Structural insights into the catalytic reaction trigger and inhibition of D-3-hydroxybutyrate dehydrogenase

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D-3-Hydroxybutyrate dehydrogenase catalyzes the reversible conversion of acetoacetate and D-3-hydroxybutyrate. These ketone bodies are both energy-storage forms of acetyl-CoA. In order to clarify the structural mechanisms of the catalytic reaction with the cognate substrate D-3-hydroxybutyrate and of the inhibition of the reaction by inhibitors, the enzyme from *Alcaligenes faecalis* has been analyzed by X-ray crystallography in liganded states with the substrate and with two types of inhibitor: malonate and methylmalonate. In each subunit of the tetrameric enzyme, the substrate is trapped on the nicotinamide plane of the bound NAD⁺. An OMIT map definitively shows that the bound ligand is D-3-hydroxybutyrate and not acetoacetate. The two carboxylate O atoms form four hydrogen bonds to four conserved amino-acid residues. The methyl group is accommodated in the nearby hydrophobic pocket so that the formation of a hydrogen bond from the OH group of the substrate to the hydroxy group of Tyr155 at the active centre is facilitated. In this geometry, the H atom attached to the C³ atom of the substrate in the *sp*³ configuration is positioned at a distance of 3.1 Å from the nicotinamide C⁴ atom in the direction normal to the plane. In addition, the donor–acceptor relationship of the hydrogen bonds suggests that the Tyr155 OH group is allowed to ionize by the two donations from the Ser142 OH group and the ribose OH group. A comparison of the protein structures with and without ligands indicates that the Gln196 residue of the small movable domain participates in the formation of additional hydrogen bonds. It is likely that this situation can facilitate H-atom movements as the trigger of the catalytic reaction. In the complexes with inhibitors, however, their principal carboxylate groups interact with the enzyme in a similar way, while the interactions of other groups are changed. The crucial determinant for inhibition is that the inhibitors have no active H atom at C³. A second determinant is the Tyr155 OH group, which is perturbed by the inhibitors to donate its H atom for hydrogen-bond formation, losing its nucleophilicity.

1. Introduction

There are two known pathways for the synthesis of acetyl-CoA, through glycolysis from glucose and through β -fatty acid oxidation from fatty acids (Laffel, 1999; Dedkova & Blatter, 2014), both of which supply the acetyl group to the TCA cycle, as shown in Fig. 1. Excess acetyl-CoA is converted to acetoacetate (AA¹) through three enzymatic steps in the liver; AA

¹ Abbreviations: HBDH, D-3-hydroxybutyrate dehydrogenase; AA, acetoacetate; HB, D-3-hydroxybutyrate; MA, malonate; mMA, methylmalonate; Nc, nicotinamide moiety of NAD⁺. Amino-acid residues are referred to using the three-letter codes in the text and the one-letter codes in the figures.

is then further converted reversibly to D-3-hydroxybutyrate (HB) by D-3-hydroxybutyrate dehydrogenase (HBDH) with NADH as a coenzyme, while a small amount of AA spontaneously converts to acetone. Fig. 2 shows the atomic numbering of the ligands. The HB, AA and acetone molecules are collectively named ketone bodies and are soluble in water. Acetone is excreted in exhaled breath. The remaining HB and AA are transported *via* the bloodstream to organs such as heart, kidney and brain as an energy source, where they are converted to acetyl-CoA. In other words, ketone bodies supply stored energy to organs that consume a large amount of energy.

An excess of ketone bodies in the blood causes ketoacidosis, as induced, for example, by diabetes mellitus (Laffel, 1999; Shimomura *et al.*, 2013). The HB level is normally higher than the AA level (Koch & Feldbruegge, 1987; Shimomura *et al.*, 2013), but when the HB level decreases the symptoms of ketoacidosis are alleviated. Therefore, some inhibitors of HBDH function have been developed as medical drugs, for example malonate (MA), methylmalonate (mMA) *etc.* (Tan *et al.*, 1975; El Kebbjaj *et al.*, 1980; Nakashima *et al.*, 2009), although mMA causes some toxic symptoms (Tan *et al.*, 1975). HBDH itself is used as a probe to detect the HB level in blood (Williamson *et al.*, 1962; Harano *et al.*, 1983; Koch & Feldbruegge, 1987; Uno *et al.*, 1987; Kwan *et al.*, 2006; Shimomura *et al.*, 2013; Pires *et al.*, 2003). Structural insights into HBDH are essential in order to understand the reaction mechanism described above.

