of DAHP). The crystal structure shows that the enzyme assembles as a functional homodimer. Each monomer is composed of an N-terminal α/β domain and a carboxy-terminal α-helical domain.

The N-terminal domain includes a Rossmann fold, but surprisingly binds NAD⁺ in an inverted orientation to that observed in all other classic Rossmann fold proteins. This domain consists of a seven-stranded β-sheet, with strand order 1296534 (Fig. 1). Strands β1 and β2 form a β-hairpin which is connected by helix α1 to a five-stranded Rossmann-fold structure12–15, comprising a βαβ unit and a βββββ unit. Strands β7 and β8 form a separate β-hairpin. Both DHQS and the classic Rossmann fold domains bind NAD(P)⁺ at the C-terminal end of the sheet. However, uniquely among known NAD(P)⁺–binding protein structures, the phosphate moieties of NAD⁺ in DHQS are associated with the glycine–rich first turn of the second βαβββ unit, rather then the first (β0)βββ unit. This places the nicotinamide moiety on the left of the labelled sheet (β1 to β9) in Fig. 1 on the side where the adenosine moiety binds in other NAD⁺–binding proteins. This orientates the NAD⁺ so that the active site is on the opposite side of the sheet to that seen in all other known Rossmann type NAD⁺–binding proteins.

The fold of the C-terminal domain of DHQS shows no detectable structural relationship to known structures16–18. Helix α6 is surrounded by helices α7 to α11 and one 3 10 helix, forming an antiparallel α-helical barrel. This is followed by a long loop with two short helices (α12 and α13), a distorted β-hairpin (β9 to β12) and a C-terminal helix α14. The active site is located in a cleft between the two domains (Fig. 1). The C-terminal domain contains most of the residues involved in catalysis and in substrate and Zn²⁺ binding (Fig. 2). The pentacoordinate Zn²⁺ ion interacts

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**Figure 1** Ribbon diagram of the DHQS dimer showing the inhibitor carbaphosphonate (yellow), NAD⁺ (green) and Zn²⁺ (orange). The N-terminal β-hairpin and connecting α-helix are shown in grey, the Rossmann fold domain is in red, the β-hairpin insertion is shown in light pink, the C-terminal domain central α-helix is shown in mid-blue, the surrounding five α-helices in dark blue and the C-terminal α-helices and a distorted β-hairpin in grey-blue. The nicotinamide mononucleotide (N) and adenosine (A) moieties of NAD⁺ are shown in light and dark green respectively. The inhibitor carbaphosphonate is labelled Car. Arg 130 is shown in pink (figures produced using BOSSCRIPT).
with Glu 194, His 271 and His 287 and two inhibitor hydroxyls (Fig. 2).

The complex multistep mechanism of DHQS involves alcohol oxidation, phosphate β-elimination, carbonyl reduction, ring opening and intramolecular aldol condensation. It has been proposed that the phosphate catalyses its own elimination and that the final steps in the mechanism could occur spontaneously, and that the enzyme might be required for the prevention of by-product formation in later steps. The structure of DHQS allows the identification of critical interactions that could be used by the enzyme in its proposed catalytic mechanism. The reaction scheme shown in Fig. 3 indicates how interactions between residues and ligands can stabilize intermediates in this complex pathway.

In the first step of the mechanism, the substrate is oxidized at C5 by NAD⁺ (Fig. 3). This involves hydride transfer from C5 of DAHP to C4 of the NAD⁺ nicotinamide moiety; in the crystal structure the nicotinamide ring is located in a suitable orientation for hydride transfer to occur. In concert, a proton is lost from the C5 hydroxyl group of DAHP. This proton could be relayed to a water molecule (Wat 7), located 2.7 Å from the O5 oxygen of the inhibitor. His 275, adjacent to this water molecule, could then accept this proton in a proton-shuffling system. One of the roles of the Zn²⁺ ion could be to facilitate hydride transfer and proton loss by polarizing the C5 hydroxyl.

The other NAD⁺-binding enzyme of known structure that has a metal ion bound to the substrate is alcohol dehydrogenase. Like DHQS, this enzyme performs a Zn²⁺- and NAD⁺-dependent oxidation of alcohol. Comparison of the folds of the catalytic domains of DHQS and alcohol dehydrogenase shows no similarity, and yet the relative positions of active-site components comprising the Zn²⁺, NAD⁺, substrate alcohol groups and proton-shuffling groups are similar in these enzymes. Thus, this appears to be a case of convergent evolution, where the chemical requirements for alcohol oxidation impose similar local stereochemistry in a completely different protein architecture.

