

## Research paper

# Crystal structure of the NADP-dependent mannitol dehydrogenase from *Cladosporium herbarum*: Implications for oligomerisation and catalysis<sup>☆</sup>

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## ABSTRACT

The ascomycete *Cladosporium herbarum* is a prominent fungal inducer of Type I allergy. The only major allergen identified so far is Cla h 8, a NADP-dependent mannitol dehydrogenase (MtDH). MtDH, a cytoplasmic protein of 28.5 kDa, belongs to the Short chain Dehydrogenases/Reductases (SDR), acting as a NADP-dependent oxidoreductase.

In this study, we found that *C. herbarum* MtDH can exist as monomers, dimers and tetramers in solution and, correspondingly, forms tetramers and higher oligomers in two crystal structures. Additionally, we identified a unique adaptive binding site for the metal ions Na<sup>+</sup> and Zn<sup>2+</sup> that were distinguished by an anomalous dispersion experiment. A Translation-Libration-Screw analysis confirmed the stabilising effect of Zn<sup>2+</sup> for the tetrameric assembly. Moreover, the zinc containing structure explains the mode of MtDH multimerisation by metal bridging of the tetramers. The formation of oligomers and higher multimers of MtDH provides a missing link to its allergenic properties. Based on the well defined active site region and a comparative analysis with related structures, we can also clarify the atypical enzymatic properties of MtDH by two alternative binding modes of the substrate to the active site.

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## 1. Introduction

The ascomycete *Cladosporium herbarum* is involved in the decay of plant material and represents one of the most prominent moulds in the outdoor environment, but is also found in the indoor environment. Fungi in general cause medical afflictions by colonizing the human body coupled with the release of mycotoxins, resulting in various allergic reactions. *C. herbarum* is one of about 80 mould genera causing Type I allergies in susceptible persons. In case of *C. herbarum* the prevalence of Type I allergy is 1.7% within the general population [1], 13% within mould allergic subjects and reaches up to 84% in the group of patients being sensitised to more

than two fungal species [2]. Due to the high protein sequence similarity, many of the homologous allergens from moulds are IgE-cross-reactive, making allergy diagnosis and therapy difficult.

Most *C. herbarum* allergens are so-called minor allergens having a prevalence of IgE-reactivity of less than 22% within persons suffering from *C. herbarum* allergy, while the only major *C. herbarum* allergen identified so far is the NADP-dependent mannitol dehydrogenase (MtDH) with a prevalence of 57% [3]. Up to now, homologues of this protein only from *C. herbarum* and *Alternaria alternata* have been shown to be an allergen [3,4]. The *C. herbarum* MtDH (GenBank accession number: AY191816) belongs to the family of short-chain dehydrogenases/reductases (SDRs). It consists of 267 residues, exhibits a molecular weight of 28.5 kDa and an isoelectric point of 5.8 and was denoted with the allergen abbreviation Cla h 8.

The majority of SDRs is oligomeric, with either homodimeric or homotetrameric quaternary structures, related by two perpendicular twofold axes. Although the protein sequence identity between different SDR-enzymes is not higher than 15–30% (Fig. 1) their members are characterised by an N-terminal coenzyme-binding motif of TGxxxGxG (Cla h 8: T<sup>26</sup>-G<sup>35</sup>) and an active site triad of S-Y-K (Cla h 8: S<sup>160</sup>-Y<sup>175</sup>-K<sup>179</sup>), covering a broad range of substrate specificities for steroids, prostaglandins, aliphatic

**Abbreviations:** MtDH, mannitol dehydrogenase; SDR, short chain dehydrogenase/reductase family; NADP, Nicotinamide adenine dinucleotide phosphate.

<sup>☆</sup> Atomic coordinates and structure factors have been deposited in the RCSB Protein Data Bank under the PDB entry code 3GDG for the native structure and 3GDF for the zinc structure.

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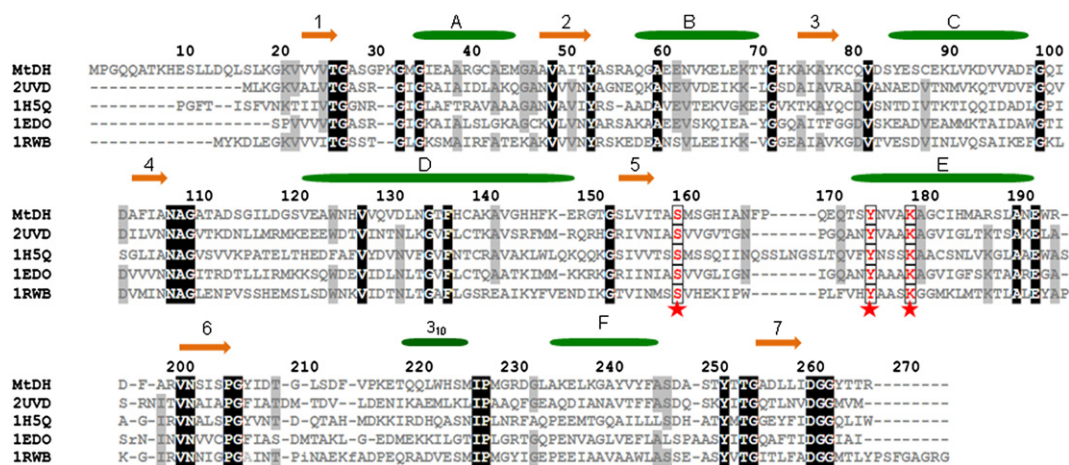


**Fig. 1.** Structure-based sequence alignment of the SDR family members. Sequences of proteins with known three-dimensional structure (2UVD–3-oxoacyl-(acyl carrier protein) reductase from *Bacillus anthracis*, 1H5Q – mannitol 2-dehydrogenase NADP<sup>+</sup> complex from *Agaricus bisporus*, 1EDO– $\beta$ -keto acyl carrier protein reductase from *Brassica napus*, 1RWB – glucose dehydrogenase from *Bacillus megaterium*) compared with *C. herbarum* MtDH. Amino acids are coloured as follows: the identical amino acids in white are highlighted with black background, the homologous ones with grey background. Amino acids forming the catalytic triad are shown as red letters in frames, indicated by a red asterisk. The residue numbering and the marked secondary structure elements ( $\beta$ -sheets and the  $\alpha$ -helices) are displayed as arrows and cylinders) correspond to *C. herbarum* MtDH and are coloured as in Figs. 3–5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

alcohols, and xenobiotics [5,6]. In the catalytically active triad, tyrosine acts as catalytic base, whereas serine is thought to stabilise the substrate and lysine interacts with the nicotinamide ribose.

*C. herbarum* MtDH was shown to catalyse the pH-dependent reduction of D-fructose to mannitol, accompanied by the oxidation of NADPH to NADP<sup>+</sup> which may be formally written as: D-fructose + NADPH + H<sup>+</sup>  $\leftrightarrow$  D-mannitol + NADP<sup>+</sup>. Among the enigmatic features of MtDH are the atypically high  $K_M$  values of the 6-carbon polyol substrates of  $1.17 \pm 0.20$  M and  $0.23 \pm 0.05$  M for D-fructose and D-mannitol, respectively [3]. These apparent  $K_M$  values are qualitatively consistent with published data determined for orthologous enzymes, albeit a factor 10 to 20 higher than those [7–9]. The  $K_M$  value for the NADPH and NADP<sup>+</sup> co-substrates (“cofactors”) are in a range typically expected for enzyme-catalysed reactions (53  $\mu$ M and 67  $\mu$ M, respectively) [3]. A further intriguing observation was the metal dependence of the MtDH enzymatic activity in *Aspergillus parasiticus*: While the activity was specifically inhibited by Zn<sup>2+</sup> ( $K_i$  of 1  $\mu$ M) and Cu<sup>2+</sup>, no change of the enzymatic activity was caused by Mn<sup>2+</sup>, Fe<sup>2+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, and La<sup>3+</sup> [10].

Currently structural data are only available for MtDH from *Agaricus bisporus* and *Pseudomonas fluorescens*. We have determined crystal structures of *C. herbarum* MtDH in two different conformations to investigate and rationalise these enzymatic peculiarities. By means of solution studies we could elucidate the adaptive oligomerisation behaviour of MtDH, which confirms and substantiates our earlier studies on the allergenic behaviour of MtDH from *C. herbarum*.