HBDH is a member of the large SDR (short-chain dehydrogenases/reductases) superfamily (Kavanagh *et al.*, 2008; Persson & Kallberg, 2013), which is widespread and originates from a common ancestor structure not only in mammals but in every living organism, including microorganisms. To understand the functional variety of protein structures from a phylogenic point of view, extensive structural studies have been performed. Therefore, several X-ray structures of HBDH have already been reported (Hoque *et al.*, 2008, 2009; Nakashima *et al.*, 2009; Paithankar *et al.*, 2007; Ito *et al.*, 2006; Guo *et al.*, 2006), but the detailed enzymatic reaction mechanism and the substrate specificity are not yet fully understood. Previously, we suggested the substrate-binding mode using the structures of *Alcaligenes faecalis* HBDH in complex with NAD⁺ and acetate (Hoque *et al.*, 2009), and our proposal was subsequently confirmed by the crystal structure

Figure 1
Flow of ketone bodies coupled to the acetyl-CoA supply system.

of the ternary complex containing the cognate substrate HB (Hoque *et al.*, 2008). However, owing to low resolution (3.0 Å) it was difficult to distinguish clearly whether the bound molecule was HB or AA. In the present study, we have succeeded in obtaining another crystal form and have analyzed its structure at higher resolution (1.19 Å). In addition, to clarify the mechanism of inhibition of the catalytic reaction, crystal structures of HBDH complexed with NAD⁺ and either of two inhibitors, MA or mMA, have been determined.

2. Materials and methods

2.1. Crystallization

HBDH was expressed and purified using the method described in a previous report (Hoque *et al.*, 2008), but the Q Sepharose FF column was renewed and the FF-type Phenyl Sepharose column was replaced by an HP-type column (GE Healthcare). The reaction activity was confirmed according to a published procedure (Shuster & Doudoroff, 1962). Referring to the previous conditions, a sample of lyophilized HBDH was dissolved at a concentration of 20 mg ml⁻¹ in 100 mM Tris-HCl buffer pH 8.5. Crystallizations of three ternary complexes, HBDH-NAD⁺-HB, HBDH-NAD⁺-MA and HBDH-NAD⁺-mMA, were carried out using the hanging-drop vapour-diffusion method by mixing equal volumes (2 µl) of the enzyme solution and of reservoir solution containing 30–34% (w/v) PEG 4000, and then equilibrating the mixed solutions against 700 µl reservoir solution in a 24-well plate (Stem Corporation, Japan) at 293 K. Crystals of the HB-containing complex (HB complex), the MA-containing complex (MA complex) and the mMA-containing complex (mMA complex) were easily obtained within a few days at 293 K when the protein solution consisted of 5 mM NAD⁺ and 200 mM sodium salt of HB, MA or mMA. The actual pH values of the droplets were within the range 7.7–8.0.

2.2. X-ray diffraction data

Fresh crystals were transferred into cryoprotectant solution (a 1:1 mixture of the reservoir solution and 80% glycerol solution) for 30 s and mounted on a Cryo-Loop (Hampton Research, California, USA) for flash-cooling in a cold nitrogen-gas stream at 100 K. X-ray diffraction from crystals of the three complexes was recorded at 100 K on a Quantum 315r detector (ADSC, California, USA) using synchrotron radiation ($\lambda = 1.00$ Å) on BL-5A of the Photon Factory (PF),

Figure 2
Chemical structures of HB, AA, MA and mMA. The numbers indicate the atomic numbering used in the text.

Table 1

Crystal data, data-collection statistics and structure-refinement statistics.

Values in parentheses are for the highest resolution shell.

