Secondary Tritium and Solvent Deuterium Isotope Effects as a Probe of the Reaction Catalyzed by Porcine Recombinant Dihydropyrimidine Dehydrogenase†

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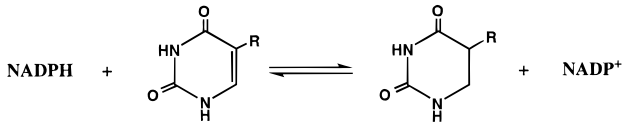
ABSTRACT: Dihydropyrimidine dehydrogenase catalyzes the rate-limiting step in the degradation of pyrimidines in mammals, the reduction of uracil or thymine to their 5,6-dihydro derivatives. The reduction of uracil by enzyme-bound reduced flavin involves both proton and hydride transfer. In order to determine whether hydride and proton transfer occur in a concerted or stepwise fashion, and to determine the nature of the transition state for the reduction, secondary tritium kinetic isotope effects were measured in H_2O and D_2O. The tritium isotope effect using 5-^{3}H-uracil is 0.90 ± 0.03 in H_2O and becomes more inverse, 0.85 ± 0.04, in D_2O. Data are interpreted in terms of a stepwise reduction at C-6 followed by protonation at C-5. A late transition state is proposed for the proton transfer at C-5 of uracil.

Dihydropyrimidine dehydrogenase (DPD,[1] EC 1.3.1.2.) catalyzes the NADPH-specific reduction of thymine and uracil to the corresponding 5,6-dihydropyrimidines according to the following equation, where R is H for uracil and methyl for thymine. The DPD reaction is the rate-limiting step in the pyrimidine degradation pathway (*1*).



The porcine recombinant enzyme is a homodimer with an M_r of 214 kDa and contains tightly associated FAD and FMN molecules and two [4Fe-4S] clusters per subunit (*2*−*4*).

A nonclassical two-site ping-pong mechanism has been proposed for the pig liver DPD (*5*). In the first site, NADPH reduces flavin and electrons are transferred (presumably via (an) FeS cluster(s)) to a flavin in the second site where uracil is reduced to 5,6-dihydrouracil. Reduction of flavin by NADPH is from the pro-*S* side of the nicotinamide ring (*6*) and is rate-limiting for the overall reaction (*7*). Reduction of uracil by the flavin in site 2 proceeds via an anti addition across the 5,6 double bond (*8*), and protonation at C-5 is postulated to occur via a cysteine residue (*7*, *9*). The recently determined three-dimensional structure of *Lactococcus lactis* dihydroorotate dehydrogenase A, homologous to DPD, shows a cysteine in the dihydroorotate binding site, properly positioned to act as a general base (*10*).

Little is known about the transition state for the two subsequent reductions in the DPD reaction, NADPH to flavin and flavin to uracil. In the present study, reduction of uracil is studied using secondary tritium kinetic isotope effects in the presence and absence of solvent deuterium. Data suggest a late transition state for the proton transfer at C-5 of uracil.

MATERIALS AND METHODS

Chemicals. NADP and NADPH were obtained from Boehringer-Mannheim. Uracil, DHU, 2,6-dihydroxypyridine, glucose dehydrogenase, and glucose-*1-d* were purchased from Sigma. DEAE-Cellulose TLC CEL 300 plates were from Macherey & Nagel. All other chemicals and reagents were obtained from commerical sources and were the highest purity available.

Secondary Tritium Isotope Effects. These assays were carried out at 30 °C in a reaction volume of 1 mL using a buffer containing 10 mM potassium phosphate, pH(D) 7.4, 8.5, and 9.5, 1 mM DTT, 60 μM NADPH, and 50 μM uracil (0.5 Ci/mol of 2-^{14}C-uracil and 40 Ci/mol of 5-^{3}H-uracil). Reaction progress was monitored by the change in absorbance at 340 nm after addition of DPD (2.3 milliunits/mL). The reaction was quenched after 5 min by adding 10 μL of NH_4OH (85%). Experiments were carried out in hextuplicate with 4 different aliquots of each sample counted. Assays were also carried out at 30 °C in a 1 mL volume containing 25 mM potassium phosphate, pD 8.4, 1 mM DTT, 60 μM NADPH, 0.5 μM 2,6-dihydroxypyridine, and variable concentrations of uracil. The concentration of D_2O was approximately 100%.

