gargaro et al., 1998; Polshakov et al., 1999; Martorell et al., 1994). The structure of the TMP trimethoxy ring is also well characterized with the 3′ and 5′-methoxy groups being in the plane of the aromatic ring and the 4′-methoxy being out of the plane and oriented mainly towards the methyl groups of L19 and L27. The latter residues, together with H22, F30 and P50 form a pocket surrounding the three methoxy-groups.

Conformation of bound NADPH and its binding site

The structures of the nicotinamide and adenine rings and their ribose moieties are all fairly well defined (RMSD values 0.65 ± 0.25) with the pyrophosphate group being less well defined (see Figure 1, and Figure 7S in the Supplementary materials). The nicotinamide carboxamide group is in the trans-conformation and its O7 atom forms a hydrogen bond with the backbone HN amide proton of A6 and its NH_2 protons form hydrogen bonds with the carbonyl oxygen atoms of residues A6 and I13 (see Figure 2b). An earlier detailed analysis of the available PDB structures of DHFR with $NADP^+$ and NADPH (Polshakov et al., 2002) showed that no large structural changes in the protein accompanies the change in oxidation state of the nicotinamide ring with the carboxyamide group in each form interacting with the same atoms on the protein and with the same pattern of hydrogen bonds. In the present structure the ribose rings are both bound in a 3′-endo conformation. The nicotinamide ribose ring is close to G17, R44 and S48 and the adenine ribose ring is close to R43, Q101 and I102. The NADPH 2′-phosphate group is close to R43, T63 and Q65. A direct interaction of this group with the R43 guanidino NHε had previously been detected from the $^1H/^{15}N$ chemical shift changes in R43 on binding NADPH (Gargaro et al., 1996) and an interaction with the T63 OH proton had been inferred on the basis of the observed long lifetime of this OH proton. The adenine ring itself is very well defined in the structure, binding in a hydrophobic slot formed by L62, H64, and V79. Although the conformation of the pyrophosphate moiety is less well defined it is observed close to R44, T45, G99, A100 and Q101. Thus the overall structure of the NADPH and its interactions with the protein are very similar to those seen for bound NADPH in the X-ray structure of *L. casei* DHFR with methotrexate and NADPH (Bolin et al., 1982).

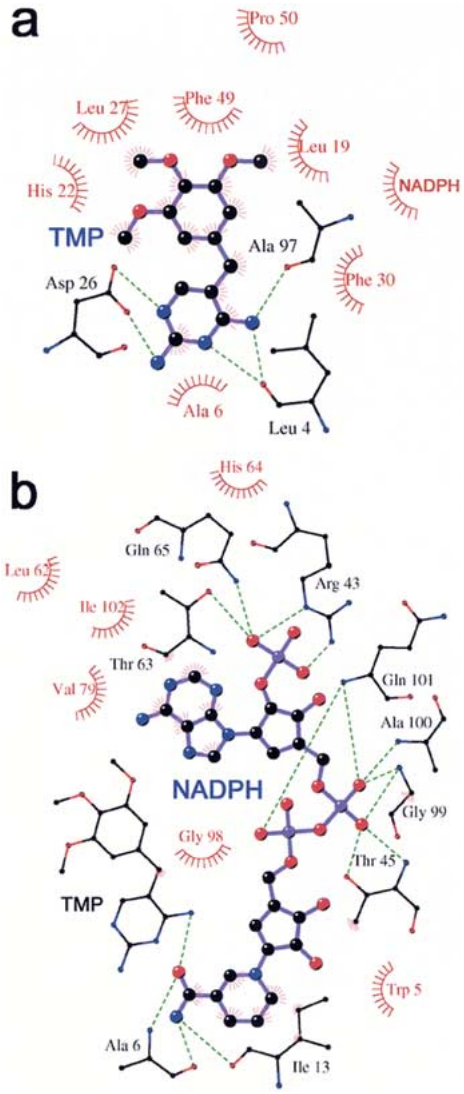

Figure 2. Interactions between DHFR and the ligands (a) TMP and (b) NADPH. H-bonds distances below 3.5 Å between heavy atoms are indicated by dashed green lines. Protein residues involved in hydrophobic interactions are also indicated, in red. The figures were prepared using LIGPLOT (Wallace et al., 1995).

Contact between bound TMP and NADPH

Parts of the bound TMP and NADPH are in close proximity to each other and the contact region between the two ligands is illustrated in Figure 1 and Figure 7S (in the Supplementary materials). The closest approach is between the TMP H71 atom and one of the NADPH nicotinamide H4 atoms (1.59 ± 0.12 Å). Other short

distances are found between the TMP trimethoxy ring 3′-OCH_3 protons and the NADPH nicotinamide ribose H2′ and 2′OH and a possible interaction is indicated between one of the TMP 4-NH_2 protons and the nicotinamide O7 atom. Whether or not the intermolecular contacts themselves result in increased or decreased binding will be difficult to assess since in earlier studies on the ternary complex formed with TMP and the oxidised coenzyme ($NADP^+$), the intermolecular interactions between the ligands appear to be repulsive (Birdsall et al., 1980).

Comparison of the structure of the DHFR-TMP-NADPH ternary complex with binary complexes of related ligands such as methotrexate (Gargaro et al., 1998) and trimetrexate (Polshakov et al., 1999a) suggest that there will not be large conformational differences between the binary and ternary complexes. Thus in order to detect any differences between the structures it is important to obtain structures of high quality. The structure reported here, obtained by combining residual dipolar coupling constants and chemical shift restraints with an extensive list of distance and angular constraints, is the highest quality structure so far reported for a DHFR complex in solution. The availability of this structure and the spectral assignments will be of value for studying antifolate drug design, ligand binding specificity and co-operativity, protein and ligand dynamics (Polshakov et al., 1999a,b), and amide HN exchange behaviour.

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