## 2. Materials and methods

### 2.1. Purification & crystallisation

The purification of *C. herbarum* MtDH was performed according to standard protocols and included ammonium sulphate precipitation, hydrophobic interaction and anion exchange chromatography. The final purification step before crystallisation trials was size exclusion chromatography which resulted in more than 95% pure protein [3]. The protein was concentrated to 10 mg/ml. Crystallisation was carried out at 277 K and 293 K with the sitting drop vapour diffusion method by mixing equal volumes of the protein and reservoir solution. Initial crystals appeared after 1 month at

277 K equilibrated against a reservoir containing 45% MPD and 0.1 M HEPES. Fine screening yielded well diffracting single crystals ( $0.1 \times 0.2 \times 0.1$  mm<sup>3</sup>) of native protein that belonged to space group C2 (unit cell constants:  $a = 82.71$  Å,  $b = 112.01$  Å,  $c = 107.29$  Å,  $\alpha = \gamma = 90^\circ$ ,  $\beta = 98.28^\circ$ ). To study the influence of zinc on the protein, native crystals were soaked overnight with a solution containing supplementary 5 mM ZnCl<sub>2</sub>, leading to a rearrangement of the protein molecules in the crystal, involving significant changes of the molecular packing and the cell constants. The exact nature of the remarkable crystal transformation is unclear, but involved a cracking and dissolving phase followed by recrystallisation. The zinc soaked crystals adopted a higher symmetry belonging to space group P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> (cell constants:  $a = 69.01$  Å,  $b = 106.53$  Å,  $c = 132.78$  Å,  $\alpha = \beta = \gamma = 90^\circ$ ).

### 2.2. X-ray diffraction data collection

The crystals were mounted with cryo-loops directly from the mother/soaking solution and flash-frozen in the nitrogen stream of 100 K. X-ray diffraction data of both native and Zn-derivative crystals were collected to 2.2 Å and 2.3 Å resolution using a MAR225 CCD detector at beamline BM14 of the European Synchrotron Radiation Facility in Grenoble. The wavelength was chosen to be 1.28 Å, corresponding to the K-edge of zinc. The data were processed using MOSFLM [11] and SCALA [12]. Data collection, processing and scaling statistics are listed in Table 1.

### 2.3. Structure determination

The structure was determined by molecular replacement performed with the CCP4i program suite and the CNS software package [13]. Initial molecular replacement was difficult due to the low sequence identity to most members of the SDR family. The tetramer of  $\beta$ -keto acyl carrier protein reductase (PDB entry 1 EDO) was used as a search model. The resulting electron density map was of good quality and enabled the incorporation of a complete model. The initial model had a crystallographic R factor of about 40%. Real space electron density averaging was performed with MAIN [14] in combination with CCP4 routines. The model was refined by CNS and corrected by using the interactive 3D graphics of the program MAIN, finally resulting in crystallographic R factors of 22.8% and

**Table 1**  
Summary of data collection and processing/scaling.

|                                           | MtDH-native                                                                                          | MtDH-Zn                                                                              |
|-------------------------------------------|------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| Space group                               | C2                                                                                                   | P2(1) 2(1) 2(1)                                                                      |
| Wavelength (Å)                            | 1.28                                                                                                 | 1.28                                                                                 |
| Unit cell dimensions (Å, °)               | $a = 82.71$ , $b = 112.09$ ,<br>$c = 107.29$<br>$\alpha = \gamma = 90^\circ$ , $\beta = 98.28^\circ$ | $a = 69.01$ , $b = 106.54$ ,<br>$c = 132.78$<br>$\alpha = \beta = \gamma = 90^\circ$ |
| Resolution range (Å)<br>(highest shell)   | 20–2.30 (2.42–2.30)                                                                                  | 20–2.5 (2.64–2.50)                                                                   |
| Number of measurements                    | 101,300 (14,722)                                                                                     | 114,2510 (16,496)                                                                    |
| Number of unique reflections              | 41,671 (6053)                                                                                        | 33,729 (4962)                                                                        |
| Average multiplicity                      | 2.4 (2.4)                                                                                            | 3.3 (2.4)                                                                            |
| Anomalous multiplicity                    | —                                                                                                    | 1.9 (1.8)                                                                            |
| $R_{\text{merge}} (\%)^a$                 | 5.6 (14.1)                                                                                           | 12.8 (45.5)                                                                          |
| $R_{\text{ano}} (\%)^b$                   | —                                                                                                    | 11.0 (37.5)                                                                          |
| $R_{\text{pim}} (\text{all } I+ \& I-)^c$ | 4.9 (10.3)                                                                                           | 7.6 (28.5)                                                                           |
| Completeness (%) (20–2.3)                 | 97.1 (96.8)                                                                                          | 98.0 (99.7)                                                                          |
| $I/\sigma$                                | 13.4 (6.8)                                                                                           | 8.6 (2.4)                                                                            |

$I$  is the intensity of an individual measurement and  $\langle I \rangle$  the corresponding mean value.

$$^a R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$$

$$^b R_{\text{ano}} = \frac{\sum_{hkl} |\langle I(+) \rangle - \langle I(-) \rangle|}{\sum_{hkl} |\langle I(+) \rangle + \langle I(-) \rangle|}$$

$$^c R_{\text{pim}} = \frac{\sum_{hkl} \sqrt{\frac{1}{N} \sum_i I_i(hkl)} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$$

18.1% and free  $R$  factors of 29.6% and 26.2% for the native data and the zinc derivative, respectively (Table 2).

#### 2.4. Gel filtration and Blue Native PAGE

For size exclusion chromatography, the concentrated sample was centrifuged at 15,000 g for 15 min at 4 °C and filtered immediately prior to gel filtration. Aliquots of 500  $\mu$ l were loaded onto a Superose 6 10/300 GL column, pre-equilibrated with SEC buffer (50 mM Tris/HCl, 25 mM NaCl, pH 7.5) using an ÄKTA FPLC system.

**Table 2**  
Summary of refinement.

|                                                            | MtDH-native        | MtDH-Zn            |
|------------------------------------------------------------|--------------------|--------------------|
| Resolution in refinement (Å)<br>(highest shell)            | 20–2.3 (2.44–2.30) | 20–2.5 (2.66–2.50) |
| Reflection used for refinement<br>(data cutoff $F = 0$ )   | 41670 (6896)       | 33705 (5358)       |
| Completeness (%)                                           | 96.9 (96.7)        | 97.5 (99.3)        |
| Working set                                                | 39592 (6577)       | 31989 (5076)       |
| Test set                                                   | 2078 (319)         | 1716 (282)         |
| $R_{\text{cryst}} (\%)^a$                                  | 22.8 (26.5)        | 18.1 (27.1)        |
| $R_{\text{free}} (\%)^b$                                   | 29.6 (36.0)        | 26.2 (33.9)        |
| rmsd bond lengths (Å)                                      | 0.010              | 0.006              |
| rmsd bond angles (°)                                       | 1.372              | 1.113              |
| Number of non-hydrogen<br>protein atoms                    | 7992               | 7992               |
| Number of ions                                             | 2 Na <sup>+</sup>  | 4 Zn <sup>2+</sup> |
| Number of solvent molecules                                | 695                | 552                |
| Overall $B$ value (Å <sup>2</sup> )                        | 19.8               | 25.9               |
| Overall $B$ -factor for protein<br>atoms (Å <sup>2</sup> ) | 19.4               | 25.7               |
| Overall $B$ for ions (Å <sup>2</sup> )                     | 21.0               | 42.6               |
| Overall $B$ for solvent atoms (Å <sup>2</sup> )            | 23.7               | 28.5               |
| Ramachandran (non-Gly, non-Pro residues)                   |                    |                    |
| Most favored regions                                       | 825 (89.5%)        | 813 (88.0%)        |
| Additional allowed regions                                 | 88 (9.5%)          | 100 (10.8%)        |
| Generously allowed regions                                 | 9 (1.0%)           | 11 (1.2%)          |
| PDB accession codes                                        | 3GDG               | 3GDF               |

$F_{\text{obs}}$  – observed structure factor.

$F_{\text{calc}}$  – calculated structure factor.

$$^a R_{\text{cryst}} = \frac{\sum_{hkl} |F_{\text{obs}}(hkl) - F_{\text{calc}}(hkl)|}{\sum_{hkl} |F_{\text{obs}}(hkl)|}$$

$^b R_{\text{free}}$  – factor calculated on a partial data set.