	HB complex	MA complex	mMA complex
Data collection			
Crystal system	Tetragonal	Tetragonal	Tetragonal
Space group	$P4_22_12$	$P4_22_12$	$P4_22_12$
Unit-cell parameters (Å)	$a = b = 62.8$, $c = 119.5$	$a = b = 62.8$, $c = 119.3$	$a = b = 62.8$, $c = 119.3$
Z	1	1	1
X-ray source	BL-5A, PF	BL-5A, PF	BL-5A, PF
Wavelength (Å)	1.00	1.00	1.00
Crystal-to-detector distance (mm)	176.6‡	118.4	117.2
Resolution (Å)	33.63–1.19 (1.25–1.19)	23.0–1.45 (1.53–1.45)	22.9–1.50 (1.58–1.50)
Observed reflections	607475 (48858)	569931 (82975)	510429 (69810)
Unique reflections	57643 (7213)	42260 (6208)	38406 (5518)
Multiplicity	12.0 (6.5)	13.5 (13.4)	13.3 (12.7)
Completeness (%)	74.0 (65.0)§	98.0 (100.0)	98.7 (99.1)
$R_{p.i.m.}$ ¶	0.047 (0.147)	0.034 (0.074)	0.023 (0.084)
R_{merge} ††	0.138 (0.385)	0.118 (0.262)	0.083 (0.294)
$\langle I/\sigma(I) \rangle$	11.2 (6.5)	17.3 (9.1)	22.2 (8.1)
Structure determination			
PDB code	5b4t	5b4u	5b4v
Resolution (Å)	24.7–1.19 (1.22–1.19)	20.8–1.45 (1.49–1.45)	21.0–1.50 (1.54–1.50)
R factor‡‡	0.157 (0.164)	0.136 (0.141)	0.142 (0.147)
R_{free} §§	0.173 (0.188)	0.151 (0.159)	0.163 (0.192)
Overall B value (Å ²)	10.8	9.1	13.7
R.m.s.d. from ideal			
Bond lengths (Å)	0.010	0.003	0.006
Bond angles (°)	1.4	0.92	1.2
No. of atoms			
HBDH	1906	1906	1906
NAD ⁺	44	44	44
Ligands	7	7	8
Water	234	332	397
Na ⁺	1	1	3
Cl [−]	—	1	1

† Number of subunits in the asymmetric unit. ‡ In the case of the HB complex, two additional data sets were collected with different exposure times (5 and 2 s) with the detector at a distance of 142.3 mm from the crystal. To measure the higher resolution data, the detector was moved to a position where it was possible to cover the half-sphere of diffraction, with the distance being 176.6 mm from the crystal. The exposure time was 10 s. The two data sets were processed by *CrystalClear* (Rigaku, Japan) and used to compensate for the overloaded reflections. § Despite the collection of two data sets, many strong reflections in the inner shell could not be processed owing to overloading. ¶ $R_{p.i.m.}$ as defined in Evans (2006). †† $R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the i th measurement of the intensity of reflection hkl and $\langle I(hkl) \rangle$ is its mean value. ‡‡ R factor = $\sum_{hkl} |F_{obs}| - |F_{calc}| / \sum_{hkl} |F_{obs}|$, where $|F_{obs}|$ and $|F_{calc}|$ are the observed and calculated structure-factor amplitudes, respectively. §§ R_{free} was calculated using a random set containing 5% of the observations that were not included throughout refinement (Brünger, 1992).

Tsukuba, Japan. Crystals diffracting to higher resolution were chosen for complete data collection. All diffraction patterns of these crystals were obtained with 1° oscillation steps with 10 s exposure per each frame; a total of 180 frames were recorded by rotating the crystal samples. In the case of the HB complex, however, the 360 frames were taken to cover the half reciprocal sphere at the highest resolution. Bragg spots were indexed and their intensities were integrated using *iMosflm* (Battye *et al.*, 2011). The collected intensity data were scaled between the frames and were then converted to amplitudes using *SCALA* (Evans, 2006) in *CCP4* (Winn *et al.*, 2011). In the case of the HB complex, some overloaded reflections were compensated for by merging with two additional data sets obtained using different exposure times at different distances from the crystals. Table 1 lists the diffraction-intensity processing statistics and the crystallographic data.

