Dihydropyrimidine Dehydrogenase

KINETIC MECHANISM FOR REDUCTION OF URACIL BY NADPH

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Steady-state and pre-steady-state kinetic data were used to determine the kinetic mechanism for bovine liver dihydropyrimidine dehydrogenase (DPDase). Steady-state kinetic data suggested a random rapid-equilibrium mechanism with $k_{m}$ values for NADPH and uracil of 0.12 μM and 0.8 μM, respectively, and a $k_{cat}$ of 1.6 s⁻¹ in Tris buffer at pH 8.0 and 37 °C. The dissociation constant of DPDase for NADPH at 25 °C in the absence of uracil (0.09 μM) was similar to the $K_{m}$ for NADPH. DPDase also catalyzed the exchange of tritium in [4S-3H]NADPH with solvent protons in the absence of uracil. DPDase inactivated by 5-ethyluracil, which covalently modifies the enzyme at the uracil binding site, catalyzed the exchange reaction at the same rate (1 s⁻¹) as native enzyme. Thus, the interaction of NADPH with DPDase was independent of the uracil binding site. Because DPDase catalyzed the exchange of deuterium in [4S-2H]NADPH with solvent protons with a rate constant of 5.4 s⁻¹, which was significantly larger than that for tritium, the analogous rate constant for exchange of the 4-hydrogen in NADPH must be significantly larger than 5 s⁻¹. Consequently, intermediates on the exchange pathway were kinetically competent to participate in the reduction of uracil by NADPH ($k_{cat} = 1.6$ s⁻¹). Rate constants for reduction of DPDase by NADPH and 5,6-dihydouracil were several orders of magnitude greater than $k_{cat}$. The rate constants for dissociation of E-NADPH (15 s⁻¹) and for dissociation of E,5,6-dihydouracil (>250 s⁻¹) were also greater than $k_{cat}$. These results supported a random rapid-equilibrium kinetic mechanism and suggested $k_{cat}$ was an internal electron transfer between enzyme prosthetic groups.

Dihydropyrimidine dehydrogenase (DPDase; EC 1.3.1.2) catalyzes the reversible reduction of pyrimidines to 5,6-dihydropyrimidines as the first step of pyrimidine catabolism to β-amino acids (1). Inhibitors of this enzyme have been shown to modulate 5-fluorouracil as a chemotherapeutic agent (2-7). We found that 5-ethyluracil is a potent suicide inactivator of DPDase that covalently modifies the enzyme at a cysteinyl residue (8, 9). Significantly, 5-ethyluracil was also shown recently to increase the efficacy and therapeutic index of 5-fluorouracil in several animal models.

The recent interest in DPDase prompted us to investigate the kinetic mechanism of the enzyme. DPDase has been purified to various degrees from several mammalian sources (9, 10-15) and from human liver (16). The purified enzyme contains FMN, FAD, and iron-sulfur centers (14-16). However, the role of these prosthetic groups in catalysis has not been demonstrated. In fact, it has been suggested that the flavin prosthetic group may be involved in the regulation of enzyme synthesis (17). Steady-state kinetic data for porcine liver DPDase are consistent with a nonclassical ping-pong kinetic mechanism in which the flavin is reduced by the substrate (18). We have investigated the kinetic mechanism for bovine liver DPDase and find that, in contrast to porcine DPDase, the steady-state kinetic data are described by a random rapid-equilibrium kinetic mechanism. The seminal finding reported herein supporting this mechanism was that DPDase catalyzed in the absence of uracil the exchange of the pro-S-4-hydrogen of NADPH with solvent.

EXPERIMENTAL PROCEDURES

Materials—Tris, diethiothreitol, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, glucose dehydrogenase, NADPH, uracil, thymine, 5-iodouracil, and 5,6-dihydouracil were from Sigma. [1-2H]Glucose (6Ci/mmol) was from Amersham Corp. [1-3H]Glucose, and 3H2O were from Aldrich. 5-Ethyluracil was synthesized at Wellcome Research Laboratories (Research Triangle Park, NC). DPDase was purified from bovine liver as described previously to a specific activity of approximately 20 units/mg (8).

