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RATIONAL DESIGN OF AN INHIBITOR OF DETHIOBIOTIN SYNTHETASE; INTERACTION OF 6-HYDROXYPYRIMIDIN-4(3H)-ONE WITH THE ADENINE BASE BINDING SITE.

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Abstract: Modelling of the H-bonding interactions involved in the binding of the adenine base component of ATP to *E.coli* dethiobiotin synthetase (DTBS) suggested that 6-hydroxypyrimidin-4(3H)-one (6-HP4) should selectively bind to this site in the enzyme. Kinetic studies show that 6-HP4 is a competitive inhibitor of DTBS and x-ray crystallography shows that 6-HP4 binds specifically in the purine base binding site of the enzyme.

Keywords: ATP binding site, enzyme inhibitor, nucleotide-protein interaction, inhibitor design, dethiobiotin synthetase, biotin biosynthesis

Introduction

Mammalian vitamins are synthesised in small amounts by both microorganisms and plants and are as essential to the metabolism of the producing organisms as they are to species which obtain them through diet. It is well established that microorganisms which are, for example, *thi*$^-$ and *bio*$^-$ cannot survive without added thiamine or biotin. Thus, inhibition of synthesis of certain vitamins in producing organisms is as potentially debilitating and/or lethal as vitamin denial to animals. A potentially useful aspect of this argument arises from the fact that in producing species the enzymes of vitamin biosynthesis are present at exceptionally low concentrations. In the case of biotin we know that, at least in *E. coli*, the production of the four biosynthetic enzymes of the pathway are strictly regulated by feedback control.[1] It has been suggested that for *E. coli* cells the requirement for biotin could be as low as 100 molecules/cell [A. Campbell, personal communication]. Sanyal *et al*[2] have

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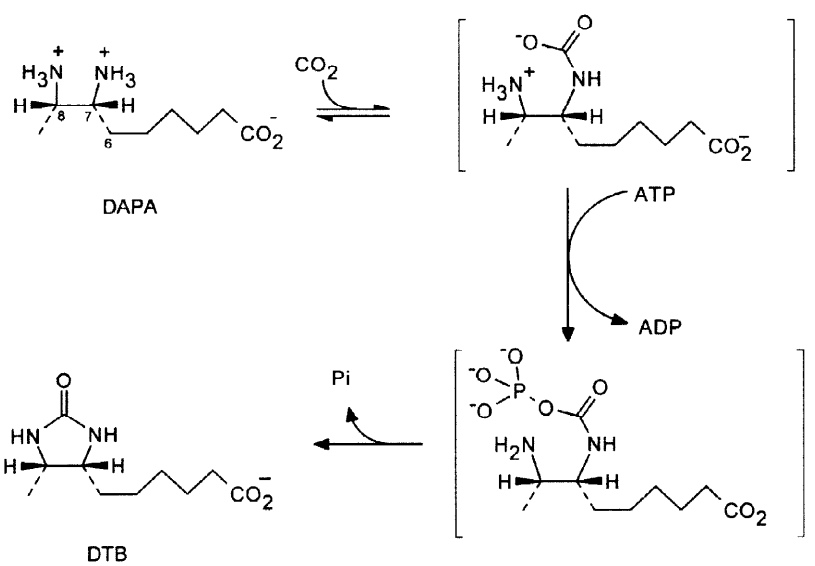
recently shown that the turnover of dethiobiotin to biotin by purified *E. coli* biotin synthase (which seems likely to be the slowest step in the biotin biosynthetic pathway) is less than 10 h^{-1} *in vitro* which might suggest that only a few molecules of the enzyme/cell may be sufficient for growth. Thus a specific inhibitor of a biotin biosynthetic enzyme could prove to be an extremely effective targeted biocide. To date, however, there have been few reports on the inhibition of the enzymes of this pathway, and so we have initiated a programme on the design of specific inhibitors.