The recombinant protein was eluted at a flow rate of 0.3 ml/min with collection of 0.5 ml fractions. Blue Native gel electrophoresis was performed as described by Krause & Seelert [15].

### 3. Results

#### 3.1. Oligomerisation behaviour in solution

MtDH could be shown to co-exist as monomer, dimer, and tetramer in solution as detected in Blue native PAGE (Fig. 2a). Tetramer formation seems to be, according to Blue native PAGE, concentration and NADP driven. Interestingly, size exclusion chromatography confirmed that *C. herbarum* MtDH exists as monomers and dimers in solution under the given experimental conditions, Fig. 2b [15]. Thus, we conclude that the tetramer is rather labile and easily dissociates and reassociates, while the dimer species that could be also observed at concentrations of 0.1–1 mg/ml is more stable. These concentrations thus translate into dissociation constants of  $K_d \approx 10 \mu\text{M}$ .

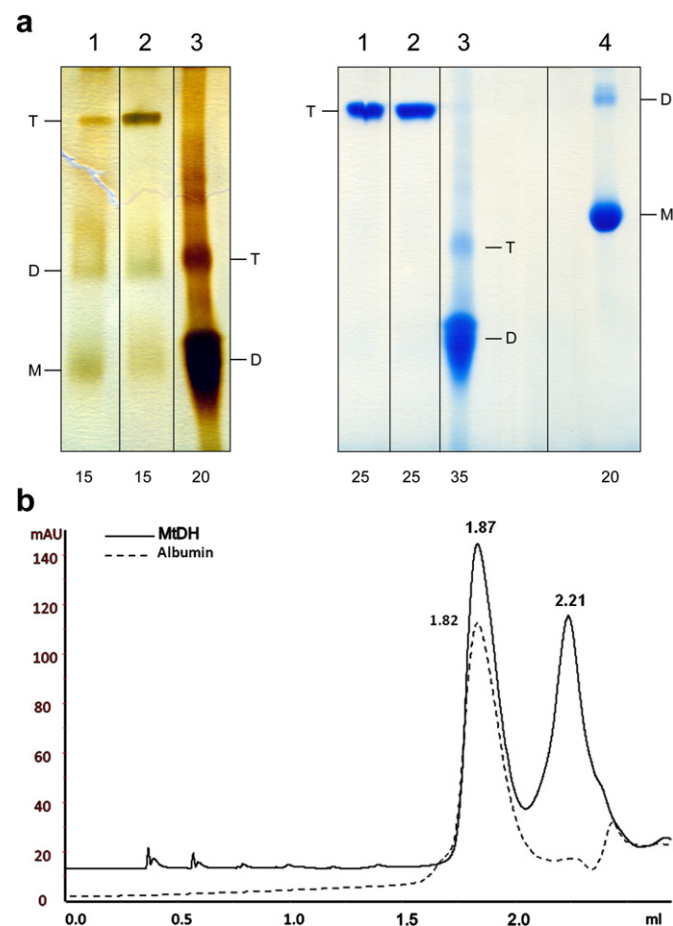


**Fig. 2.** *C. herbarum* MtDH oligomerisation behaviour in solution. (a) Blue native gel electrophoresis. *C. herbarum* MtDH was separated on 13% Blue native polyacrylamide gels. In lane 1 native protein was loaded, whereas in lane 2 *C. herbarum* MtDH has been pre-incubated with 250  $\mu\text{M}$  NADP<sup>+</sup>. The molecular weight (MW) of the monomer (M) is 28.5 kDa, and the respective tetramer (T) has a MW of 114 kDa. In lane 3 purified native Alt 1, the major allergen of the mould *A. alternata*, was used as control. Alt 1 forms a dimer of 30 kDa and a tetramer of 60 kDa. In lane 4 bovine serum albumin (monomer 66 kDa, dimer 132 kDa) was loaded as second control. [29]. The protein amount (given in  $\mu\text{g}$ ) loaded in the individual lanes is depicted below the lanes. (b) Size exclusion chromatography – the gel filtration chromatogram of *C. herbarum* MtDH (continuous line) and albumin (dotted line) indicate that in solution MtDH coexists as a mixture of monomers and dimers. Numbers at peaks denote the exact elution volume.

### 3.2. Crystal structure analysis of MtDH

#### 3.2.1. Tetramers are formed as dimers of dimers

We obtained two different crystal forms of MtDH in space groups C2 and P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>, both of which contained a homotetramer as building block. The crystal structure analysis confirms that MtDH exists as tetramer obeying a D<sub>2</sub> point group symmetry with three orthogonal twofold axes interrelating the subunits A–B, A–C, and A–D, (Fig. 3a, b). Two dominant interfaces are formed by A–B

(and C–D) with contact areas of  $\sim 2200 \text{ \AA}^2$  and by A–C(B–D) with  $\sim 1680 \text{ \AA}^2$ , whereas the third interaction interface formed by A–D is rather small ( $\sim 370 \text{ \AA}^2$ ).

#### 3.2.2. Subunit contacts

**3.2.2.1.  $\beta$ -Sheet extension forms the mechanism of A–B (or C–D) dimerisation.** The two central seven-stranded parallel  $\beta$ -sheets of subunits A and B or C and D align along their seventh strand to form a 14-stranded parallel beta-pleated sheet (Figs. 3a and 4a, b).

