BIOCHEMISTRY
including biophysical chemistry & molecular biology

RAPID REPORT

pubs.acs.org/biochemistry

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

Claudia A. McDonald and Bruce A. Palfey*

Department of Biological Chemistry, University of Michigan Medical School, 1150 West Medical Center Drive, Ann Arbor, Michigan 48109-0606, United States

Supporting Information

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 mutant Class 1A 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 Leu71Phe mutation 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 phylogenetically classified into Class 1 and Class 2.[1] The Class 1 DHODs are further grouped into Class 1A and Class 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 that 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 *Lactococcus lactis* Class 1A Cys130Ser mutant enzyme oxidizes DHO ~5 orders of magnitude more slowly than the 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 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.

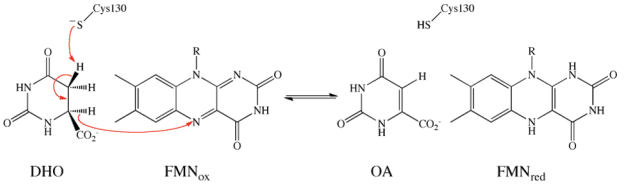

Figure 1. Reaction of a Class 1A DHOD with DHO. A hydride from C6 of DHO is transferred to N5 of FMN, while the proton from C5 of DHO is removed by the active site base, Cys130. In Class 2 DHODs, the active site base is serine.

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 the base. This was tested by making the Class 1A Leu71Phe/Cys130Ser/Val133Thr triple mutant. 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 (~1 month) that its k_{red} is not reported. Thus, installation of a proton-transfer network next to Ser130 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 3 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 −273 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 mutation of three residues.

The *Escherichia 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 for 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).

Received: February 19, 2011
Revised: March 11, 2011
Published: March 14, 2011

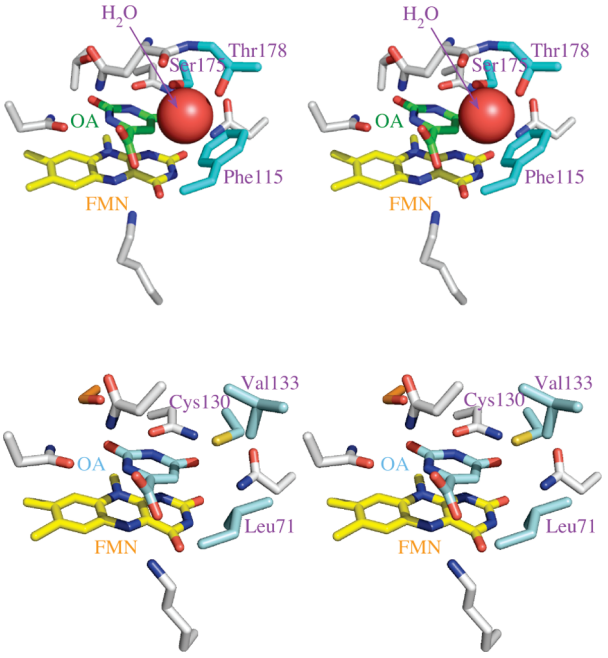

Figure 2. Stereoview of Class 2 and Class 1A DHODs. Class 2 DHODs (top) have a proton-transfer network consisting of Thr178, Ser175, a water molecule, and Phe115 (lavender). Class 1A DHODs (bottom) do not have a proton-transfer network; however, mutations of Val133, Cys130, and Leu71 (lavender) were introduced to incorporate a Class 2 proton-transfer network into the Class 1A enzyme.

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 that these residues actually inhibit the function of the residue at position 130 of the Class 1A enzyme was investigated. Therefore, the Leu71Phe/Val133Thr double mutant was created. On the basis of the reduction potential of the double mutant (−231 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 that of the triple mutant but still drastically slower than those of 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, ~8-fold tighter than that of the wild-type Class 1A enzyme and nearly the same as that of the 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 (−238 mV) was also almost the same as that of the wild-type Class 1A enzyme. The Val133Thr mutant enzyme

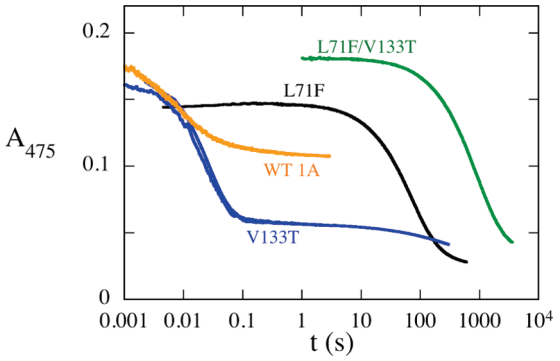

Figure 3. Reduction of Class 1A variants. The reduction of the wild-type Class 1A enzyme and the Leu71Phe, Val133Thr, and Leu71Phe/Val133Thr variants was studied by mixing anaerobic solutions of the oxidized enzyme with anaerobic solutions of DHO in a stopped-flow spectrophotometer. The wild-type was not reduced fully because the preparation had significant amounts of damaged enzyme, but the kinetics were the same as reported in ref 7. All reactions were conducted in 27.8 mM Taps and 22 mM KCl (pH 8.5) at 4 °C. Note the logarithmic time-scale.

