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Insight into the role of halogen bond in the activity of D-mandelate dehydrogenase toward halogenated substrates

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ABSTRACT

Chlorine substitution in mandelate turned its derivatives into less active substrates for D-mandelate dehydrogenase (DMdh). To improve the catalysis of chloro-mandelate, a halogen bond was introduced into the protein–substrate complex by site mutation. The catalytic activity of the resulting mutant A89H toward *o*-chloromandelate was improved by 5 times. The effect of halogen bond was also observed in the dehydrogenation of *m*-chloromandelate and *p*-chloromandelate. Based on these results, a new insight was proposed for the role of halogen bond in enzyme activity, and the introduction of halogen bond was shown as an efficient strategy to optimize the catalytic activity toward halogenated substrates.

Halogenated aromatic systems are common scaffolds in medicinal chemistry[1–3] due to their improved permeability and catabolic stability compared to the non-substituted counterparts.[4] In the past few years, enzymatic synthesis has received increasing interest in the production of halogenated pharmaceuticals.[5–7] However, for most proteins, the natural substrates have non-substituted structures. When applied in the reaction with halogenated compounds, the catalytic properties of proteins change significantly owing to the prominently different steric and electronic properties of halogen from hydrogen.[8–10]

As a key synthetic block of clopidogrel, the bioproduction of enantiomerically pure *o*-chloromandelate has attracted extensive attention in recent years.[11–15] However, the chlorine substitution at *ortho*-position turns this molecule into a less active ligand, impeding the enzymatic catalysis.[16–19] In particular, the dehydrogenation efficiency of *o*-chloromandelate was significantly lower than that toward mandelate. In the preparation of *S*-mandelic acid by *Pseudomonas putida* ECU1009, both the enzymatic activity and enantioselectivity decreased when the substrate was switched from mandelic acid to *o*-chloromandelic acid.[11] This reduced catalytic performance was also encountered in the dehydrogenation of *o*-chloromandelate by *Pseudomonas aeruginosa* ZJB1125[17] and D-mandelate dehydrogenase from *Lactobacillus brevis* (LbDMDH).[19]

To eliminate the activity inhibition exerted by chlorine substitution at *ortho* position and improve the biocatalysis of *o*-chloromandelate, rational protein engineering could be adopted as a promising strategy considering its extensively demonstrated effectiveness in improving enzyme catalytic properties.[20–23] In protein design, the substrate-specific interaction between the protein and the substrate which stabilizes the protein–substrate complex has been shown advantageous in optimizing enzymatic catalysis and the introduction of this interaction was proposed as an efficient strategy for specific improvement of catalytic efficiency.[24] In the enzymatic catalysis of halogenated substrates, halogen bond is a specific electrostatic force-based interaction recently revealed to be established by one halogen atom acting as lewis acid and another as lewis base.[25] This interaction has been reported to exhibit high intensity and directionality in many macromolecular systems and could be regarded as one of the stabilizing factors for the halogen-containing systems.[26–28] Therefore, halogen bond can be chosen as the candidate in protein redesign for improved catalytic performance toward chlorinated substrates (Fig. 1).

In the present work, to investigate the role of halogen bond in the protein–ligand complex structure and furthermore to explore its application in expanding protein engineering strategy, the oxidation of *o*-chloromandelate by D-mandelate dehydrogenase (DMdh) was employed as a model reaction (Scheme 1), due to the explicit mechanism of enzymatic dehydrogenation[29,30] and the available X-ray structure of the protein (PDB ID: 2W2L). As the first step, the catalytic activities of the wild-type DMdh toward the natural substrate mandelate (**1a**), the target substrate *o*-chloromandelate (**1b**) and two other chlorinated substrates *m*-chloromandelate (**1c**) and *p*-chloromandelate (**1d**) were determined, respectively (Table 1). Other halogenated substrates were not

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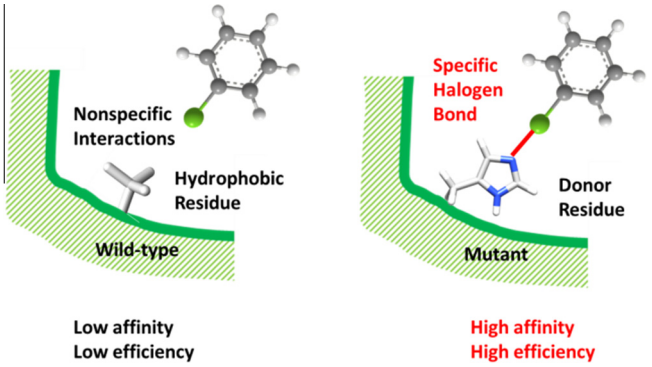

Figure 1. Strategy for enzyme design by introduction of a halogen bond. In the wild-type DMdh, only nonspecific interactions to the halogenated substrate could be formed between the substrate and the hydrophobic residues, leading to the low affinity and catalytic efficiency of the enzyme. The electron donor residues introduced would form specific halogen bond with the halogen in the substrate and thus stabilize the enzyme–substrate complex, resulting in improved affinity and efficiency.

involved due to the following reasons. Fluoro-substituted substrates were not employed because fluorine atom is unable to form halogen bond,[25–28] while bromo- and iodo-substituted substrates were excluded because of the destructive steric inhibition from their large volume in *para*-position. The wild-type protein exhibited a relatively high activity toward the *R*-enantiomer of the non-substituted **1a** while no activity was detected with the *S*-isomer (data not shown), which was consistent with the reported enantioselectivity of DMdh.[31] In the catalysis of chloro-substituted substrates, it was found that chlorine substitution at different positions depressed the activity of DMdh to different extents. Among these substrates, *ortho*-substituted **1b** was the most detrimental ligand of DMdh with only 1.5% of the activity remaining.

