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# Substrate Binding and Reactivity are Not Linked: Grafting a Proton-Transfer Network into a Class 1A Dihydroorotate Dehydrogenase†

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## Abstract

Adding the two residues comprising the conserved proton-transfer network of Class 2 dihydroorotate dehydrogenase (DHOD) to the Cys130Ser Class 1A DHOD did not restore the function of the active site base or rapid flavin reduction. Studies of triple, double, and single Class 1A mutant enzymes showed that the network actually prevents cysteine from acting as a base and that the network residues act independently. Our data show that residue 71 is an important determinant of ligand binding specificity. The mutation of Leu61Phe tightens dihydrooroate binding, but weakens the binding of benzoate inhibitors of Class 1A enzymes.

Dihydroorotate dehydrogenases (DHODs) catalyze the only redox reaction in the biosynthesis of pyrimidines. DHODs convert dihydroorotate (DHO) to orotate (OA). An active site base from DHOD removes a proton at C5 of DHO, and a hydride is transferred from DHO at the C6 position to FMN, reducing the flavin (Figure 1). The DHODs are phylogentically classified into Class 1 and Class 2 (1). The Class 1 DHODs are further grouped into 1A and 1B. The oxidizing substrate used by DHOD is different for each phylogenetic class; Class 2 DHODs use ubiquinone (2), Class 1A DHODs use fumarate (3), and Class 1B DHODs use NAD (4). The location of the DHODs in the cell is also different; Class 2 DHODs are membrane-bound (2) while Class 1 DHODs reside in the cytosol (3). The oligomerization state is also different, Class 2 DHODs are monomers (2), Class 1A DHODs are dimers (3) and Class 1B DHODs are $\alpha_2\beta_2$ heterotetramers (4).

The structure of the pyrimidine binding site appears to be nearly the same in all DHODs (Figure 2), except that the active site bases which deprotonate DHO are different for the DHOD classes (5,6). The Class 2 DHODs use a serine as their active site base while the Class 1 DHODs use a cysteine. Despite the nearly identical active sites, the two bases are not interchangeable. The *L. lactis* Class 1A Cys130Ser mutant enzyme oxidizes DHO ~5 orders of magnitude slower than wild-type enzyme (7). This suggests that residues not immediately in contact with DHO or FMN are critical for reactivity. The serine of the Class 2 enzyme passes a proton from DHO to solvent via a proton-transfer network. A threonine and a water molecule form the network, and a phenylalanine helps orient the hydrogen

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Supplementary table and experimental procedures. This material is available is available at http://pubs.acs.org

bonds (8). The Class 1A enzyme does not have a proton-transfer network (9). Its active site base is more exposed to solvent than the Class 2 enzyme.

The absence of the proton-transfer network in the Class 1A enzyme (9) may be the critical factor for the lack of reactivity of serine as base. This was tested by making the Class 1A triple-mutant Leu71Phe/Cys130Ser/Val133Thr. The usual red-shift (10) was seen upon addition of DHO. Therefore DHO binds. The triple-mutant reacted excruciatingly slowly, with a $k_{red}$ of $9.7 \times 10^{-6} \pm 3 \times 10^{-7}$ $s^{-1}$ at 25 °C. The reduction of the triple-mutant with DHO at 4 °C took so long (~one month) that its $k_{red}$ is not reported. Thus, installation of a proton-transfer network next to serine 130 did not restore rapid reduction. The very slow reactivity of DHO allowed aerobic titrations. Interestingly titrations of the triple-mutant with DHO at 25 °C showed that it had a $K_d$ of 57 ± 2 μM which is about three times tighter than the wild-type Class 1A enzyme. The reduction potential of the triple-mutant was determined by the method of Massey (11) using phenosafranine as the indicator dye and found to be −264 mV, very similar to that of the wild-type Class 1A enzyme (−245 mV) (10), thus confirming that the active site had not been severely disrupted despite mutating three residues.