Thin-Layer Chromatography. DEAE-Cellulose plates (20 × 20 cm) were used for separation of uracil and DHU. Aliquots of 5 μL of the samples were spotted at the origin,

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[1] Abbreviations: DPD, dihydropyrimidine dehydrogenase; DTT, 1,4-dithiothreitol; TLC, thin-layer chromatography; DEAE, diethylaminoethyl; Ur, uracil; DHU, 5,6-dihydrouracil; DHOD, dihydroorotate dehydrogenase.

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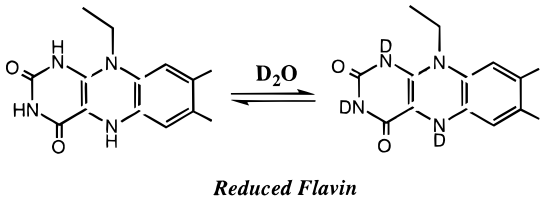

FIGURE 1: Solvent exchangeable positions in reduced flavin.

and plates were developed by ascending chromatography (about 4.5 h) using a mobile phase composed of t-butanol−methylethyl ketone−H_2O−NH_4OH (40:30:20:10 v/v). Under the given conditions, uracil and DHU have R_f values of 0.73 and 0.88, respectively. Radioactivity was detected using a radioscanner. Spots containing uracil and DHU were scraped and counted using a scintillation counter.

The isotope effect was calculated according to the following equation (*11*).

$$\log({}^{T}V/K) = \log[(1 - f)/(1 - f[{}^{3}H/{}^{14}C]_f/[{}^{3}H/{}^{14}C]_o)] \quad (1)$$

RESULTS AND DISCUSSION

Secondary Tritium Isotope Effects. The secondary tritium isotope effect for the DPD reaction was obtained using 5-^{3}H-uracil at saturating concentrations of NADPH. The isotope effect is 0.90 ± 0.03, independent of pH from 7.5 to 9.5, a pH range over which the V/K_{uracil} decreases with increasing pH. The pH independence of the tritium isotope effect is consistent with either selective binding of the correctly protonated forms of enzyme and reactant to give a productive Michaelis complex or rate-limiting interconversion of the enzyme−uracil and enzyme−DHU complexes (*12*). In the case of selective binding of the correctly protonated forms of reactant and enzyme, V is predicted to be pH-independent, and this is not the case for DPD (*7*). Thus, interconversion of the binary reactant and product complexes must be rate-limiting for the second half reaction. However, data do not necessarily indicate rate-limiting reduction of uracil by the flavin to DHU, since there may be (a) slow conformational change(s) once the binary complex is formed prior to the catalytic step(s) that limit the overall reaction.

In order to determine whether the chemical step limits the second half reaction, the tritium isotope effect was repeated in D_2O. An inverse solvent deuterium isotope effect of 0.67 was previously measured on the V/K for uracil, thought to reflect the involvement of a cysteine residue in catalysis (*7*). The inverse nature of the measured isotope effect strongly suggests a reactant state effect (*13*). A thiol has a fractionation factor of 0.4−0.6 in D_2O. However, there are two likely sources for the overall measured solvent deuterium isotope effect. The first is, as suggested above, the inverse reactant state effect attributed to thiol ionization. The second is a transition state effect that accompanies transfer of a hydride from N-5 of the reduced flavin to C-6 of uracil. (In D_2O, the protium at N-5 will exchange with deuterium, Figure 1.) The observed solvent deuterium isotope effect under these conditions will be a combined effect, the magnitude of which will depend on the transition-state structure for hydride transfer and the fractionation factor for the enzyme thiol.

The secondary kinetic tritium isotope effect obtained in D_2O is more inverse than that obtained in H_2O, Table 1.

Table 1: Secondary Tritium Isotope Effects Measured with 5-^{3}H-Uracil for DPD[a]

	f	$({}^{3}H/{}^{14}C)_o$	$({}^{3}H/{}^{14}C)_f$	$^{T}(V/K)$
pH 7.4	0.1	0.81	0.919	0.87
	0.116	0.849	0.95	0.89
	0.108	0.811	0.946	0.86
	0.118	0.818	0.91	0.89
	0.115	0.819	0.894	0.91
	0.121	0.857	0.923	0.92
pH 9.5	0.12	0.84	0.888	0.95
	0.095	0.825	0.915	0.89
	0.12	0.808	0.87	0.91
	0.127	0.83	0.858	0.94
	0.127	0.805	0.88	0.91
	0.125	0.83	0.89	0.9
				av: 0.90 ± 0.03
pD 8.5	0.054	0.808	0.925	0.88
	0.047	0.785	0.98	0.80
	0.053	0.795	0.965	0.83
	0.053	0.80	0.912	0.89
	0.056	0.818	0.95	0.86
	0.057	0.80	0.96	0.81
				av: 0.85 ± 0.04

[a] All experiments were carried out at 30 °C. f is the fractional completion of the reaction, $({}^{3}H/{}^{14}C)_o$ and $({}^{3}H/{}^{14}C)_f$ are the ratios of the starting uracil and the isolated product DHU at f fractional reaction, and $^{T}(V/K_{Ur})$ is calculated according to eq 1.