Assay of DPDase—DPDase activity was determined by two methods. Diethiothreitol (2 mM), which enhanced the activity of DPDase approximately 3-fold (8, 9), was a constituent of both assays. Method I assayed DPDase by monitoring the oxidation of NADPH or reduction of NADP⁺. The standard assay for DPDase activity monitored spectrophotometrically at 340 nm the decrease in absorbance associated with the oxidation of NADPH by thymine (8). Enzyme was added to reference and sample cuvettes that contained 200 μM NADPH and 2 mM diethiothreitol in 0.05 M Tris-HCl at pH 7.7. After 2 min at 37 °C, the reaction was initiated by addition of thymine to the sample cuvette. Because the reference cuvette with thymine and NADPH blanked out any rate resulting from the NADPH oxidase activity of DPDase, the observed rate was due to oxidation of NADPH by thymine. DPDase oxidation of 5,6-dihydouracil was monitored by following NADPH formation spectrophotometrically ($\Delta\epsilon_{340}$ 10 μM) at 340 nm or spectrophotometrically (NADP⁺ 10 μM) with an excitation wavelength of 340 nm and an emission wavelength of 460 nm. The observed rates of reduction of NADP⁺ in the complete reaction mixture were corrected for the rate of reduction of NADP⁺ by 2 mM diethiothreitol in the absence of 5,6-dihydouracil.

Method II monitored DPDase activity by the absorbance decrease at 260 nm ($\Delta\epsilon_{260}$ = 6.5 μM⁻¹ cm⁻¹) associated with reduction of uracil. The assay contained 2 units/ml glucose-6-phosphate dehydrogenase and 1 mM glucose-6-phosphate to regenerate NADP⁺ from NADPH. The time courses for substrate disappearance were analyzed by the integrated rate equation (see below).

Method II was used to determine the steady-state kinetic parameters for the reduction of uracil. Because the $K_{m}$ of DPDase for NADPH in this reaction was small, it was necessary to demonstrate that the regenerating enzyme was not perturbing the concentration of free NADPH. The $K_{m}$ of NADPH for glucose-6-phosphate was determined.
from steady-state kinetic to be greater than 5 μM, whereas the \( K_{\text{m}} \) for NADP\(^+\) was 0.6 μM. The \( k_{\text{nuc}} \) for glucose-6-phosphate dehydrogenase was estimated to be over 150 s\(^{-1}\) from the rate of reduction of 1 μM NADP\(^+\) by 1 mM glucose 6-phosphate in the presence of excess glucose-6-phosphate dehydrogenase. Thus, 2 units/mL glucose-6-phosphate dehydrogenase was approximately 0.2 μM for the definition of a unit (1 μmol/min) and a \( k_{\text{nuc}} \) of 150 s\(^{-1}\). Because the concentration of glucose-6-phosphate dehydrogenase was only 0.12 μM in the DPDase assay and the \( K_{\text{m}} \) of glucose-6-phosphate dehydrogenase was over 5 μM, NADPH was not sequestered significantly by glucose-6-phosphate dehydrogenase under these conditions.

The enzyme-catalyzed bound flavin was determined from the absorbance of the enzyme at 427 nm and an extinction coefficient of 31 mM\(^{-1}\) cm\(^{-1}\) (8). The active site concentration was estimated from titration of enzymatic activity with 5-iodouracil to be 0.31 times the concentration of enzyme-bound flavin (8). Alternatively, the enzymatic activity was titrated stoichiometrically with 5-ethylnyluracil as described previously (9).

**DPDase-catalyzed Exchange of Tritium in [4S-2H,4R-1H]NADPH with Solvent**—The DPDase-catalyzed exchange of tritium in [4S-2H,4R-1H]NADPH was monitored by the time-dependent decrease in its specific activity. [4S-2H,4R-1H]NADPH was generated in situ with glucose dehydrogenase, [1-2H]glucose, and NADP\(^+\) (19). A solution of 200 μM glucose, 8 units/mL glucose dehydrogenase, 2 mM dithiothreitol, 100 μM NADP\(^+\), and 1 mM 5-[1-2H]glucose was incubated at 37 °C until all of the NADP\(^+\) was reduced to [4S-3H,4R-1H]NADP\(^+\). The exchange reaction was initiated with DPDase. Samples (0.5 ml) were removed from the reaction at 5-min intervals and applied to a small column of Whatman DE-52 resin made with 0.5 ml of a 50% slurry of resin equilibrated in 0.05 M Tris-HCl at pH 8.0. The column was washed with 6 ml of buffer. The tritium content in the subsequent 2 ml of buffer wash was determined for an estimate of background. NADP\(^+\) and NADPH were eluted from the column with 2 ml of 0.2 M KCl in 0.05 M Tris-HCl at pH 8.0. The recovery (\(R_{\text{pK}}\)) of NADP\(^+\) and NADPH was greater than 95%. The concentration of NADPH was determined by the absorbance of the solution at 340 nm and an \( \epsilon_{260} \) of 6.22 mM\(^{-1}\) cm\(^{-1}\) (20). The ratio of the absorbance at 340 nm to that at 260 nm indicated that greater than 95% of the adenine dinucleotide was NADPH. The specific activity of the NADP\(^+\) was reduced to [4S-3H,4R-1H]NADP\(^+\). The exchange rate of enzymatic activity with 5-iodouracil to be 0.31 times the concentration of the substrate at an initial concentration \([S]\) were analyzed by the integrated rate equation (22) to give values for \(K_{\text{m}}\) and \(V_{\text{m}}\) (Equation 1).