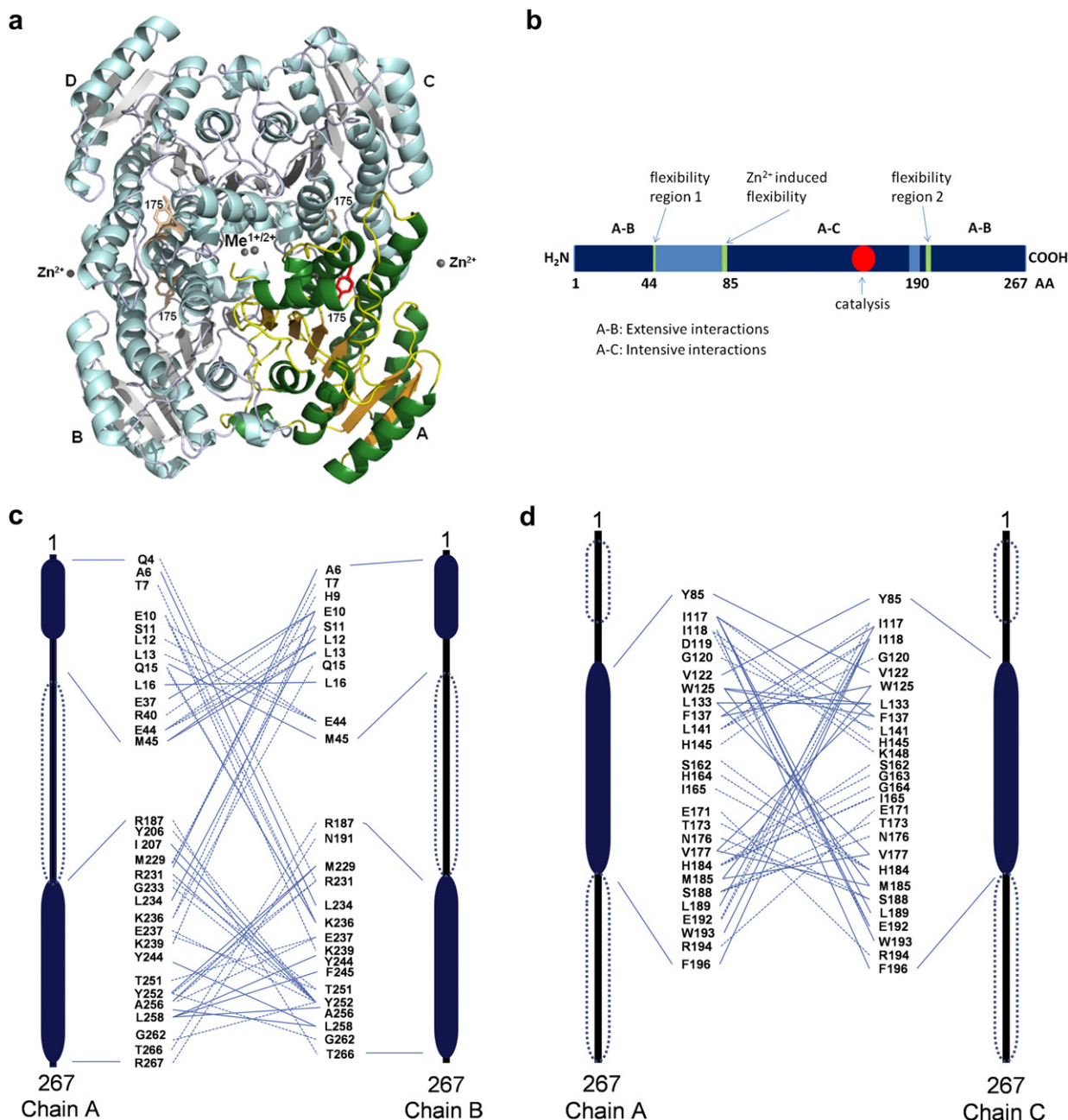


**Fig. 3.** (a) Ribbon representation of the tetrameric *C. herbarum* MtDH structure. The tetramer is positioned along the molecular 2-fold axis with the same orientation for monomer A as in Fig. 4b for monomer A, while the other indicated subunits B–D are depicted with lighter shades. The two zinc ions coordinating the C-termini of subunits A and D and B and C as well as intermolecular zinc ions are depicted as grey spheres. The catalytic residues Y<sup>175</sup> are shown as red or wheat coloured sticks, respectively. (b) A linearised mapping of contacts that illustrates the interactions for one monomer molecule within the dimers present in the tetramer of MtDH. The regions coloured by navy blue are extensive interactions between monomers A and B ( $\beta$ -sheet extension) and intensive interactions between monomers A and C ( $\alpha$ -helical stacking). Three flexible regions (green) and the catalytic center (red) are marked. Schematic presentation of interactions within A–B (c) and A–C (d) interfaces in the tetrameric MtDH. Hydrophobic and polar interactions, the latter comprising hydrogen bonds and electrostatic interactions, are illustrated as continuous and dotted lines, respectively. The polypeptide chains are represented as vertical sticks and the areas of interactions are shown as navy blue sections. For better visualisation of all interactions within the tetramer other interactions are depicted as dotted white sections. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

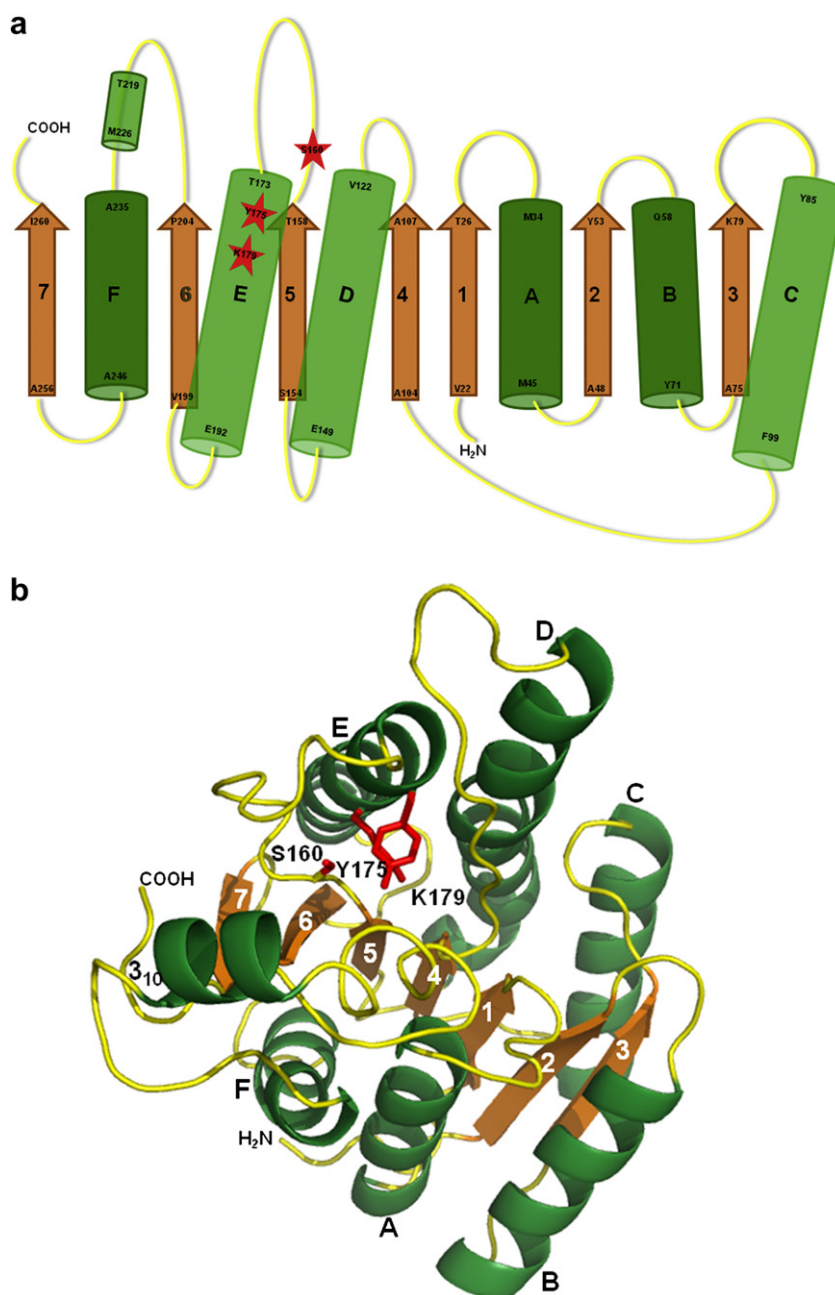


**Fig. 4.** (a) Topology diagram illustrating the monomer structure of *C. herbarum* MtDH. The seven-stranded  $\beta$ -sheet comprises seven parallel strands. The strand topology is 7-6-5-4-1-2-3 for the central sheet that is flanked by three  $\alpha$ -helices on each side denoted as A, B, F and C, D, and E positioned below and on top of the sheet. The short  $3_{10}$  helix is shown and the catalytic amino acids are illustrated by a red asterisk. (b) Ribbon representation of the overall fold of the monomer unit of *C. herbarum* MtDH. Green – helices ( $\alpha$  or  $3_{10}$ ), orange –  $\beta$ -strands, yellow – loops. Strand numbering and letter codes for the helices correspond to the topology diagram of Fig. 4a. The N- and C-termini are indicated, the catalytic triad amino acids are shown in red as stick model. The monomer is positioned along the molecular 2-fold axis with the same orientation as monomer A in Fig. 3a. The figure was prepared with PyMOL (<http://pymol.sourceforge.net/>). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

In addition, predominantly hydrophobic side chain contacts contribute to this interface formation, as is detailed in the contact matrix (Fig. 3b).

**3.2.2.2.  $\alpha$ -Helical stacking builds the A–C (or B–D) dimer.** The dimerisation of the alternative A–C (or B–D) dimer is mediated by  $\alpha$ -helical stacking of the helices C, D, and E. The corresponding interaction matrix reveals that relatively few, yet intensive contacts contribute to this dimer interface, whereas the A–B interface is built by more amino acids. Thus, the A–B interaction would be more tolerant towards single site mutations located in the interface

than the A–C interaction. Furthermore, the structure shows that helices and loops flanking the NADPH-binding site contribute to the A–C interface.

The interaction schemes for A–C and A–B interfaces illustrate the type and amount of intersubunit contacts in MtDH (Fig. 3b–d). Interestingly, both interfaces are mixed hydrophobic and polar, comprising of hydrogen bonds and electrostatic interactions. Overall, more interactions contribute to the A–B interface than to the A–C interface. These observations are in line with the observed difference in the interaction interface area ( $2200 \text{ \AA}^2$  for A–B versus  $1680 \text{ \AA}^2$  for A–C), both providing experimental

support for a higher stability of the A–B dimer than of the A–C dimer in solution.