2.3. Structure determination

Approximate phase angles for the three data sets were derived by the molecular-replacement method with *MOLREP*

(Vagin & Teplyakov, 2010) and *AMoRe* (Navaza, 1994) in *CCP4* using a subunit structure of tetrameric HBDH (PDB entry 3vdr; Hoque *et al.*, 2009) as a reference structure. To find the remaining bound molecules, the atomic parameters were refined using *REFMAC5* (Murshudov *et al.*, 1997, 2011) and *CNS* (Brünger *et al.*, 1998; Brünger, 2007). After the addition of NAD⁺ and HB, MA or mMA molecules, the atomic parameters of each HBDH complex were further refined together with additional solvent water molecules and ions. The statistics of structure refinement are summarized in Table 1. *Coot* (Emsley *et al.*, 2010) from the *CCP4* suite was used for the manipulation of electron-density maps. The final $F_o - F_c$ OMIT maps of HB, the Nc moiety of NAD⁺, MA and mMA are shown in Fig. 3. These figures were generated by *DINO* (Philippsen, 2003) and Figs. 4–8 were generated by *PyMOL* (DeLano, 2002), with the exception of Fig. 7(a), which was drawn using *Coot*. A Ramachandran plot shows that the ϕ/ψ angles in favoured and allowed regions are 98.2 and 1.8%, respectively, for the HB complex, 97.7 and 1.9%, respectively, for the MA complex and 98.2 and 1.6%, respectively, for the mMA complex.

3. Results and discussion

3.1. Overall structures

When the present HB complex is compared with the previously published HB complex (Hoque *et al.*, 2009), it is notable that the two crystal forms are quite different, even though they were obtained under similar conditions. The space group and unit-cell parameters of the previous structure are $P4_12_12$ and $a = b = 91.6$, $c = 261.0$ Å, while those of the present structure are $P4_22_12$ and $a = b = 62.8$, $c = 119.5$ Å, respectively. In the present work, the crystal structures of the HB complex, MA complex and mMMA complex have been determined at higher resolutions of 1.19, 1.4 and 1.5 Å, respectively. Therefore, as shown in Fig. 3, their $F_o - F_c$ OMIT maps are well resolved to identify the molecular structures; in particular, the HB complex shows electron density at the atomic level, in contrast to the previous 3.0 Å resolution map.

Fig. 4(a) shows the overall tetrameric structure of HBDH composed of four subunits related by crystallographic 222 symmetry around $(\frac{1}{2}, \frac{1}{2}, 0)$ (for the side view see Supplementary Fig. S1). Each subunit is divided into two parts: the large domain (residues 1–190 and 220–260) and the small movable domain (residues 191–219). In the present complexes, all of the subunits adopt a closed conformation as the NAD^+ -bound state, as shown in Fig. 4(b). This feature is common to the three complexes, and the r.m.s.d.s on C^α atoms on superimposition are 0.11 Å between the HB complex and the MA complex and 0.15 Å between the HB complex and the mMMA complex, as shown in Fig. 4(c). Since the pK_a values of HB, AA, MA and mMMA are 4.41, 4.02, 2.43 and 2.48, respectively, their carboxy groups must be dissociated in the crystalline state.

3.2. Substrate binding and catalytic reaction

The final OMIT map (Fig. 3a) definitively shows that the bound molecule is D-3-hydroxybutyrate (HB) and not aceto-

acetate. Consistently, the OMIT map of the Nc moiety (Fig. 3b) is planar, indicating that the cofactor is NAD^+ and not NADH. HB is trapped on the nicotinamide plane of the NAD^+ bound in the central part of HBDH, as shown in Figs. 4(b), 5(a) and 5(b). The two carboxylate O atoms of HB interact with HBDH through four hydrogen bonds. One O atom accepts the two carbamoyl NH groups of Gln94 and Gln196; Gln196 belongs to the small movable domain. Another O atom also forms two hydrogen bonds to Lys152 and His144. These hydrophilic interactions are the same as those found in the HBDH- NAD^+ -acetate complex (Hoque *et al.*, 2008).