\[
V_{\text{n}} = -K_{\text{m}} \ln \left[ \frac{[S]}{[S]_0} \right] + \left( [S]_0 - [S] \right)
\]

(Eq. 1)

The time course for substrate disappearance was calculated by numerical integration using a fourth-order Runge-Kutta method (23) that was implemented on a Macintosh II personal computer (Equation 2) with the values calculated for \(V_{\text{n}}\), \(K_{\text{m}}\), and \(V_{\text{m}}\) by Equation 1.

\[
\frac{d[S]}{dt} = V_{\text{n}} - [S] = \frac{V_{\text{m}}}{K_{\text{m}}} \left[ \frac{[S]}{[S]_0} \right] + \left( [S]_0 - [S] \right)
\]

(Eq. 2)

If the concentration of substrate was much greater than the enzyme concentration, the Michaelis-Menten equation was fitted to the steady-state kinetic data. If the enzyme concentration and the dissociation constant or \(K_{\text{m}}\) of the substrate were such that a significant fraction of the substrate was bound to the enzyme, Equations 3 and 4 were used to estimate the \(K_{\text{m}}\) or \(K_d\).

\[
[S]_0 = [S]_0 + [E] + K_{\text{m}}
\]

(Eq. 3)

\[
[S]_0 = [S]_0 + [E] + K_{\text{d}}
\]

(Eq. 4)

**Fluorescence Titration Data Analysis**—Titration of DPDase by NADPH was monitored by following the quenching of NADPH fluorescence at an emission wavelength of 450 nm with an excitation wavelength of 340 nm. Glucose-6-phosphate dehydrogenase (2 units/mL) and glucose 6-phosphate (1 mM) were present to insure that any NADP\(^+\) formed by the slow oxidase reaction was rapidly reduced to NADPH. The fluorescence emission spectrum of 1 μM NADPH was determined in the presence of DPDase (1.3 μM) and 540 μM NADP\(^+\). NADP\(^+\) prevented binding of NADPH to DPDase. Glucose-6-phosphate dehydrogenase was not added to this solution. The fluorescence emission data were corrected for inner and outer filter effects resulting from the absorbance of the enzyme at the excitation and emission wavelengths. Titration of DPDase with NADPH resulted in a biphasic increase in NADPH fluorescence. The slope of the plot of fluorescence versus addition of NADPH was linear at high concentrations of NADPH and similar to that for addition of NADPH to a solution free of DPDase. The early phase of this plot was the result of titrating DPDase with NADPH. Because the dissociation constant of DPDase for NADPH was comparable to the concentration of enzyme used in the titration experiment, the concentration of free NADPH was calculated as the difference between the concentration of added NADPH ([S]) and that of E-NADPH ([E]·[S]). E[S] was calculated according to Equation 4. The dependence of the fluorescence of NADPH (\(F_{\text{E[S]}}\)) is given by Equation 5, in which \(F_{\text{E[S]}}\) is the fluorescence of E-NADPH, and \(F_{\text{E[S]}}\) is the fluorescence of free NADPH.

\[
P([S]) = F_{\text{E[S]}}([E]·[S]) + F_{\text{E[S]}} - [E]·[S])
\]

(Eq. 5)

**Transient Kinetic Data Analysis**—Equation 6 was fitted to the time course of first-order changes in NADPH fluorescence to yield a pseudo-first-order rate constant (\(k_{\text{obs}}\)).

\[
A(t) = (A_0 - A) \exp(-k_{\text{obs}}t) + A_0
\]

(Eq. 6)

The signal at the end of the reaction is \(A_0\), and the amplitude of the signal change is (\(A_0 - A_0\)).