Crytallisation conditions, gel filtration, and native gels allow for an estimation of the dissociation constants ( $K_{\text{diss}}$ ) of MtDH tetramers and dimers (Fig. 2). In the crystallisation samples with 10 mg/ml protein, corresponding to 350  $\mu\text{M}$  concentration, 100% tetramers of MtDH are present. Also, in Blue native gels with 0.5 mg/ml (12.5  $\mu\text{g}/25 \mu\text{l}$ , corresponding to  $\sim 18 \mu\text{M}$ ) about 100% tetramers are observed. In contrast, a reduced loaded volume of MtDH (7.5  $\mu\text{g}/15 \mu\text{l}$ ) shows clearly that only about one third of MtDH still forms tetramers, while dimers and monomers are also present at roughly 33% each. This effect is due to dilution of the protein concentration during the gel-run. Given the lower effective concentration of the latter experiment, we estimate the  $K_{\text{diss}}$  for both tetramers and dimers to be around 10  $\mu\text{M}$ . The relatively large area of the A–B and A–C interfaces does not exclude dissociation constants in the micromolar range [16]. These findings are qualitatively in line with gel filtration runs with MtDH concentrations of 10–30  $\mu\text{M}$  demonstrated that relatively stable dimers exist under these special conditions (Fig. 2b). Interestingly, in the native gel run the co-substrate  $\text{NADP}^+$  lead to a stabilisation of the MtDH tetramer and probably of the dimer (Figs. 2a and 3a). Consequently, we expect that  $\text{NADP}^+$  significantly influences the overall oligomerisation behaviour of MtDH. Conversely,  $\text{NADP}^+$  exchange may be assisted by monomer to tetramer transformation.

### 3.2.3. Metal cations stabilise oligomer formation

Both dimer interactions (A–B and A–C) make a contribution to the formation of the tetramer that is configured as a dimer of dimers (Fig. 3a). Tetramerisation is further supported by a metal-bridge based interaction between the C-terminal carboxy termini ( $\text{R}^{267}$ ) of the A and D chain on the top as well as of the B and C chain at the bottom of Fig. 3a; (See also Fig. 5a, b). Both mono- and divalent cations are able to bind to the adaptive binding site formed by the C-termini, as indicated by the labels  $\text{Me}^{1+/2+}$  in Fig. 3a. In the C2 crystal structure we find a tetragonal-bipyramidal (close to octahedral) coordination sphere around the metal ion with average bond-distances of 2.4–2.5 Å. Together with the occupancy refinement of the electron density, this cation can be sensibly interpreted as sodium [17]. The protein derived ligands to  $\text{Na}^+$  are positioned at the apical positions of the bipyramid with four water molecules completing the equatorial coordination sphere. By contrast, in a second crystal form obtained in the presence of 5 mM  $\text{ZnCl}_2$ , a trigonal-bipyramidal coordination sphere was observed around the central metal; besides the (mono-dentate) carboxylate ligands derived from  $\text{R}^{267}$  three water molecules complete the coordination with average bond-distances of about 1.8–2.0 Å, inconsistent with sodium at the metal site [17]. Additionally, an occupancy analysis of the bridging metal ions clearly excluded that  $\text{Na}^+$  (10 electrons) could account for the observed strong electron density. Instead, the electron density and coordination geometry suggested that  $\text{Zn}^{2+}$  replaced  $\text{Na}^+$  in this crystal form. We could unambiguously confirm this hypothesis by an anomalous diffraction measurement at the zinc K-edge, eventually showing strong zinc occupancy in an anomalous Fourier map at this site (Fig. 5b, c). In a control experiment, no anomalous electron density was observed for the “native”  $\text{P2}_12_12_1$  crystal structure, consistent with our interpretation of the metal density as sodium (Fig. 5a). In consequence, we have identified a binding site with adaptive metal valences, for which no precedence has been described to our knowledge.

Surprisingly, the inspection of the anomalous difference electron density map revealed two additional  $\text{Zn}^{2+}$  ions bound to the protein. These ions are positioned on the protein surface and bridge tetramers via A–D and B–C interactions. The first intermolecular bridging zinc ion is approximately tetrahedrally coordinated by the

side chains of  $\text{H}^{127}$  and  $\text{E}^{123}$  derived from the A monomer and  $\text{E}^{89}$  and  $\text{E}^{86}$  provided by the D monomer from the neighbouring tetramer. Likewise, the second intermolecular zinc interaction involves  $\text{H}^{127}$  and  $\text{E}^{123}$ , derived from monomer B, as well as  $\text{E}^{89}$  and  $\text{E}^{86}$ . In the crystal lattice, this zinc-mediated bridging results in the formation of an infinitely linked multimer (Fig. 5c).

### 3.2.4. Monomer structure

The monomer subunit of MtDH is composed of a Rossmann fold-like  $\beta\alpha\beta$  unit displaying a single-domain architecture (Fig. 4a, b). This domain is built up from twisted parallel  $\beta$ -sheets consisting of seven  $\beta$ -strands. The central sheet is flanked by three  $\alpha$ -helices from each side denoted as A, B, F and C, D, and E positioned below and on top of the sheet in Fig. 4a, respectively. The strand topology is 7-6-5-4-1-2-3 with an extended loop-helix(C)-loop crossover between strands 3 and 4. The latter motif is characteristic for the nicotinamide binding capacity of enzymes within the SDR family.

Helix  $\alpha\text{E}$  harbours the catalytic residues  $\text{Y}^{175}$  and  $\text{K}^{179}$ . The third residue of the catalytic triad,  $\text{S}^{160}$ , is located in the loop region preceding E. This loop ( $\text{M}^{161}$ – $\text{S}^{174}$ ) and in particular the neighbouring loop ( $\text{Y}^{206}$ – $\text{A}^{235}$ ) are relatively long and contain three short segments with  $\phi$  and  $\psi$  values characteristic for ideal  $3_{10}$  helical turns. Overall, the subunit structure of MtDH shares the typical fold with the related short chain dehydrogenases (Fig. 4b).

### 3.2.5. Active site architecture and possible substrate binding modes

Mutagenesis analysis of members of the SDR family revealed that four residues are crucial for catalysis. Three of them form the catalytic triad, which in the *C. herbarum* MtDH molecule consists of residues  $\text{S}^{160}$ – $\text{Y}^{175}$ – $\text{K}^{179}$ . The side chain of  $\text{Y}^{175}$  acts as the catalytic acid/base (reductase/dehydrogenase activity), whereas  $\text{S}^{160}$  is thought to stabilise and polarise the carbonyl substrate group (of open-chain D-fructose). The  $\text{K}^{179}$   $\epsilon$ -amino group provides the positively charged environment to lower the  $\text{pK}_a$  of the  $\text{Y}^{175}$ –OH.

Unfortunately, attempts of co-crystallisation and soaking experiments to obtain a structure of MtDH in complex with D-mannitol or/and with  $\text{NADP}^+$  have failed. To understand the binding of the substrate to the active site, D-mannitol, D-fructose,  $\text{NADP}^+$  and  $\text{NADPH}$  were modelled into the active site cavity. In particular, our modelling studies revealed two alternative binding modes of fructose compatible with the active site geometry, if fructose binds in the open-chain form (Fig. 6b). Given the planar C2 centre, both binding orientations can be productive.

### 3.2.6. Conformational variability of MtDH

The function of a protein is not only determined by its static structure, but also by its conformational dynamics. The latter information can be extracted from a crystal structure analysis by a detailed comparison of the atomic scattering power, which is encoded in the so-called temperature factor (B-factor). Therefore, we studied the conformational flexibility of the apoenzyme by using the Translation-Libration-Screw Motion Determination method (“TLS-MD”) [18]. The results of the molecular dynamics simulations suggest the occurrence of distinct regions with varying flexibility, summarised in Fig. 3b. A high variability is observed in the N-termini of the chains, in a segment comprising residues between 60 and 70, and in a more C-terminal segment starting around residue 220 (Fig. 3b). Interestingly, the flexibility distribution is changed and less pronounced in the  $\text{Zn}^{2+}$  complex crystal, where an additional segment around residue 120 shows increased flexibility (data not shown). The TLS analysis confirms the more ordered conformation at the C-termini of the protein chains in the native ( $\text{Na}^+$ -bound) and  $\text{Zn}^{2+}$  complexed structures, reflecting the rigidifying effect of metal bridging. Moreover, these results indicate an enhanced C-terminal stabilisation by  $\text{Zn}^{2+}$  compared with  $\text{Na}^+$ ,