Near to the reaction centre, the hydroxy group attached to the C^3 atom of HB is specifically recognized through two hydrogen bonds, one accepting the hydroxy group of Ser142 and the other donating its hydroxy group to Tyr155, as shown in Fig. 5(b). The remaining methyl group attached to the C^3 atom of HB is accommodated in a hydrophobic pocket surrounded by the methyl group of Ala143 and the planar moieties of the Trp187, Trp257 and His144 residues. Thus, the space around the C^3 binding site seems to be suitable for the C^3 atom to adopt the sp^3 configuration. Based on this geometry, it is easy to estimate the position of the H atom attached to C^3 , as shown in Fig. 5(b) as an arrow towards the C^4 atom of Nc. The C^3 –H bond vector is almost perpendicular to the Nc plane and points in the direction of the C^4 atom of Nc, so that the two atoms, C^3 of HB and C^4 of Nc, closely approach each other at a distance of 3.1 Å, suggesting a trigger of the catalytic reaction.

It can also be noted that the hydroxy O atom of Tyr155 forms another hydrogen bond to the hydroxy $\text{O}^{2'}$ atom attached to $\text{C}^{2'}$ of the NAD^+ ribose. Since the $\text{O}^{2'}$ atom accepts an $\text{O} \cdots \text{HN}$ hydrogen bond from the amino group of Lys159, this $\text{O}^{2'}$ atom could donate an H atom as a donor for hydrogen-bond formation. Therefore, the hydroxy O atom of Tyr155 must form two hydrogen bonds to bifurcated acceptors

Figure 3

OMIT $F_o - F_c$ maps of HB (stereo pair) (a), Nc (b), MA (c) and mMMA (d). Electron density is contoured at $2.5-3\sigma$. The asterisk in (c) indicates an isolated water O atom that was incorporated to fill the void space of the hydrophobic pocket upon MA binding.

from the ribose OH group and from the HB OH group, because the latter accepts the OH group of Ser142 (see Figs. 6*a* and 6*c*). These hydrogen bonds suggest ionization of the Tyr155 residue, releasing a proton at the crystallization pH, as shown in Fig. 6. This subtle change might be related to the movement of H atoms during catalysis. The H atom of C³ could easily attack the π orbital of the C⁴ atom of Nc, while the H atom of the hydroxy group of HB could move to the

deprotonated O atom of Tyr155 following conversion from HB to AA. The reversed double H-atom movements at C³ and Tyr155 are also applicable to the reverse reaction from AA to HB. Thus, the geometrical features of the substrate-binding site can accept only D-form 3-hydroxybutyrate with the *R* configuration at the C³ atom. Even if the L-form (L-HB) is bound, the catalytic reaction does not proceed because the H atom of C³ is located in the opposite direction to Nc, as

Figure 4

Overviews of the tetrameric HBDH structure with 222 symmetry viewed along (1, −1, 0) (*a*) and the subunit (*b*). The small movable domain (violet) interacts with NAD⁺ (yellow) and HB (cyan). Thin lines indicate the side chains surrounding HB. For conformational comparison (*c*), the C α atoms of the MA complex (red) and the mMMA complex (blue) are superimposed on those of the HB complex (black). Their r.m.s.d. values are 0.11 and 0.15 Å, respectively, suggesting no significant conformational changes upon complex formation with substrate/inhibitors.

observed in a complex with the L-form (Nakashima *et al.*, 2009). These residues shown in Figs. 5 and 6 are highly conserved between organisms as the essential amino acids for the catalytic reaction of the enzyme (Mountassif *et al.*, 2010).

In the structural analysis of the previous HB complex ($P4_12_12$ form; Hoque *et al.*, 2009), it was difficult to distinguish the configuration of the bound molecule between HB and AA owing to low resolution. In contrast, the present HB-complex crystal ($P4_22_12$ form) indicates that HB is not converted to AA in the crystalline state even near the optimal pH of the catalytic condition. In addition to the space groups, the packing densities also differ, as is apparent from the solvent contents: 51.2% ($V_M = 2.52 \text{ \AA}^3 \text{ Da}^{-1}$) for the $P4_12_12$ form and 43.3% ($V_M = 2.17 \text{ \AA}^3 \text{ Da}^{-1}$) for the $P4_22_12$ form. It is of further interest to speculate why the catalytic reaction does not occur in the present crystal. In the purification of the present HBDH sample, two chromatographic columns were renewed. As soon as fresh crystals appeared, they were quickly cooled for X-ray data collection. These treatments, especially the purity of the HBDH sample, may have caused the difference in the crystal form.