**Analysis of Enzyme-catalyzed Exchange**—The enzyme-catalyzed exchange of isotope in the 4-position of NADPH with solvent is a first-order process. For example, the exchange of the 4-\(H\) of NADPH with \(\text{H}_2\text{O}\) can be represented by Equation 7, in which NADPH and NADP\(^+\) bind to DPDase with equal affinity and the first-order rate constant for exchange in E-NADPH is \(k_{\text{ex}}\).

\[
E + \text{NADPH} \rightarrow E + \text{NADPH}
\]

(Eq. 7)

The pseudo-first-order rate constant (\(k_{\text{ex}}\)) for formation of NADP\(^+\)
from NADPH is given by Equation 8, where \( k_{\text{cat}} \) is the exchange rate constant and \([\text{NADPH}]_c\) is the total concentration of NADPH and \( E_c \) is the total concentration of enzyme.

\[
\frac{k_{\text{obs}}}{E_c} = \frac{k_{\text{cat}}[E_c]}{[\text{NADPH}]_c + K_d}
\]  
(Eq. 8)

If \([\text{NADPH}]_c\) is much greater than \( K_d \), then

\[
k_{\text{obs}} = \frac{k_{\text{cat}}[E_c]}{[\text{NADPH}]_c}
\]  
(Eq. 9)

Data Fitting—Sigma Plot (Jandel Scientific, Corte Madera, CA) was used to fit the appropriate equations to the data by nonlinear least squares procedures. The weighting factors were unity.

RESULTS

Steady-state Kinetic Parameters for DPDase—The kinetic mechanism for reduction of uracil by NADPH was established from the dependence of the \( K_m \) for uracil on the concentration of NADPH. The \( K_m \) for uracil was determined from the time course for uracil reduction (Fig. 1), following the decrease in uracil absorbance at 260 nm (Method II). The apparent \( V_m \) of the reaction had a limiting value of 0.054 ± 0.005 μmol/s at saturating concentrations of NADPH (Fig. 2A). Because the \( K_m \) for NADPH was small (<0.150 μM), the fraction of the added NADPH bound to DPDase (0.034 μM) was corrected for by Equation 4. The \( K_m \) for uracil was independent of NADPH concentration and had an average value of 0.8 ± 0.1 μM (Fig. 2B). The \( K_m \) for NADPH was 0.12 ± 0.01 μM, and \( k_{\text{cat}} \) was 1.6 ± 0.1 s⁻¹. The \( K_m \) for NADPH was also independent of uracil concentration. These results were consistent with a random rapid-equilibrium mechanism for reduction of uracil by NADPH in which the \( K_m \) values for uracil and NADPH were equal to their respective dissociation constants.

The rate at which DPDase catalyzed the reduction of uracil by NADPH was decreased when the solvent was changed from H₂O to ²H₂O (24). Under our experimental conditions, \( k_{\text{cat}} \) decreased from 1.6 s⁻¹ to 0.44 s⁻¹, whereas the \( K_m \) values for uracil were 0.68 ± 0.04 μM in H₂O and 0.5 ± 0.1 μM in ²H₂O, respectively.

The \( K_m \) for 5,6-dihydrouracil at a saturating concentration of NADP⁺ (86 μM) was determined to be 240 ± 40 μM from initial velocity data collected at seven concentrations of 5,6-dihydrouracil (125–1500 μM). The \( K_m \) for NADP⁺ at 1 mM 5,6-dihydrouracil was 0.40 ± 0.02 μM, which was calculated from initial velocity data collected by Method I at six concentrations of NADP⁺ (0.25 μM to 5.3 μM). \( k_{\text{cat}} \) was 0.40 ± 0.02 s⁻¹.

Because the pre-steady-state data were collected at 25 °C (see below), \( k_{\text{cat}} \) values for uracil reduction and 5,6-dihydrouracil oxidation were also determined at 25 °C for direct comparison. The \( k_{\text{cat}} \) for reduction of 100 μM uracil by 100 μM NADPH was 0.9 s⁻¹, and the \( k_{\text{cat}} \) for oxidation of 500 μM 5,6-dihydrouracil by 100 μM NADP⁺ was 0.35 s⁻¹.