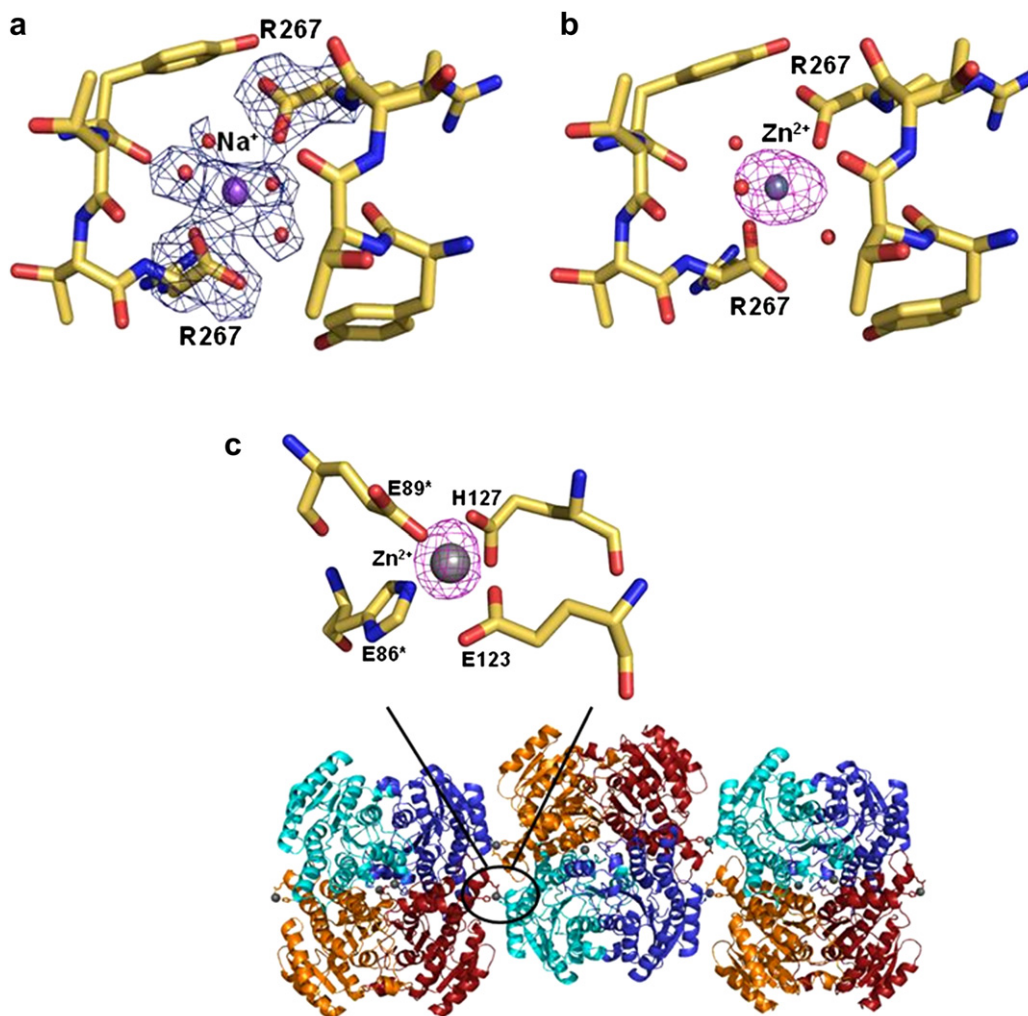


**Fig. 5.** Close-up views of the sodium/zinc binding sites of *C. herbarum* MtDH. (a) Subunit A–D, B–C bridging by  $\text{Na}^+$  (purple sphere) coordination at the C-termini in the native structure. C-terminal residues including the sodium ion coordinating carboxylate of R267 are displayed as stick models, while water molecules are shown as red balls. The final  $2\text{Fo}-\text{Fc}$  density surrounding the  $\text{Zn}^{2+}$ , the C-terminal carboxylates, and four water molecules is shown as dark blue grid contoured at  $1\sigma$ . (b) Subunit A–D, B–C bridging by  $\text{Zn}^{2+}$  (grey sphere) in the zinc MtDH structure, C-terminal residues, including the zinc ligand R267 are displayed as stick models and water molecules are shown as red balls. The anomalous Fourier map for zinc is depicted in magenta and contoured at  $5\sigma$ . (c) Close-up insert of A–D, B–C bridging by intermolecular  $\text{Zn}^{2+}$  (grey spheres) in the zinc structure. Residues E123, H127, E86 and E89 are shown as stick models. The asterisks indicate the amino acids from the neighbour molecules. The arrangement of three MtDH tetramers explains the intermolecular bridging zinc in the crystal lattice. Zinc is surrounded by an anomalous Fourier map (magenta grid contoured at  $5\sigma$ ). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

which can be explained by stronger electrostatic interactions of the doubly charged cation. The ordering effect of  $\text{Zn}^{2+}$  on nearly all regions of chains A, B, C and D, may be partially caused by the C-terminally bound  $\text{Zn}^{2+}$ , but also by the surface bound, tetramer bridging  $\text{Zn}^{2+}$ , which effectively stabilises the whole crystal lattice.

In this context, one might be tempted to ask whether the presence of  $\text{Zn}^{2+}$  in our crystal structure provides a mechanism how  $\text{Zn}^{2+}$  may contribute to allergenicity. However, the literature provides no corresponding evidence for such a relation, while other metals such as  $\text{Ni}^{2+}$  are well known elicitors of allergic contact dermatitis.

## 4. Discussion

### 4.1. Modelling the substrate binding

One of the most puzzling features of MtDH is its extremely poor substrate affinity. The  $K_M$  values for D-mannitol (dehydrogenase

activity) and D-fructose (reductase activity) are  $\sim 230$  mM and  $\sim 1300$  mM, respectively [3].

Due to its characteristic pH-dependence, the dehydrogenase activity of MtDH is unlikely to be of physiological significance in *C. herbarum*. The question then arises how D-fructose, having an approximately 1 M  $K_M$  value, can be specifically recognized and turned over by MtDH. The crystal structure of MtDH highlights an important aspect of substrate recognition: Only the open-chain form of D-fructose is able to bind to the active site, whereas fructose is mainly present in the hemiketal ring forms in aqueous solution (99.99%) [19,20] (Fig. 6b). The predominant ring form lacks an electrophilic C2 carbon, and would therefore inhibit the enzyme upon binding (Fig. 6c). Consequently, the apparent  $K_M$  of fructose underestimates the effective binding affinity of the open-chain fructose by about 10,000-fold, leading to effective  $K_M$  values in the submillimolar range. Given the stereo-selectivity of the initial hydrogen donation step, these discrete binding modes result in either D-mannitol or D-sorbitol as product [10], consistent with our two-binding mode hypothesis.