In a comparison of the two crystal structures, it has been found that the molecular interactions around the reaction centres are quite different. Fig. 7(a) shows that the movable

domain composed of the helix–turn–helix is extruded into the solvent region without any interactions in the $P4_12_12$ form. In the $P4_22_12$ form (Fig. 7b), however, each HBDH subunit of a tetramer is associated with the subunit of a neighbouring tetramer related by crystallographic twofold symmetry. In this association, the movable small domain interacts with a helix of the remaining large domain belonging to another tetramer through several direct hydrogen bonds (see Supplementary Fig. S2) and hydrophobic contacts. These interactions would affect the position of the small domain that carries Gln196, which is essential for substrate recognition. This situation is shown in Fig. 6(b), which shows 13 superposed HBDH subunits: five in complex with NAD and HB, and the remaining eight in the apo state (PDB entries 2yz7 and 3vdq). Although the small domain is highly variable in the apo state, this domain is moved to close the active site in the complex with the ligands, and the Gln196 side chain moves closer to the substrate to form a hydrogen bond (for details, see Fig. 6c). When the domain movement is affected by the molecular packing, its influence will further propagate to the interaction of HB and Nc. Here, it is interesting to speculate that the $C^3 \cdots C^4$ distance will change depending on the movement of the small domain. In other words, the flexibility of the small domain might be coupled to the catalytic reactions in both

Figure 5
(a) A stereo-pair diagram of the HB-bound state of HBDH. (b–d) Detailed interaction geometries of the substrate/inhibitor-binding sites found in the HB complex (b), MA complex (c) and mMMA complex (d) projected from the back side of (a). Broken lines indicate possible hydrogen bonds. Dotted half circles show the hydrophobic pocket surrounded by His144, Ala143, Trp187 and Trp257 that accepts the methyl group of HB in the HB complex, the carboxylate O atom of MA and a water molecule in the MA complex and a methyl group of mMMA. One-letter codes are employed to specify the amino-acid residues. The numbers on the spheres indicate the atom numbering. An H atom in a blue circle, postulated to fill the tetrahedral configuration of the C^3 atom, points towards the C^4 atom of Nc in (b), with a $C^3 \cdots C^4$ distance of 3.1 Å.

directions: from HB to AA and from AA to HB. Some differences in the flexibility between the two forms can be estimated from the atomic r.m.s.d. values of their structures. When the four subunits of the $P4_22_12$ form are superimposed on the present subunit, the values are 0.89 Å between all atoms including the side chains and 0.53 Å between the C^α atoms. Indeed, the latter value is significantly larger than the corresponding values of 0.11 and 0.15 Å when compared with the MA complex and mMMA complex (see Fig. 4c) crystallized in the same $P4_22_12$ form. Therefore, it could be concluded that the molecular interactions suppress the catalytic reaction in the $P4_22_12$ packing with the help of the excess amount of substrate molecules (200 mM compared with 5 mM NAD^+).

In order to confirm the configurational change of the HB C^3 atom, X-ray/neutron analyses could visualize the stepwise

structural changes during the catalytic reaction along with pH changes in the crystalline state. In addition, if the HB-complex crystal diffracts well beyond 1 Å resolution, the H-atom movement described above could be demonstrated by X-ray/neutron analysis.

3.3. Inhibitor binding and inhibition of the catalytic reaction

As shown in Fig. 2, MA and HB differ from each other in the attachment of carboxy or 1-hydroxyethyl functional groups to the C^2 atom of acetate. MA and AA are similar to each other in molecular shape, but the latter contains a large hydrophobic methyl group at C^3 . Since HB should adopt the *R* configuration (*D*-form) at the C^3 atom, the *L*-form acts as an inhibitor (Nakashima *et al.*, 2009). Furthermore, mMMA and MA differ in the presence or absence of a methyl group at the C^2 atom. Compared with the HB-complex crystals, there are no significant changes in the conformation of the HBDH folding and of the bound NAD^+ molecules between MA-complex and mMMA-complex crystals.