The *E. coli* wild-type Class 2 enzyme does not bind the ligands 3,4-dihydroxybenzoate (3,4-diOHB) or 3,5-dihydroxybenzoate (3,5-diOHB), while the *L. lactis* wild-type enzyme does (12,13). The binding affinity of the *L. lactis* Class 1A triple-mutant with these ligands was tested by aerobic titrations at 25 °C. Surprisingly, the triple-mutant did not bind 3,4-diOHB and had weak binding for 3,5-diOHB ($K_d$ = 392 ± 38 μM). The installation of the proton-transfer network changed the ligand selectivity and in this regard made the triple-mutant more like the Class 2 enzyme. However, installing a proton-transfer network into a Class 1A DHOD does not convert it completely into a Class 2 DHOD because its rate of reduction is slow.

It was surprising that the leucine to phenylalanine and valine to threonine substitutions did not make the serine a functional base. The possibility was investigated that these residues actually inhibit the function of the residue at position 130 of the Class 1A enzyme. Therefore the double-mutant Leu71Phe/Val133Thr was created. Based on the reduction potential of the double mutant (−262 mV) the integrity of the active site seems to be preserved despite the two mutated residues. Installation of the proton-transfer network inhibited reduction. The reaction of the double-mutant with DHO had a $k_{red}$ of 0.0010 ± 0.0001 $s^{-1}$ at 4 °C (Figure 3) which was faster than the triple-mutant but still drastically slower than the wild-type Class 1A and Class 2 enzymes. Despite the large decrease in the speed of reduction with DHO, the $K_d$ was 22 ± 0.9 μM, about eight-fold tighter than the wild-type Class 1A enzyme and nearly the same as wild-type Class 2 enzyme. The double-mutant, like the triple-mutant, also does not bind 3,4-diOHB and weakly binds 3,5-diOHB ($K_d$ = 453 ± 27 μM). Adding the two residues of the proton-transfer network when the base is cysteine resulted in a mutant that had more Class 2-like properties, like the triple-mutant, except for reactivity.

To determine whether one residue or both residues in the proton-transfer network were responsible for the behavior of the triple and double-mutants described above, the two residues were mutated individually. The reduction potential of the Val133Thr mutant enzyme (−266 mV) was also almost the same as wild-type Class 1A enzyme. The Val133Thr mutant enzyme was just a little slower ($k_{red}$ = 41 ± 0.8 $s^{-1}$ at 4 °C; Figure 3 and 100 ± 1.9 $s^{-1}$ at 25 °C) than the wild-type Class 2 enzyme ($k_{red}$ = 46 ± 0.3 $s^{-1}$ at 4 °C). Binding of DHO to the Val133Thr mutant enzyme was much weaker ($K_d$ = 84 ± 0.9 μM) than the Class 2 wild-type enzyme but tighter than the wild-type Class 1A enzyme ($K_d$ = 145 ± 50 μM). The Val133Thr mutation did not inhibit binding of 3,4-diOHB as had been

observed in both the double and triple Class 1A mutants. Therefore the Val133Thr mutation did not change the ligand binding behavior to that of the Class 2 enzyme. The Val133Thr mutant had weak binding for 3,4-diOHB and 3,5-diOHB with $K_d$ values of 640 ± 40 and 156 ± 4 μM, respectively. The Val133Thr mutant enzyme-3,4-diOHB complex had charge-transfer absorbance, as seen by large spectral increases from 550–800 nm, as does the wild-type Class 1A-3,4-diOHB complex, and none was observed in the presence of 3,5-diOHB, similar to the wild-type Class 1A enzyme (13).

The Leu71Phe mutant enzyme had a slower rate of reduction with DHO ($k_{red}$ = 0.015 ± 0.002 $s^{-1}$ at 4 °C; Figure 3 and 0.07 ± 0.001 $s^{-1}$ at 25 °C) compared to both the wild-type Class 1A and Class 2 enzymes. The $k_{obs}$ did not vary with DHO concentration, indicating that the $K_d$ of DHO was much less than 125 μM - tighter than the wild-type Class 1A enzyme but not the wild-type Class 2 enzyme. The Leu71Phe enzyme did not bind 3,4-diOHB and had weak binding for 3,5-diOHB with a $K_d$ of 417 ± 17 μM. The reduction potential of Leu71Phe was −259 mV. These data show that residue 71 is partly responsible for ligand-binding specificity, in particular that of 3,4-diOHB.