Further kinetic analysis was conducted for the enzymatic dehydrogenation of **1a** and its halogenated derivatives on the basis of the ordered bi–bi mechanism.[32] As shown in Table 2, kinetic constants of the non-substituted and chloro-substituted substrates pointed to distinct difference in catalytic activities. In the catalysis of the chloro-substituted **1b**, **1c**, and **1d**, the protein affinity of both the substrate and the coenzyme was dramatically suppressed compared to that in the dehydrogenation of **1a**, which could be ascribed to the larger volume of chlorine than hydrogen. Among all the substrates tested, *ortho*-substituted **1b** had the lowest affinity to the enzyme. Furthermore, the significantly decreased catalytic constant indicated that the electron transfer process during the reaction was also hindered by the chloro-substitution due to the intense electron-withdraw effect of the substituent. As a result of these steric as well as electronic effects exerted by the chlorine substituent, the catalytic efficiency of both the substrate and the coenzyme dropped remarkably.

Scheme 1. Dehydrogenation of mandelate and chloro-substituted mandelate by D-mandelate dehydrogenase (DMdh).

Table 1
Catalytic activities of the wild-type DMdh and the mutant A89H toward mandelate and its chloro-substituted derivatives

Entry	Protein	Substrate	Specific activity[a] (U/mg)
1	WT	*R*-**1a**	30.06 ± 11.97
2	WT	*R*-**1b**	0.46 ± 0.35
3	WT	*R*-**1c**	9.98 ± 2.47
4	WT	*R*-**1d**	6.58 ± 2.79
5	A89H	*R*-**1a**	14.75 ± 5.09
6	A89H	*R*-**1b**	2.36 ± 0.15
7	A89H	*R*-**1c**	14.41 ± 1.85
8	A89H	*R*-**1d**	4.30 ± 0.87

[a] The specific activities were determined spectrophotometrically by monitoring the formation of NADH at 340 nm with the reaction condition of 30 °C and Glycine–NaOH buffer (0.15 M, pH 9.5). The concentrations of the substrate and the coenzyme were 10 mM and 0.5 mM, respectively. 1 U was defined as the amount (μmol) of NADH formed per mg enzyme per minute.

To provide a molecular insight into the reason why chloro-substitution decreased the reactivity of the halogenated substrates, molecular dynamics simulations were conducted. The trinary complexes of protein–coenzyme–substrate were investigated to reveal the molecular basis for the dramatically different catalytic activities toward **1a** and **1b**. As shown in Figure 2A and B, stabilized by a set of hydrophilic residues, there was no difference in the pattern of coenzyme binding to the protein between the DMdh-substrate complexes of **1a** and **1b**. However, the location that the benzene ring of the substrates embedded into the protein active pocket was different. The binding of **1b** was apparently impeded by the chlorine substitution and consequently the substrate was pushed out of the protein. The study on the ligand binding conformation verified that the chlorine substitution at *ortho*-position inactivated **1b** by reducing the protein–substrate affinity, in good agreement with the kinetic analysis.

To investigate the role of halogen bond in enzyme activity, mutants were designed by introducing this specific interaction into the complex formed by DMdh and the most inactive substrate **1b** through site mutagenesis. Based on the directionality of the halogen bond, the residues located within 6 Å of the chlorine atom in the extension direction of C—Cl bond in the substrate were taken as the mutation hot-spots. As shown in Figures S3A and S3B, Alanine at site 89 was designated as the mutation site, and five amino acids which have the potential to form halogen bond with the substrate, including histidine, serine, cysteine, aspartic acid, and asparagine, were proposed as the lewis base atom donor to replace the 89A.[28]

All five mutants were tested virtually by molecular dynamics simulations, and the substrate binding as well as halogen bond formation was examined. Judged from the orientation of the residue at site 89 which faced the coenzyme and apart from the substrate, only the histidine substitution with two nitrogen atoms had the potential to form a halogen bond with the chlorine in **1b**. Simulation was performed for the complex of mutant A89H with **1b** and the results are shown in Figure 2C and D. The binding conformation of coenzyme and substrate in the mutated protein was similar to that of the wild type with a slightly changed binding location. This alteration was caused by the newly formed interactions between His89 and the ligands. A halogen bond was shown to be established by the conformation of the complex with a distance (Cl···N_ε) of 4.5 Å and an angle (C—Cl···N_ε) of 161.7° (Table S2). Additionally, a hydrogen bond was introduced between the protein and NAD^+, which was considered as beneficial to the catalysis in two ways, to further stabilize coenzyme binding, and more importantly to help constrain the orientation of the imidazole ring in His89 so as to guarantee the accession of N_ε to Cl. Besides, the interaction energy between the residue at the mutation site and the substrate in the typical conformations extracted from MD