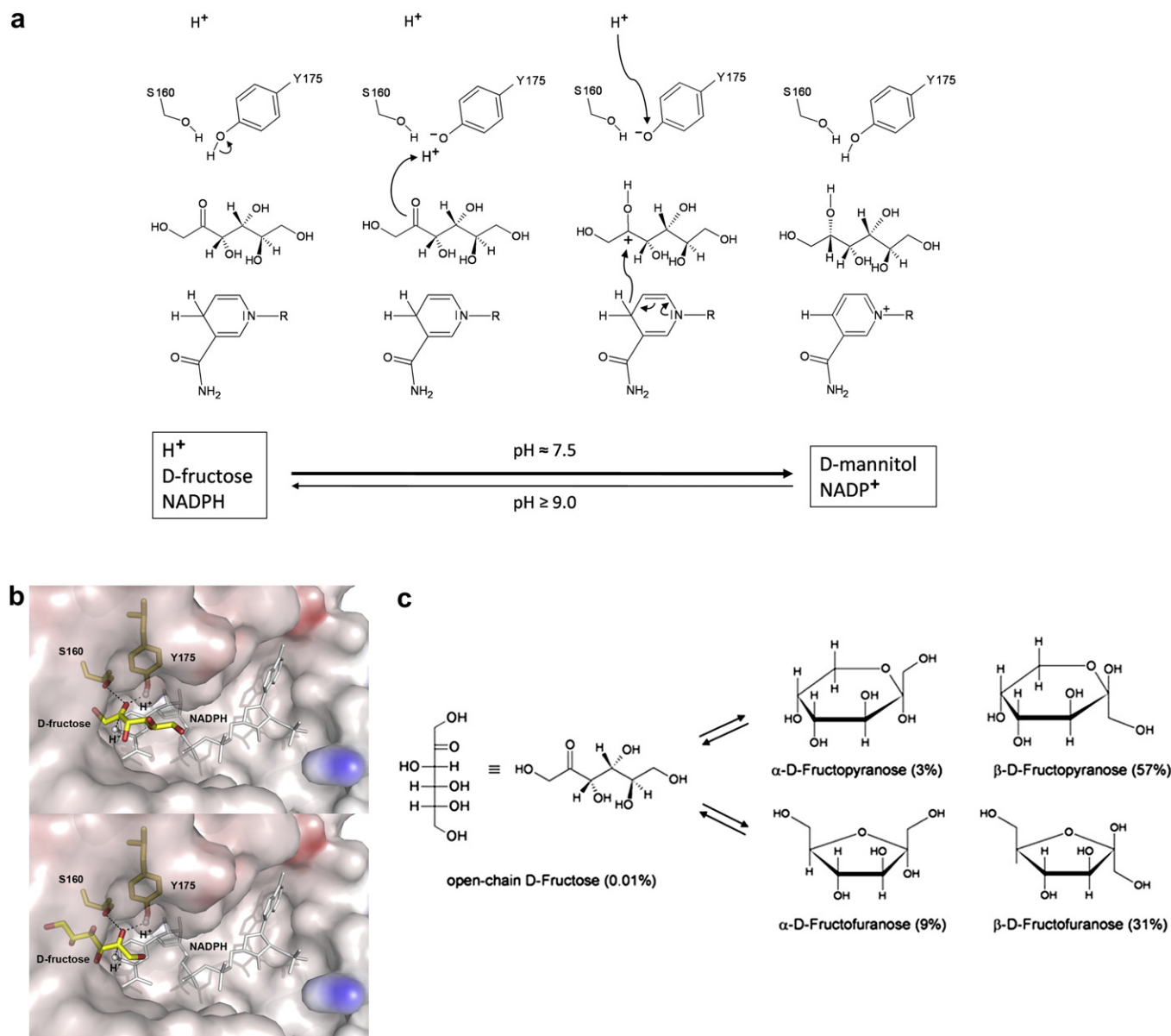


**Fig. 6.** Catalytic mechanism and substrate binding model of the active centre of *C. herbarum* MtdH and equilibrium scheme for open chain and ring forms of D-fructose. (a) Proposed catalytic mechanism of *C. herbarum* MtdH. The catalytic residues S<sup>160</sup> and Y<sup>175</sup> and the nicotinamide moiety are shown. Most likely, the tyrosine 175-OH that is stabilised by the Ser<sup>160</sup>-OH group acts as proton donor to the carbonyl O at C2 of D-fructose. Concomitantly, the C4 of the NADPH ring donates a hydrogen with a full electron pair (hydride) to the C2 atom of D-fructose, forming H-C-OH at the C2 atom of D-mannitol [30]. (b) Transparent molecular surface representation with electrostatic potential (blue positive, red negative) of the active centre cleft with modelled substrate. Two alternative binding modes of D-fructose to the active centre of *C. herbarum* MtdH are illustrated. D-fructose and the catalytic S<sup>160</sup> and Y<sup>175</sup> as well as the co-substrate NADPH are shown as stick models. The distance between the O2 oxygen of fructose and the C4 position of the nicotinamide ring and stabilising hydrogen bonds to S<sup>160</sup> are indicated with dotted lines. The figure was prepared with GRASP [30] and PyMOL (<http://pymol.sourceforge.net/>). (c) Schematic solution equilibrium of D-fructose with percentages for the open chain form (0.01%) and for the  $\alpha$ - and  $\beta$ -D-fructopyranose and fructofuranose ring forms. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

#### 4.2. Catalytic mechanism

For the reductase activity of MtdH, we assume Y<sup>175</sup> to be protonated in the resting state (Fig. 6a). Y<sup>175</sup> hereby acts as the central acid-base catalyst that donates (or abstracts) a proton to (from) the substrate (in brackets: dehydrogenase activity). The proton donation to the substrate's O2 atom by Y<sup>175</sup> further enhances the electrophilicity of the C2 carbon, thus accepting the NADPH-derived hydride ion at H2 position. Consequently, D-fructose is reduced to either D-mannitol or D-sorbitol, depending on its binding mode to the active site (Fig. 6b). In parallel, the nicotinamide ring will be oxidised by a double electron transfer, generating a positively charged ring system (Fig. 6a).

For completion of the catalytic cycle, the tyrosinate state of Y<sup>175</sup> needs to return to the uncharged protonated state, which we suggest to be mediated by a solvent-derived proton (H<sup>+</sup>) at neutral pH. Similarly, the oxidised co-substrate NADP<sup>+</sup> ("co-factor") needs to be replaced. This replacement is likely to occur after protonation of the tyrosine as a negatively charged tyrosinate would electrostatically stabilise the NADP<sup>+</sup> bound to the enzyme.

#### 4.3. Oligomerisation

Our interaction area analysis suggests that the dimer observed in solution is more likely formed by the A–B interaction. This conclusion is conflicting with earlier studies on related enzymes of

the SDR family that suggested the alternative A–C dimer to prevail in solution [21,22]. The rationale of this suggestion by Yamamoto is based on the dominance of hydrogen bonds at the A–C dimer interface (“Q-axis”), whereas the interaction surfaces for the two alternative dimers extend over comparable areas (~1600 Å each). Clearly, the final decision awaits additional experimental evidence.

Interestingly, both mono- and divalent cations contribute to the stabilisation of the tetramer by cross-linking the C-termini of MtDH. Furthermore, the MtDH tetramers can be cross-linked in the crystal by  $\text{Zn}^{2+}$  to form infinitely extended multimers. Although involving only oligomers consisting of not more than four monomers, a similar bridging by various cations ( $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ) at a given metal binding site resulted in oligomerisation of an engineered cytochrome [23,24].

#### 4.4. Relating the MtDH structure to its allergenicity

The molecular principles underlying the allergenicity of a protein are very complex, involving properties of the allergen on the one side and of the immune response of the allergic patient on the other side. It is established that the three-dimensional structure is crucial to the allergenic properties of an allergen, as heat-induced denaturation typically abolishes its allergenicity [25–27]. As has been pointed out by Magler and colleagues, molecular metamorphosis is a widespread conformational feature shared by almost all allergens studied so far [28]. Molecular metamorphosis indicates the unusual, allergen-specific property to adopt different conformations, often induced by oligomerisation transitions. Indeed, this capacity for metamorphosis is also shared by MtDH which exists in a conformational ensemble of monomers, dimers, and tetramers (Figs. 2 and 3). Additionally, in the crystalline state, even higher oligomers are observed in a concentration- and metal-dependent manner. The metamorphosis hypothesis is particularly attractive as it provides a straightforward explanation how a relatively small allergen is able to cross-link IgE antibodies on mast cells, a prerequisite of allergic reactions. Nevertheless, the elucidation of the exact molecular determinants that govern IgE binding to MtDH in its various oligomerisation states awaits further experiments.

#### Acknowledgements

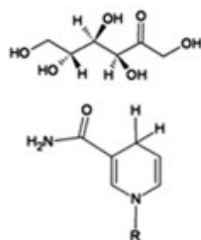
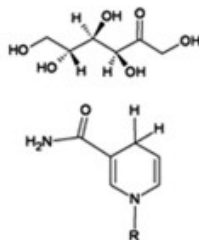
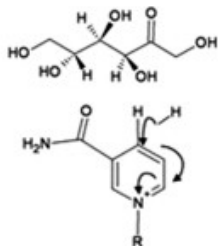
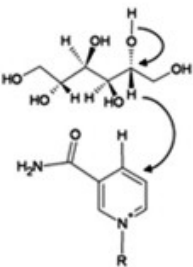
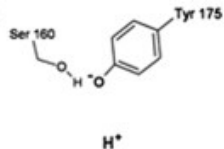
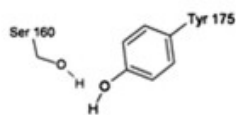
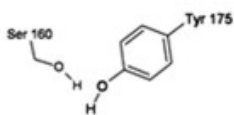
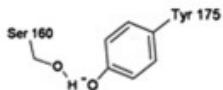
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#### Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biochi.2010.04.012.