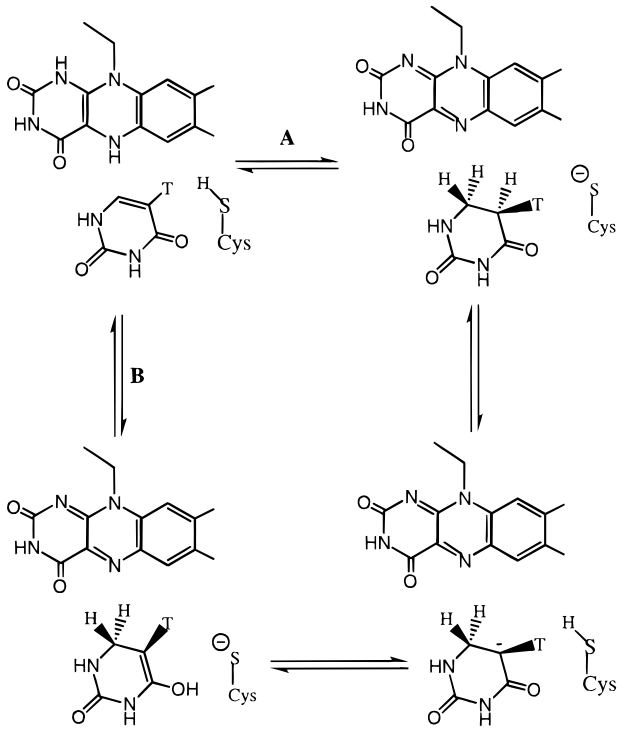

FIGURE 2: Possible mechanism for the reduction of uracil to DHU by reduced flavin in the dihydropyrimidine dehydrogenase reaction. (A) Concerted hydride transfer to C-6 and proton transfer to C-5 of uracil. (B) Stepwise reaction with hydride transfer to C-6 accompanied by protonation of the C-4 carbonyl and followed by tautomerization and protonation of the resulting C-5 carbanion.

Data are indicative of at least two possibilities. The first possibility is a concerted hydride transfer from N-5 of the flavin to C-6 of uracil and proton transfer to C-5 of uracil, so that the hybridization changes at C-5 and C-6 occur in the same transition state, Figure 2A. The second possibility is a stepwise mechanism in which reduction at C-6 and its accompanying hybridization change occur in a step separate

from the hybridization change at C-5, Figure 2B. In the stepwise mechanism, the C-4 carbonyl is protonated, as electrons are delocalized to give an enolamine species. A tautomer of the enolamine species is the C-5 carbanion, which can then be protonated by the thiol. The observed change in the inverse tritium secondary kinetic isotope effect and the ground state solvent isotope effect are most consistent with a stepwise reaction. As a logical extension of the information above, the change in the secondary tritium effect to become more inverse suggests that the slow step overall is protonation of C-5, and that the hydride transfer step comes to equilibrium at least in D_2O. The inverse fractionation factor for the thiol would increase the rate of the hydride transfer step, increasing the rate limitation of the C-5 protonation step. Thus, the observed tritium isotope effect can be interpreted in terms of the transition state for reduction of uracil by flavin at the second site of DPD (*5*).

Interesting is the fact that a concerted removal of hydride and proton from dihydroorotate has been proposed by Hines and Johnston (*14*) for bovine dihydroorotate dehydrogenase using multiple isotope effects. Using the multiple isotope effect approach, the authors determined the effect of deuterium substitution at one primary position (C-5 or C-6) on the primary deuterium isotope effect at the other position (C-6 or C-5). In both cases, Hines and Johnston concluded there was no significant decrease in $^{D}(V/K)$ when deuterium was substituted for protium at the second position. However, the errors on these effects are relatively large and the isotope effect at the 6 position is small (1.3 on *V*/*K*) compared to that obsered for the 5 position (3 on *V*/*K*), making it difficult to see a decrease. Data are suggestive of a finite stickiness (partitioning of the E−dihydroorotate complex toward product rather than dissociation to E plus dihydroorotate) for dihydroorotate. The *V* isotope effect on the Hines and Johnston study, however, are much more easily interpreted. The value of ^{D}V at C-5 decreases from about 3 with protium at C-6 to a value of 2.45 with deuterium at C-6, while the value of ^{D}V decreases from a value of 1.7 at C-6 with protium at C-5 to a value of 1.3 with deuterium at C-5. Data are more consistent with a stepwise proton abstraction preceding hydride transfer than with a concerted reaction.