The best studied of the enzymes of biotin biosynthesis is dethiobiotin synthetase (DTBS, E.C. 6.3.3.1), which is responsible for the penultimate step in the pathway. The sequence of reactions catalysed by DTBS involves (a) formation of an enzyme bound carbamate (from (7R,8S) 7,8-diaminononanoate (DAPA) and CO_2); (b) phosphorylation of the carbamate to give a phosphoric acid mixed anhydride; and (c) intramolecular substitution of the phosphate by the second amino group of the substrate to afford the final product (Scheme).[3-5] The crystal structure of DTBS has been solved[6,7] and its interactions with its substrate, DAPA, the intermediate DAPA carbamate, a synthetic DAPA carbamate mimic and ATP analogues characterised in some detail.[6-10]



Scheme: The reaction sequence catalysed by dethiobiotin synthetase.

A much vaunted, but as yet infrequently realised, justification of structural biochemistry is the use of accurate crystal structure data to design active site inhibitors of metabolically important enzymes which are structurally unrelated to the actual enzyme substrates. Thus, given the data established in previous studies, we wished to design a simple ligand which would target the nucleoside base binding site in the DTBS protein and thereby inhibit its action. Here we describe design of a minimal "adenine replacement" ligand, 6-hydroxypyrimidin-4(3H)-one (6-HP4); the inhibition of DTBS activity by 6-HP4; and the characterisation of the interaction of 6-HP4 with the purine binding site of DTBS by x-ray crystallography.

Results and discussion

The goal we set in this study was to design, from the detailed knowledge of the ATP binding site of DTBS, a small inhibitor molecule which would bind specifically to the purine binding elements of the DTBS protein and to no other sites on the protein surface. The data available from crystallographic studies[6-10] were that three atoms of the adenine base moiety of ATP (and of ADP and the ATP analogue AMP-PNP), namely the N1, N7 and the 6-amino nitrogen, formed specific hydrogen bonds to the enzyme as shown in Fig. 1 (top).

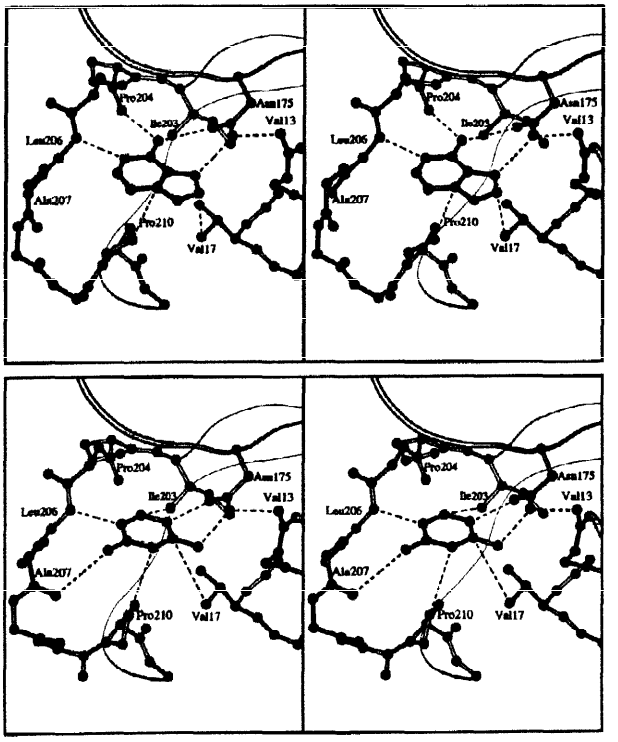


Figure 1: Stereodiagram of the nucleotide binding pocket in dethiobiotin synthetase showing the H-bonding contacts for the adenine of ATP (top) and for 6-hydroxypyrimidin-4(3H)-one (6-HP4) (bottom). The continuity of the main chain around residue 175, before 203 and after 210 is indicated by a continuous thread. Only those amino acid side chains which make direct contact with the ligands are shown.

The 6-amino nitrogen is bound between the carbonyl of Pro204 (at rear) and the carbonyl of the Asn175 side chain amide. The amine of Asn175 is hydrogen-bonded both to the N7 of adenine and to the main chain carbonyl of Val13. The Asn175-Val13 H-bond ensures that the side chain of Asn175 is in the optimal