Fig. 8(a) shows the structures of the bound-state HB, MA and mMMA molecules superimposed on the principal carboxylate groups for comparison. In the MA complex (Fig. 5c), MA is trapped in the central part of HBDH, where the principal carboxylate group corresponding to that of HB is firmly

Figure 6

(a) Postulated donor–acceptor relationship of hydrogen bonds around Tyr155 of HBDH. The two arrows suggest H-atom movement in the catalytic reaction. (b) A comparison of 13 superposed HBDH monomers. Thin lines indicate Gln196 and thick lines indicate C^α chains. When NAD^+ and HB are bound to HBDH, the small domain moves to close the active site (red) from the open form (black) by forming a hydrogen bond between Gln196 and HB. Stick lines and thin lines indicate C^α chains and Gln196, respectively. As shown in (c), which is viewed from the back side of (b), the participation of the small domain in forming an additional hydrogen bond appears to facilitate the approach of the H atom of C^3 to C^4 of Nc.

bound through four hydrogen bonds in the same way as described above for the HB complex. A remarkable difference is found in one of the two O atoms, O³ and O⁴, of the second carboxylate group. The O⁴ atom occupies the hydrophobic pocket, similar to the methyl group of HB. In addition, it is accompanied by an isolated water molecule to form a hydrogen bond. In other words, MA mimics HB in shape with the help of an isolated water molecule to fill the void space. Therefore, the conformation of MA is similar to that of HB.

MA and mMMA differ in the presence or the absence of a methyl group at the C² atom. In the case of the mMMA complex (Fig. 5*d*) the principal carboxylate group is similarly bound to

Gln94, Gln196 and Lys152 but not to His144. An H atom, postulated geometrically to be the N^δ atom of His144, points in the direction of the O⁴ atom of mMMA. The clearest difference from the MA complex is found in the methyl group, which is bound in the hydrophobic pocket. It implies that the hydrophobic effect is preferred over other hydrophilic interactions to comfortably fit mMMA in the pocket. For this, as shown in Fig. 8(*a*), the conformation of the second carboxylate group is largely changed by a rotation of 79° around the C¹—C² bond so that the methyl group approaches the position of the methyl group of HB and O⁴ is left near the O³ atom of MA. As a result, the O⁴ atom forms two hydrogen bonds to

Figure 7
Comparison of the effects of the surroundings on the movable domain composed of a helix–turn–helix motif (H–t–H) in the *P*₄₂₁₂ form (*a*) and the *P*₄₂₁₂ form (*b*). In (*a*) the four subunits (violet, blue, green and brown) are drawn as C^α chains. The movable domain (red) is extruded into the solvent region, but it does not directly interact with the other tetramers. In (*b*) (ribbon drawing) the two subunits of the neighbouring tetramers are associated at the interface (grey line), where the movable domain interacts with a rigid helix of another tetramer through direct hydrogen bonds and hydrophobic effects. Thin lines indicate side chains protruding from the ribbon models and dotted spheres indicate contacts between the movable domain and the main domain.

Figure 8
HB (cyan), MA (green) and mMMA (purple) molecules bound in HBDH (*a*) and a possible H-atom arrangement for the hydrogen-bonded network of the MA complex (*b*) and the mMMA complex (*c*). In (*a*), the principal carboxylate groups of MA and mMMA are superimposed on that of HB for comparison of conformational change. The second carboxylate group of mMMA is rotated to accommodate the methyl group in the hydrophobic pocket.

Ser142 and His144, and the O³ atom then forms a hydrogen bond to Tyr155.

Based on the above structural features, it is interesting to consider how MA and mMMA inhibit the catalytic reaction. The main reason is simply that their carboxy groups positioned near the reaction centre have no active H atom to attack the C⁴ atom of Nc. Another factor involves the hydroxy group of Tyr155. Since the second carboxy groups of MA and mMMA are also dissociated, they can behave as acceptors (see Figs. 8*b* and 8*c*). To form another hydrogen bond, Tyr155 must accept the ribose hydroxy group. In both the MA complex and the mMMA complex, therefore, the hydroxy group of Tyr155 should be undissociated to donate an H atom for hydrogen-bond formation to the carboxylate O atoms. In the case of L-HB, although it can bind to HBDH with a similar geometry and Tyr155 may be ionized in the hydrogen-bonded network as deduced from the deposited PDB data (PDB entry 2zt1; see Supplementary Fig. S3), the H atom of C³ is unfortunately not positioned in the direction of the Nc C⁴ atom.

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