The second step of the mechanism is β-elimination of the phosphate group. The phosphonate oxygens of the inhibitor are bound to Wat 33 and the side chains of Lys 152, Asn 268, His 275 and Lys 356 and Arg 130 from the other monomer (Fig. 3). During phosphate elimination, a proton is removed from C6 of DAHP. Although the phosphate group itself could act as the base in the removal of this proton, the enzyme may catalyse the phosphate elimination by providing a phosphate-binding pocket, formed by Lys 152, Asn 162, Asn 268, His 275 and Lys 356. The structure shows...
that these interactions could force the phosphate oxygens into a position where they can remove the proton from C6. The enzyme may also selectively stabilize an E1cb-like transition state or reactive intermediate.

In the third step, the C5 ketone of intermediate 3 in Fig. 3 is reduced by NADH. This is a reversal of the first step, with hydride ion from C4 of NADH being transferred back to C5 of the substrate. Using the proton-shuffling system, a proton could be transferred to the ketone oxygen from His 275, via Wat 7, creating intermediate 4 (Fig. 3).

The final two steps of the reaction consist of ring opening and intramolecular aldol condensation (Fig. 3). The ring-opening step involves deprotonation of the hydroxyl at C2. The crystal structure indicates that Wat 20 could participate in this step because the only nearby side chain, Asn 268, is unlikely to act as a proton acceptor. Indications that Wat 20 could participate in this step because the only nearby side chain, Asn 268, is unlikely to act as a proton acceptor.

Methods

Data collection and reduction. Expression, purification and crystallization of DHQS are to be described elsewhere. Two orthorhombic crystal forms, A and B, in space group P212121, were obtained for the complex between DHQS, NAD+ and carbaphosphonate (Table 1). Data were collected either with a rotating-anode source and an AXI3IX detector, or at the SRE, CCLRC Daresbury laboratory, UK, on beamlines 7.2.9, 9.5 or 9.6. A single mercurocetic acid derivative was obtained by soaking a form-A crystal for 3 h in 0.5 M mercuroacetate in mother liquor and data were collected using a wavelength of 0.87 Å. Data were integrated, scaled and reduced using DENZO and SCALEPACK, the CCP4 suite of crystallographic programs was used for subsequent steps.

Single isomorphous replacement phasing. Heavy-atom sites were identified with the program HRESSP, Patterson maps and difference Pattersons. Initial phases from eight Hg sites and anomalous data were refined using MLPHARE.

Density modification and refinement. Six-fold averaging (with the dimers in the two form-A and one form-B datasets) and solvent flattening using DMUMUT gave an interpretable map. The structure was built using the program O (ref. 26) in a map generated from the 3 Å dataset; the model was then extensively rebuilt using the 25.0–1.8 Å data from the 1.7 Å dataset. Refinement proceeded using TNT27 and REFMAC24, with a total of 64,534 reflections (3,385 reflections were excluded from the refinement for crossvalidation28). Towards the end of refinement, the non-crystallographic symmetry restraints were removed. Electron density was visible for residues A4 to A223, A231 to A391, B3 to B223, B235 to B393.

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Correspondence and requests for materials should be addressed to K.A.B. (e-mail: k.brown@ic.ac.uk). Coordinates have been deposited with the Brookhaven Databank under accession number 1dqs.

addendum

A prolactin-releasing peptide in the brain

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The prolactin-releasing peptide cDNA sequence data have been submitted to the DDBJ/EMBL/GenBank databases. The accession numbers are as follows: AB015417, Bos taurus mRNA for preproprolactin-releasing peptide; AB015418, Rattus norvegicus mRNA for preproprolactin-releasing peptide; AB015419, Homo sapiens mRNA for preproprolactin-releasing peptide.

correction

Engineering cyclophilin into a proline-specific endopeptidase

Eric Quéméneur, Mireille Moutiez, Jean-Baptiste Charbonnier & André Ménez


The efficiency value (kcat/Km) of cyproceptin 1 is equal to 0.2 × 104 M−1 s−1, and not to 0.7 × 104 M−1 s−1 as published. Also, the histidine at residue 104 titrates with a pK equal to 6.47 ± 0.16 and 6.74 ± 0.15 when measuring Kcat and kcat/Km, respectively, and not to 6.74 ± 0.16 and 6.74 ± 0.15.