conformation for the nucleotide binding. The main chain nitrogen of Leu206 (on the left) is bound to the N1 of adenine. Leu206 just precedes the 208-210 loop which is significantly disordered in the native protein and in all the complexes which have been examined so far. The flat adenine moiety is sandwiched between the hydrophobic Pro210 and Val17 and the loop Asn175-Thr178, the side chains of which are roughly parallel to the adenine ring (not shown). The structural specificity for adenine base binding is ensured by the shape of the binding pocket, by the hydrophobic interactions from the top and from the bottom of the flat purine ring and by the hydrogen bonds formed to the nitrogens N1, N6 and N7 in the plane of the adenine ring. The specific requirement for adenine is consistent with early enzymatic work[11] which has shown that while GTP and CTP can substitute for ATP in the DTBS catalysed reaction, these are relatively poor substrates. If guanine is modelled into the adenine-binding site then the position of the N6-amine of the adenine H- which is bonded to two cabonyls is taken by the O6-carbonyl group of guanine and this seriously disrupts the binding. In the case of cytosine the same position is occupied by the N4-amine but no other hydrogen bonds between the base and the enzyme can be formed. While consideration of the geometric constraint requirements imposed by the adenine base binding site of the enzyme shows that it can accommodate a variety of rigid monocyclic or bicyclic ligands, the H-bonding criteria focused our attention on the pyrimidine nucleus as the smallest template from which to start.

The structurally simplest candidate molecule which could satisfy the majority of geometric, H-bonding and charge distribution criteria proved to be 6-hydroxypyrimidin-4(3H)-one (6-HP4). .Two of the proposed binding modes of 6-HP4 in the adenine-binding pocket are shown in Fig. 2.

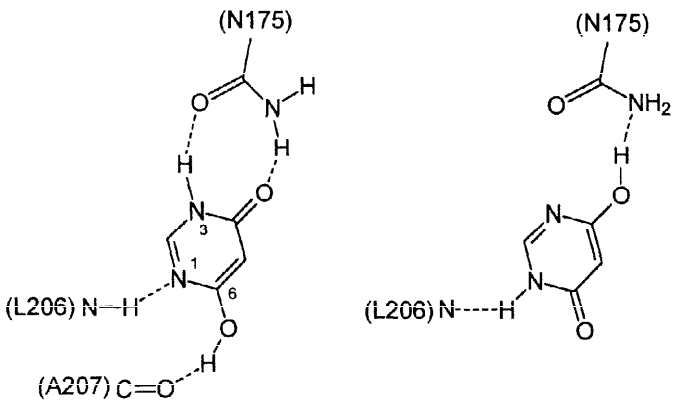


Figure 2: Schematic view of potential interactions of the 6-HP4 tautomers with DTBS protein residues. It should be noted that whilst a variety of donor-acceptor combinations is possible, that shown in (a) is almost certainly the major contributor.

We have soaked 6-HP4 into the crystals of DTBS and shown by x-ray crystallography that this ligand is bound specifically in the adenine-binding pocket. Kinetic measurements showed that 6HP4 is a competitive inhibitor for DTBS albeit with a fairly high K_i (11.2 ± 1.0 mM). As 6-HP4 does not have a charged sugar-

phosphate tail, it is to be expected that the 6-HP4 would bind much more weakly than ATP and, indeed, the inhibition constant of the ATP analogue AMP-PNP is about 10^3 times better (K_i *ca* 50μM). It is more appropriate to compare this to that of adenine (K_i = 2.2 ± 0.5 mM), which is only 5 times less than that of 6-HP4. An indication of the ΔΔG for binding can be estimated from the K_i values and, at *ca* 4 kJ/mol, this is consistent with the incomplete replacement of the adenine amino H-bond by the 6-hydroxyl H-bond. Thus 6-HP4 appears to successfully mimic adenine in its binding site.