Enzyme-catalyzed Exchange of Isotope in the 4-Position of NADPH with Solvent—The steady-state kinetics described above for the bovine liver enzyme were consistent with a random rapid-equilibrium kinetic scheme in which NADP⁺ remained bound to the reduced enzyme. If NADPH transferred a hydride to a position on an enzyme-bound prosthetic group that was exchangeable with solvent (i.e., the flavin), DPDase could potentially catalyze the exchange of the 4-H of NADPH with solvent. We tested this by following the exchange of tritium in [4S-⁵H,4R-¹H]NADPH with solvent and by following spectrophotometrically the exchange of the 4-deuterium in [4S-³H,4R-¹H]NADPH with solvent protons. The exchange of the 4-proton in NADPH with deuterium in ²H₂O was also monitored spectrophotometrically.

DPDase catalyzed the exchange of tritium in [4S-³H,4R-¹H]NADPH with solvent. The relative specific activity of 100 μM [4S-³H,4R-¹H]NADPH, which was generated in situ from [1-³H]glucose and glucose dehydrogenase, decreased in the presence of 0.03 μM DPDase in a first-order process with a rate constant of (1.9 ± 0.1) × 10⁻² min⁻¹ (Fig. 3). In the absence of uracil, the hydrogen transferred from NADPH to the DPDase
The specific activity at the specified times to the specific activity at time

The decay of the relative specific activity (the ratio of

The first-order rate constant for exchange of 86 μM [4-1H]NADPH with 2H2O in the presence of 0.06 μM DPDase was 0.18 ± 0.01 min⁻¹ (Fig. 4) yielding a kcat of 4.3 s⁻¹ (Equation 9), which was 10-fold larger than kcat in 2H2O (0.44 s⁻¹).

Spectral Changes Associated with Reduction of DPDase
Reduction of the enzyme-bound flavin in DPDase by 5,6-dihydrouracil, and dithiothreitol should be detectable by spectroscopic methods. However, the fractional change in absorbance could be relatively small because each subunit contains multiple flavins in which only one appears to be catalytically active (8). Accordingly, NADPH produced small changes in the visible absorbance of DPDase (Fig. 5A). 5,6-Dihydrouracil and dithiothreitol produced similar changes (Fig. 5B). The difference spectrum between untreated enzyme and enzyme treated with reducing substrate had maxima at approximately 460 and 360 nm (Fig. 5B) that were similar to those between oxidized and reduced flavin. The pronounced shoulder at 485 nm in the difference spectrum for enzyme reduced by dithiothreitol indicated that additional reactions were occurring. The enzyme was reduced to a greater extent with NADPH than with either 5,6-dihydrouracil or dithiothreitol. In all cases the fractional absorbance changes resulting

could be transferred to O2 (oxidase activity) or could exchange with solvent protons (exchange reaction). Free NADP⁺ generated through the oxidase activity of DPDase was reduced to radiolabeled NADPH with the same specific activity as the starting NADPH by the 0.2 mM [1-3H]glucose and 6 units/ml glucose dehydrogenase in the assay. Thus, the loss in relative specific activity of NADPH was the result of the exchange activity of the enzyme. The value of kcat for [4S-2H,4R-1H]NADPH was calculated to be 1 s⁻¹ from these data and Equation 9. DPDase inactivated by 5-ethynyluracil, which covalently modifies the enzyme at the uracil binding site (9), catalyzed the exchange at the same rate as native enzyme.

The absorbance maximum of [4S-2H,4R-1H]NADP⁺ was calculated to be 1.6 s⁻¹ in 2H2O.

The first-order rate constant for exchange of the deuterium in 100 μM [4S-2H,4R-1H]NADP⁺ with solvent protons by 0.068 μM DPDase was 0.22 ± 0.02 min⁻¹ for a kcat of 5.4 s⁻¹ (Equation 9) compared to a kcat of 1.6 s⁻¹ in 2H2O.
from treatment of the enzyme with reducing substrates were small.

The concentration of flavin reduced by NADPH was estimated to be 3.0 \( \mu \text{M} \) from the absorbance difference at 460 nm (Fig. 5B) and a difference extinction coefficient between oxidized and reduced flavin of 11 mm\(^{-1}\)
cm\(^{-1}\) (25). This estimate for active flavin concentration was similar to the active site concentration (3.7 \( \mu \text{M} \)) estimated from the titration of the enzyme with 5-ethyluracil.