The reference value for secondary kinetic isotope effects is the equilibrium secondary isotope effect for the hybridization change monitored (*15*). Fractionation factors for many heavy atom substituted molecules with respect to water have been compiled by Cleland (*16*). An sp^2 to sp^3 change, such as the one that occurs when uracil is reduced to 5,6-dihydrouracil, will have a predicted deuterium equilibrium isotope effect of 0.88, and the tritium value is calculated by raising the deuterium value to the 1.44 power, that is 0.83 (*17*). Thus, an early transition state for reduction of uracil would be expected to have a value close to unity for the secondary tritium kinetic isotope effect, while a late transition state would be expected to give a value equal to the full equilibrium isotope effect of 0.83. The measured value of the secondary kinetic tritium isotope effect in D_2O is 0.85, close to the value of 0.83, suggesting a late transition state for reduction of uracil.

The stereochemistry of uracil reduction is known to be anti across the 5,6 double bond (*8*). In order to accurately depict the transition state for reduction of uracil by reduced flavin, it is preferable to have structural information, so that the stereochemistry of hydride transfer from flavin to uracil can be accurately represented. The sequences of the DPD from human and pig liver have been published and the identity between the two is 92% (*3*). Sequences for 18 dihydroorotate dehydrogenases (DHOD) have been aligned and can be separated into 3 groups (*10*). The sequence of DPD is very similar (greater than 30% identity) to the DHOD-A and DHOD-B enzymes from *L. lactis*. The 3D structure of the homodimeric DHOD-A containing FMN has recently been published and shows the *re* face of the flavin resting on the protein surface with the *si* face exposed to solvent (*10*). The high sequence similarity of the DHOD-A subunit is limited to the C-terminal portion of DPD (residues 528−894), representing the uracil binding site (*3*). In the active site of DHOD-A, the substrate, 5,6-dihydroorotate, is interposed between FMN and cysteine-130, thought to act as a general base (*7*, *9*). Cys-130 in DHOD-A is equivalent to Cys-671 in DPD (*3*). The DHOD-A structure can be utilized as a reasonable model for the uracil binding site of DPD.

On the basis of the active site of DHOD-A, it is proposed that a hydride is transferred from N-5 of the reduced flavin's *si* face (*10*) to C-6 of uracil at the *si* face and is protonated at C-5 of uracil at the *si* face, Figure 1. The hydride and proton are likely transferred in two steps as shown in Figure 2B, and the protonation at C-5 exhibits a late transition state. Of interest is the similarity of the flavin site of DPD used to reduce uracil to other flavin-dependent oxidases. Specifically, in the case of glycolate oxidase (*18*), flavocytochrome b_2 (*19*), and trimethylamine dehydrogenase (*20*) the *si* face of the FMN is exposed to solvent. The similarity of flavin binding in the above 4 enzymes may suggest a common ancestral FMN-binding protein.

There have been questions raised concerning the kinetic mechanism of DPD, at least in the case of the mammalian enzyme. Podschun et al. (*5*) proposed a two-site ping-pong kinetic mechanism for the pig liver enzyme based on initial velocity studies in the absence and presence of products and dead-end inhibitors and isotope exchange at equilibrium, while Porter and Spector (*21*) proposed a rapid equilibrium random kinetic mechanism for the bovine liver enzyme based on initial velocity and pre-steady-state kinetic studies and exchange of tritium between NADPH and solvent. The closely related DHOD from bovine liver is proposed to have a two-site ping-poing kinetic mechanism (*14*) similar to that proposed by Podschun et al. (*5*) for pig liver DPD. The structural data available for the DHOD-A (primary and three-dimensional) and DPD (primary structure) support a two-site ping-pong kinetic mechanism as proposed previously (*5*, *14*). It is interesting to note that DHOD-A (10) and DHOD-B (*22*), which are similar to the C-terminal part of DPD, contain FMN. The sequence of DPD that is not similar to that of DHOD-A must code for the binding sites of the other flavin, NADPH, and FeS clusters. DPD also contains a FAD which must thus be located in the NADPH binding site. In agreement with this hypothesis, the other subunit of DHOD-B, the pyrK gene product, contains FAD and binds NADH (*22*). The reason for the difference in kinetic mechanism between the pig and bovine liver DPDs is not known.

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