The crystallographic results show that 6-HP4 is embedded in the base binding pocket of the enzyme (see Figs. 3 and 4) with a multiplicity of ligand-protein interactions. In the detail available from our x-ray experiment it is possible to identify some of the hydrogen bond donors and acceptors and hydrophobic interactions involved in binding. The ligand is H-bonded, as we expected, to both the side chain carbonyl and NH_2 of Asn175 and to the nitrogen of Leu206 and the carbonyl of Ala207. It also forms hydrophobic contacts similar to that of adenine (compare the top and bottom sections of Fig. 1). The major difference between the binding of adenine and 6-HP4 is around the carbonyl of Pro204 which forms a good H-bond to the 6-amine of adenine. This interaction is missing in the 6-HP4 complex in that the CO of Pro204 is unsatisfied (see Fig. 1). However, there is a hydrophobic interaction between the ligand C2 and the side chain of Ile203. Furthermore, 6-HP4 makes a long H-bond to the carbonyl of Ala207. The arrangement of the side chain of Asn175 is shown in the Fig. 1 with the amide nitrogen shared by two obligate acceptors, the carbonyls of Val13 and of the ligand. This geometry allows the carbonyl of Asn175 to be the obligate proton acceptor in the hydrogen bond that it forms with the nitrogen of the 6-HP4. It implies that the nitrogen N3 is protonated as shown in the Fig. 2(a). This tautomeric form then has an acceptor at N1 for the donor main chain NH of Leu206 and the 6-hydroxyl proton bonds with the main chain CO of Ala207. So, although 6-HP4 could potentially bind to the adenine site in several different tautomeric forms and orientations, binding of the keto-enol tautomer shown in Fig.2(a) produces the maximum number of satisfactory interactions and must be considered to be effectively the only one present.

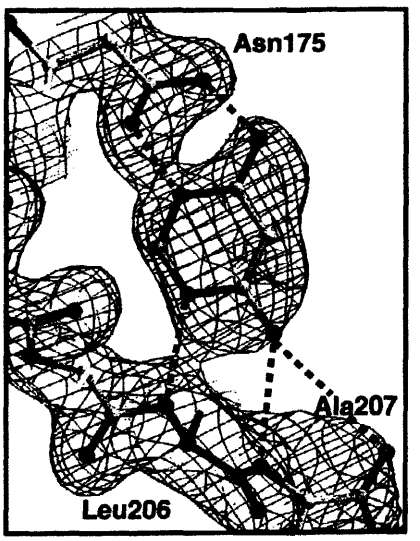


Figure 3: The ($3F_{obs}$-$2F_{calc}$, PHI_{calc} electron density map of the binding site of the 6-HP4 - DTBS complex countered at the 1.5σ level. The ligand and the amino acid residues H-bonded to it are superimposed.

The K_D value for binding of ATP to DTBS could not be obtained directly but can be approximated from the K_i value of the ATP analogue AMP-PNP (*ca* 50μM) which we have shown binds to the ATP site. While the K_i of 6-HP4 for DTBS is high compared to that of AMP-PNP it should be noted that 6-HP4 is a 'minimal' ligand lacking the ribose and highly charged phosphate groups largely responsible for the strong binding of ATP and AMP-PNP. Indeed the participation of these latter functionalities normally dominate ATP analog binding suggesting that many ATP-utilising enzymes can bind, to some extent, any nucleotide triphosphate analogue. A more specific comparison lies between the comparable binding of 6-HP4 and adenine to DTBS.. The important result here is that 6-HP4, a ligand 'designed' to bind to the adenine site in the DTBS protein, behaves as a classical competitive inhibitor and targets only to the designated site. This can be considered as a first step, the production of a new lead compound, in designing an inhibitor specific to the adenine element of the ATP binding site of DTBS. The next step will be to construct, from the 6-HP4 core, molecules which will only form contacts to this protein and are totally enzyme specific.

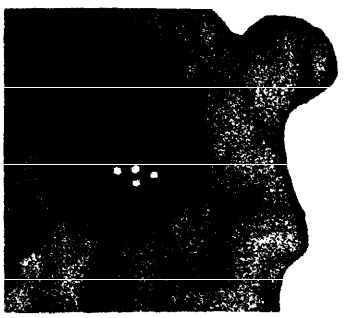


Figure 4: Part of the exclusion surface of DTBS showing 6-hydroxypyrimidin-4(3H)-one (6-HP4) occupying the the nucleotide binding pocket.

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Experimental

Enzyme Purification. The cloning and sequence of *E. coli* DTBS has been fully described.[5,12] In our work DTBS was obtained from overexpressing *E. coli* cells (harbouring the pET16b derived plasmid pET16b/BioD) by sonication, $(NH_4)_2SO_4$ precipitation and sequential chromatography on Sephacryl S-200HR and Q-Sepharose. DTBS was precipitated from active fractions from the final ion exchange step using $(NH_4)_2SO_4$ and the precipitated enzyme redissolved in Tris.HCl (50mM, pH7.8) containing $MgSO_4$ (5mM) and DTT (1mM) and stored at 4°C. High purity DTBS was obtained as required by gel permeation of the resuspended precipitate. An alternative purification procedure for DTBS has been described.[5]