Mutating the residues at positions 71 and 133 had different effects on the enzyme. The changes in free energies of activation for the double and both single mutants were calculated from transition state theory in order to decide whether position 71 interacts with position 133. In order to compare changes in free energies of activation the rate constants from 4 °C were used because the Class 1A wild-type enzyme reduces too quickly to measure at higher temperatures. The change in Gibbs free energy caused by mutating wild-type Class 1A enzyme to Leu71Phe ($\Delta\Delta G^{\ddagger}$ = 5.1 ± 0.4 kcal $mol^{-1}$) and then from Leu71Phe to Leu71Phe/Val133Thr ($\Delta\Delta G^{\ddagger}$ = 1.49 ± 0.07 kcal $mol^{-1}$) was no different than that obtained when taking the Class 1A wild-type enzyme and mutating it to Val133Thr ($\Delta\Delta G^{\ddagger}$ = 0.80 ± 0.4 kcal $mol^{-1}$) and then creating the double-mutant ($\Delta\Delta G^{\ddagger}$ = 5.83 ± 0.01 kcal $mol^{-1}$). The free energy analysis shows additive effects on the two mutations – a total of 6.6 ± 0.4 kcal $mol^{-1}$ for the double-mutant, obtained directly or by either sequence of mutations. The two residues are noninteracting.

Overall, installing a proton-transfer network into a Class 1A DHOD does not cleanly convert it into a Class 2 DHOD. The mutations had a range of effects on the binding of different ligands. Installing the proton-transfer network caused DHO to bind tighter, mimicking Class 2 enzymes. However, the binding of only one of a pair of benzoate ligands became more like Class 2. Curiously the binding of OA was never perturbed by these mutations (Table 1). The difference between OA and DHO is that OA is planar, but DHO is not. Apparently a Phe at position 71 is better suited to accommodate the non-planar ligand, although it is not yet possible to say why. Leu71, a key conserved residue in Class 1A enzymes, is critical for the binding of 3,4-diOHB but not as important for the binding of 3,5-diOHB and its mutation had large effects on reactivity. The proton-transfer network that enhances reactivity in the Class 2 enzymes inhibits reactivity when grafted into the Class 1A enzyme yet seems to be responsible for the tight DHO binding of Class 2 DHODs. Thus substrate binding is not linked to reactivity; the residues of the proton-transfer network are important for transition-state stabilization rather than reactant stabilization.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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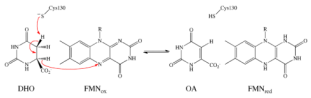


**Figure 1.**

The reaction of a Class 1A DHOD with DHO. A hydride from C6 of DHO is transferred to N5 of FMN, while the proton from C5 of DHO is removed by the active site base, Cys130. In Class 2 DHODs, the active site base is serine.