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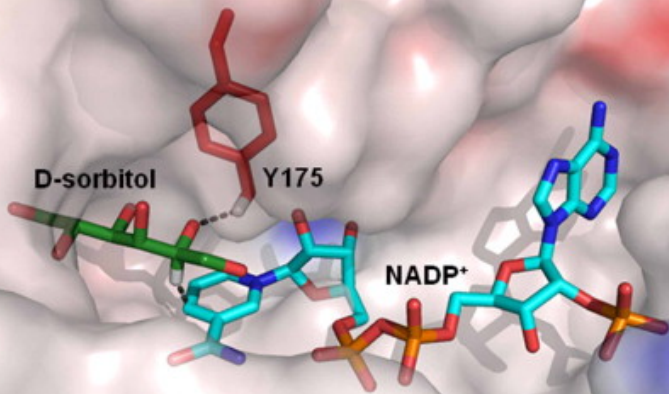


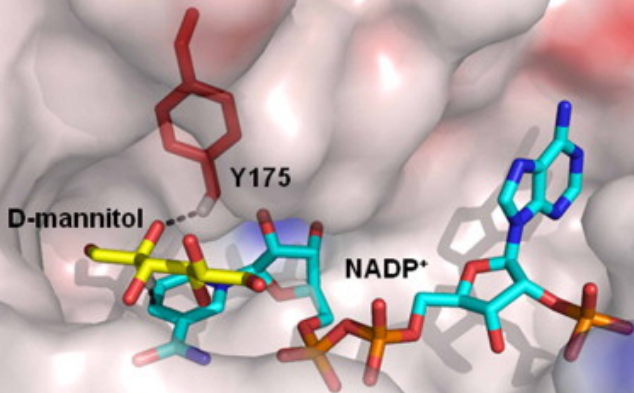
D-mannitol  
NADP<sup>+</sup>

pH  $\geq$  9.0

pH  $\approx$  7.5

$H^+$   
D-fructose  
NADPH





## SUPPORTING INFORMATION

### **Figure S1.** Catalytic mechanism and substrate binding model of the active centre of *C. herbarum* MtDH

(a) Proposed catalytic mechanism for the oxidation of D-mannitol. The residues S<sup>160</sup> and Y<sup>175</sup> directly involved in the catalysis and the nicotinamide moiety are shown. It is suggested that the tyrosinate<sup>175</sup>-O<sup>-</sup> that is stabilised by the Ser160-OH group acts as proton acceptor for the C2-OH hydrogen of D-mannitol. Concomitantly, the C4 of the positively charged NADP<sup>+</sup> ring abstracts hydrogen with a full electron pair (hydride) from the C2 atom of D-mannitol, forming the keto group of D-fructose. Potential binding modes of (b) D-mannitol and (c) D-sorbitol at the active centre of *C. herbarum* MtDH. D-Mannitol, D-sorbitol and the catalytic Y<sup>175</sup> are shown as stick models. The distance between the O2-oxygen of mannitol and the C4 position of the nicotinamide ring is indicated with a dotted line. The figure was prepared with GRASP [27] and PyMOL (<http://pymol.sourceforge.net/>).

Table S1

| Protein-Protein Hydrophobic Interactions             |         |       |                  |       |
|------------------------------------------------------|---------|-------|------------------|-------|
|                                                      | Residue | Chain | Residue          | Chain |
| 1                                                    | A6      | A     | L234             | B     |
| 2                                                    | L13     | A     | M45              | B     |
| 3                                                    | L16     | A     | L16              | B     |
| 4                                                    | M45     | A     | L12, L13         | B     |
| 5                                                    | I207    | A     | Y252             | B     |
| 6                                                    | L234    | A     | A6               | B     |
| 7                                                    | Y244    | A     | L258             | B     |
| 8                                                    | A256    | A     | L258             | B     |
| 9                                                    | L258    | A     | Y244, F245, A256 | B     |
| Protein-Protein Main Chain-Main Chain Hydrogen Bonds |         |       |                  |       |
| 1                                                    | G262    | A     | Y252             | B     |
| 2                                                    | G262    | B     | Y252             | A     |
| Protein-Protein Main Chain-Side Chain Hydrogen Bonds |         |       |                  |       |
| 1                                                    | L12     | A     | E44              | B     |
| 2                                                    | L13     | A     | E44              | B     |
| 3                                                    | Q15     | A     | K236             | B     |
| 4                                                    | R231    | A     | T251             | B     |
| 5                                                    | G233    | A     | Y252             | B     |
| 6                                                    | K236    | A     | T7, H9           | B     |
| 7                                                    | K239    | A     | E10              | B     |
| 8                                                    | Y252    | A     | R231, E237       | B     |
| 9                                                    | T266    | B     | R187             | B     |
| 10                                                   | L12     | B     | E44              | A     |
| 11                                                   | L13     | B     | E44              | A     |
| 12                                                   | Q15     | B     | K236             | A     |
| 13                                                   | R231    | B     | Q4               | A     |
| 14                                                   | K236    | B     | T7               | A     |
| 15                                                   | K239    | B     | E10              | A     |
| 16                                                   | Y252    | B     | Y206, R231, E237 | A     |
| 17                                                   | T266    | B     | R187             | A     |
| Protein-Protein Side Chain-Side Chain Hydrogen Bonds |         |       |                  |       |
| 1                                                    | S11     | A     | E44              | B     |
| 2                                                    | E37     | A     | E10              | B     |
| 3                                                    | K239    | A     | E10              | B     |
| 4                                                    | T251    | A     | E237             | B     |
| 5                                                    | R267    | A     | N191             | B     |
| 6                                                    | E10     | B     | E37              | A     |
| 7                                                    | S11     | B     | E44              | A     |
| 8                                                    | T251    | B     | E237             | A     |
| 9                                                    | R267    | B     | N191             | A     |
| Protein-Protein Ionic Interactions                   |         |       |                  |       |
| 1                                                    | E10     | A     | K236, K239       | B     |
| 2                                                    | R40     | A     | E10              | B     |
| 3                                                    | K236    | A     | E10              | B     |
| 4                                                    | K239    | A     | E10              | B     |
| Protein-Protein Aromatic-Sulphur Interactions        |         |       |                  |       |
| 1,2                                                  | Y252    | A, B  | M229             | B, A  |
| Protein-Protein Cation-Pi Interactions               |         |       |                  |       |
| 1,2                                                  | Y252    | A, B  | R231             | B,A   |

| Protein-Protein Hydrophobic Interactions             |         |       |                        |       |
|------------------------------------------------------|---------|-------|------------------------|-------|
|                                                      | Residue | Chain | Residue                | Chain |
| 1                                                    | Y85     | A     | V122                   | C     |
| 2                                                    | I117    | A     | F137, L189, W193       | C     |
| 3                                                    | L118    | A     | F196                   | C     |
| 4                                                    | V122    | A     | Y85                    | C     |
| 5                                                    | W125    | A     | L133, F137, M185       | C     |
| 6                                                    | L133    | A     | L133, V177             | C     |
| 7                                                    | F137    | A     | I117, W125             | C     |
| 8                                                    | V177    | A     | L133, M185             | C     |
| 9                                                    | M185    | A     | W125, V177             | C     |
| 10                                                   | L189    | A     | I117                   | C     |
| 11                                                   | W193    | A     | L118                   | C     |
| 12                                                   | F196    | A     | L118                   | C     |
| Protein-Protein Main Chain-Side Chain Hydrogen Bonds |         |       |                        |       |
| 1                                                    | I117    | A     | E192                   | C     |
| 2                                                    | L118    | A     | E192                   | C     |
| 3                                                    | K141    | A     | I117, L118, G120       | C     |
| 4                                                    | H145    | A     | L118                   | C     |
| 5                                                    | H184    | A     | S162, G163, H164, H165 | C     |
| 6                                                    | I117    | C     | E192                   | A     |
| 7                                                    | L118    | C     | E192                   | A     |
| 8                                                    | K141    | C     | I117, G120             | A     |
| 9                                                    | H145    | C     | L118                   | A     |
| 10                                                   | I165    | C     | H184                   | A     |
| 11                                                   | H184    | C     | S162, H164, H165       | A     |
| Protein-Protein Side Chain-Side Chain Hydrogen Bonds |         |       |                        |       |
| 1                                                    | T173    | A     | S188                   | C     |
| 2                                                    | N176    | A     | S188                   | C     |
| 3                                                    | S188    | A     | N176                   | C     |
| 4                                                    | R194    | A     | E171                   | C     |
| 5                                                    | T173    | C     | S188                   | A     |
| 6                                                    | N176    | C     | S188                   | A     |
| 7                                                    | S188    | C     | N176                   | A     |
| 8                                                    | R194    | C     | E171                   | A     |
| Protein-Protein Ionic Interactions                   |         |       |                        |       |
| 1                                                    | D119    | A     | K148                   | C     |
| 2                                                    | E171    | A     | R194                   | C     |
| 3                                                    | R194    | A     | E171                   | C     |
| Protein-Protein Aromatic-Aromatic Interactions       |         |       |                        |       |
| 1                                                    | F137    | A     | W125                   | C     |
| 2                                                    | W125    | C     | F137                   | A     |