was just a little slower [k_{red} values of 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 that of the Class 2 wild-type enzyme but tighter than that of the wild-type Class 1A enzyme (K_d = 145 ± 50 μM). The Val133Thr mutation did not inhibit binding of 3,4-diOHB, which had been observed in the Class 1A double and triple 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 to 800 nm, as does the wild-type Class 1A enzyme−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} values of 0.015 ± 0.002 s^{-1} at 4 °C (Figure 3) and 0.07 ± 0.001 s^{-1} at 25 °C] compared to those of both the wild-type Class 1A and Class 2 enzymes. 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 −257 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 the free energies of activation for the double mutant and both single mutants were calculated from transition-state theory to determine whether position 71 interacts with position 133. To compare changes in the free energies of activation, the rate constants at 4 °C were used because the Class 1A wild-type enzyme reduces too quickly to be measured at higher temperatures. The change in the Gibbs free energy caused by mutating the 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

dx.doi.org/10.1021/bi200258y |*Biochemistry* 2011, 50, 2714–2716

Table 1. Reduction Rate Constants at 4 °C and Dissociation Constants at 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 to be determined	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.

wild-type enzyme and mutating it to Val133Thr ($\Delta\Delta G^{\ddagger} = 0.80 \pm 0.4$ kcal mol^{-1}) and then creating the double mutant ($\Delta\Delta G^{\ddagger} = 5.83 \pm 0.01$ 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 nonplanar 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.

■ ASSOCIATED CONTENT

Supporting Information. Supplementary table and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Biological Chemistry, University of Michigan Medical School, 1150 W. Medical Center Dr., Ann Arbor, MI 48109-0606. Phone: (734) 615-2452. Fax: (734) 764-3509. E-mail: brupalf@umich.edu.

Funding Sources

This work was supported by National Institutes of Health Grant GM61087 to B.A.P. C.A.M. was supported by a University of Michigan Rackham Merit Fellowship and National Institutes of Health Grant GM61087-08-S1.

■ REFERENCES

(1) Björnberg, O., Rowland, P., Larsen, S., and Jensen, K. F. (1997) *Biochemistry 36*, 16197–16205.

(2) Jones, M. E. (1980) *Annu. Rev. Biochem. 49*, 253–279.

(3) Nagy, M., Lacroute, F., and Thomas, D. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8966–8970.

(4) Nielsen, F. S., Andersen, P. S., and Jensen, K. F. (1996) *J. Biol. Chem. 271*, 29359–29365.

(5) Rowland, P., Nielsen, F. S., Jensen, K. F., and Larsen, S. (1997) *Structure 5*, 239–252.

(6) Norager, S., Jensen, K. F., Björnberg, O., and Larsen, S. (2002) *Structure 10*, 1211–1223.

(7) Fagan, R. L., Jensen, K. F., Björnberg, O., and Palfey, B. A. (2007) *Biochemistry 46*, 4028–4036.

(8) Small, Y. A., Guallar, V., Soudackov, A. V., and Hammes-Schiffer, S. (2006) *J. Phys. Chem. B 110*, 19704–19710.

(9) Kow, R. L., Whicher, J. R., McDonald, C. A., Palfey, B. A., and Fagan, R. L. (2009) *Biochemistry 48*, 9801–9809.

(10) Norager, S., Arent, S., Björnberg, O., Ottosen, M., Lo Leggio, L., Jensen, K. F., and Larsen, S. (2003) *J. Biol. Chem. 278*, 28812–28822.

(11) Massey, V. (1990) A simple method for the determination of redox potentials. In *Flavins and Flavoproteins* (Curti, B., Ronchi, S., and Zanetti, G., Eds.) pp 59−66, Walter de Gruyter, Berlin.

(12) Wolfe, A. E., Thymark, M., Gattis, S. G., Fagan, R. L., Hu, Y. C., Johansson, E., Arent, S., Larsen, S., and Palfey, B. A. (2007) *Biochemistry 46*, 5741–5753.

(13) Palfey, B. A., Björnberg, O., and Jensen, K. F. (2001) *J. Med. Chem. 44*, 2861–2864.

(14) Palfey, B. A., Björnberg, O., and Jensen, K. F. (2001) *Biochemistry 40*, 4381–4390.