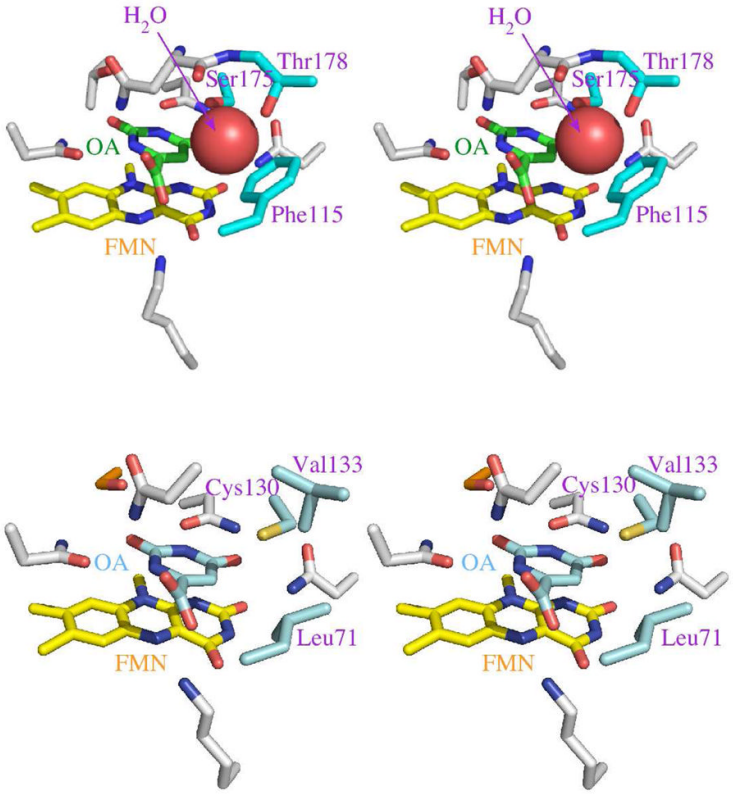


**Figure 2.**

Stereoview of Class 2 and Class 1A DHODs. Class 2 DHODs (Top) have a proton-transfer network that is made up of Thr178, Ser175, a water molecule, and Phe115 (shown in lavender). Class 1A DHODs (Bottom) do not have a proton-transfer network, however mutations were made to residues Val133, Cys130, and Leu71 (shown in lavender) to incorporate a Class 2 proton-transfer network into the Class 1A enzyme.

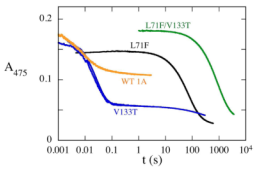


**Figure 3.**

Reduction of Class 1A variants. The reduction of the wild-type Class 1A enzyme and the Leu71Phe, Val133Thr, Leu71Phe/Val133Thr variants was studied by mixing anaerobic solutions of the oxidized enzyme with anaerobic solutions of DHO in a stopped-flow spectrophotometer. The wild-type did not reduce fully because the preparation had significant amounts of damaged enzyme but the kinetics were the same as reported in reference (7). All reactions were in 27.8 mM Taps 22 mM KCl, pH 8.5 at 4 °C. Note the logarithmic timescale.

**Table 1**

Reduction rate constants at 4 °C and dissociation constants 25 °C

| Enzyme | $k_{red}$ (DHO) ($s^{-1}$) | $K_d$ (DHO) (μM) | $K_d$ (OA) (μM) | $K_d$ (3,4-diOHB) (μM) | $K_d$ (3,5-diOHB) (μM) |
|---|---|---|---|---|---|
| *E.coli* wild-type Class 2 | 46 ± 0.3[a] | 20 ± 1[a] | 3.4 ± 0.8[b] | No binding[c] | No binding[c] |
| *L.lactis* wild-type Class 1A | 170 ± 12 | 145 ± 54[d] | 13.1 ± 0.7[e] | 19 ± 2[c] | 18 ± 0.2[c] |
| Leu71Phe | 0.015 ± 0.002 | ≪ 125[f] | 13.5 ± 0.8[g] | No binding[g] | 417 ± 17[g] |
| Val133Thr | 41 ± 0.8 | 84 ± 9[d] | 25 ± 0.7[g] | 640 ± 40[g] | 156 ± 4[g] |
| Leu71Phe/Val133Thr | 0.0010 ± 0.0001 | 22 ± 0.9[g] | 12 ± 0.6[g] | No binding[g] | 453 ± 27[g] |
| Leu71Phe/Cys130Ser/Val133Thr | Too slow TBD | 57 ± 2[g] | 16 ± 1.1[g] | No binding[g] | 392 ± 38[g] |

[a] Data taken from ref (14).

[b] Data taken from ref (9).

[c] Data taken from ref (13).

[d] Data taken from stopped-flow experiments.

[e] Data taken from ref (1).

[f] Binding value too low to be determined.

[g] Data taken from aerobic titrations.