Kinetic Measurements. The steady-state kinetic parameters for DTBS were determined using an assay based

on the pyruvate kinase / lactate dehydrogenase linked assay procedure for ADP originally described by Rosing *et al.*[13] At the concentrations used none of the inhibitors inhibited pyruvate kinase to a measurable extent; but K_i values were also determined using a radiochemical assay, essentially similar that described by Gibson *et al* [5] based on conversion of DAPA and $^{14}CO_2$ (from [^{14}C] sodium hydrogen carbonate) to [*ureido*-^{14}C]-DTB. The values obtained were in agreement with those obtained using the linked assay. The inhibition of DTBS by 6-hydroxypyrimidin-4(3H)-one (6-HP4), AMP-PNP and adenine were examined at 1.5mM enzyme concentration, pH 7.8 and 25°C. The changes in initial rate coefficients were determined over ATP concentrations 2-8mM and inhibitor concentrations 0-20mM (6-HP4 and adenine) or 0-100mM (AMP-PNP). Each of the other substrates, DAPA and CO_2, were held at concentrations of *ca.* 5 x K_M. Kinetic parameters were evaluated using Lineweaver-Burk and Dixon plots.

Crystallisation, data collection and analysis: Crystals of DTBS of about 0.2mm in size were grown from $(NH_4)_2SO_4$ as previously described.[12] The crystals were soaked in the crystallisation-well solution plus 50mM 6-hydroxypyrimidin-4(3H)-one for 1 hour prior to measurement. The x-ray diffraction data were collected on station 7.2 at the SRC Daresbury Laboratory, UK. The wavelength was 1.488Å and the maximum resolution 2.0Å. The data were collected from 2 crystals using a MAR image plate system. They were processed with DENZO and merged with SCALEPACK.[14] The data set is 99.4% complete between 30.0 and 2.0Å resolution, the outer resolution shell is 99.1% complete, the ratio of <I/sigI> for the outer resolution shell is 6.0 and the average multiplicity is 5.1. The final $R_{merge} = \Sigma (|(I - <I>)|) / \Sigma (I) = 7.9\%$. There are 13533 unique reflections, with 12343 structure factor amplitudes being over the 3σ level. The overall temperature factor of the data set is 22.7Å^2. The space group is C2 and the unit cell parameters are different from those of the native unit cell (see Table).

The R-factor on structure factors between the native and the inhibitor data sets was 14.0%. The difference Fourier map revealed a major peak at 6.3σ located in the nucleotide binding site in the same place as the adenine moiety of ATP.[6-10] The difference density peak contoured at the 3σ level was flat and was consistent with the pyrimidine ring of the designed inhibitor 6-HP4.

Table: Unit cell parameters ((differences are shown in brackets):

protein	a (Å)	b (Å)	c(Å)	β (deg.)
native	72.91	49.04	61.44	106.47
+ 6-HP4	73.63(+0.72)	49.38(+0.34)	61.71(+0.27)	106.71(+0.24)

Refinement of the 6-HP-DTBS structure: The model for 6-HP4 was built into the difference Fourier map and the structure refined with XPLOR. As the unit cell parameters are significantly different from those of the

native, the orientation of the inhibitor was not clear in the initial difference Fourier map. We assigned zero occupancy to the inhibitor atoms and refined the positional parameters of the structure to an R-factor of 19.7% starting from the native model. The occupancies of all protein atoms were fixed at 1.00 and the occupancies of the ions and water molecules were refined. Finally the B-factors of all atoms were refined with restraints on atoms bonded to each other. The difference Fourier map with the coefficients (|Fobs|-|Fcalc|) clearly revealed the details of the inhibitor binding. The 6-HP4 molecule was docked into the difference electron density and the structure was then refined to a final R-factor of 16.5%. The final model includes 1715 protein atoms, 201 water molecules and 3 sulfate ions from the crystallisation medium. The loop 208-210 that was disordered in the native structure is still disordered in the complex with the inhibitor. The average temperature factor of the refined model is 28.0Å^2 (27.3 for the protein atoms and 32.7 for the water molecules). The refined group occupancy of the inhibitor atoms is 0.7 and their average temperature factor is 36.1Å^2.

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