Titrations of DPDase with NADPH—The fluorescence of 1 \( \mu \text{M} \) NADPH was quenched by 1.3 \( \mu \text{M} \) DPDase\(^6\) (Fig. 6). If 540 \( \mu \text{M} \) NADP\(^+\) were added to DPDase prior to addition of 1 \( \mu \text{M} \) NADPH, NADPH binding to DPDase was prevented and the fluorescence emission spectrum was similar to that of free NADPH.

The quenching of the fluorescence of NADPH bound to DPDase relative to that of free NADPH was used to monitor the titration of DPDase by NADPH. The initial incremental increase in fluorescence with each addition of NADPH was small. After titration of the DPDase sites with NADPH, the incremental increase in fluorescence for each addition of NADPH was equal to that for free NADPH (Fig. 6). Equation 5 was fitted to these titration data with the active site concentration fixed at a value of 3.3 \( \mu \text{M} \), which was determined by titration of the enzyme with 5-ethyluracil. The apparent \( K_d \) for NADPH was 0.09 ± 0.03 \( \mu \text{M} \). DPDase quenched the fluorescence of NADPH by approximately 80%.

\( ^6 \) In aerobic solutions, NADPH fluorescence was quenched by its slow conversion to NADP\(^+\) by the oxidase activity of DPDase. This side reaction was eliminated by including 5 units/ml glucose-6-phosphate dehydrogenase and 1 mM glucose-6-phosphate in the solution to rapidly convert the NADP\(^+\) back to NADPH. Consequently, the observed fluorescence quenching was the result of formation of E-NADPH and E\(^{-}\)-NADP\(^+\).

Kinetics for Reaction of NADPH with DPDase—The small spectral changes associated with the reduction of DPDase by NADPH or 5,6-dihydrouracil (Fig. 5B) should have permitted us to monitor the time course of these reactions. However, the reaction of 40 \( \mu \text{M} \) NADPH with 2 \( \mu \text{M} \) enzyme was too rapid to detect a reasonable absorbance change at 460 nm with the stopped-flow spectrophotometer. Fortunately, the fluorescence changes associated with this reaction were large enough to measure at lower concentrations of NADPH and DPDase and to confirm that reduction of DPDase by NADPH was very rapid.

DPDase (0.68 \( \mu \text{M} \)) was reduced by 5 or 2.5 \( \mu \text{M} \) NADPH in a first-order reaction with rate constants of 204 ± 6 s\(^{-1}\) (Fig. 7) and 170 ± 4 s\(^{-1}\), respectively. At lower concentrations of NADPH, the reaction was no longer first-order because the NADPH concentration was not significantly greater than the enzyme concentration. At higher concentrations of NADPH, the relative fraction of the total signal quenched became small and the rate constant became large. Consequently, the pseudo first-order rate constant was determined over a narrow concentration range of NADPH. The pseudo first-order rate constant at both concentrations of NADPH was over 2 orders of magnitude larger than \( k_{\text{obs}} \) (0.9 \( \text{s}^{-1} \)) at 25 °C.

Kinetics for Reduction of DPDase by 5,6-Dihydrouracil—5,6-Dihydrouracil bleached the visible absorbance spectrum of DPDase (Fig. 5B). However, the reaction of 300 \( \mu \text{M} \) 5,6-dihydrouracil (\( K_a = 240 \mu \text{M} \) at 37 °C) with 2 \( \mu \text{M} \) DPDase, which was monitored at 460 nm, was complete within the mixing time of the stopped-flow spectrophotometer (\( k_{\text{obs}} > 500 \text{s}^{-1} \)). Since \( k_{\text{obs}} \) is the sum of the dissociation rate constant and the pseudo first-order association rate constant and since these rate constants are approximately equal at this concentration of 5,6-dihydrouracil, the dissociation rate constant for 5,6-dihydrouracil must be large (>250 s\(^{-1} \)).

Kinetics for Dissociation of NADP\(^+\) from E-NADP\(^+\)—The first-order rate constant for dissociation of E-NADP\(^+\) was determined by trapping NADP\(^+\) as E-NADP\(^+\) dissociated. NADP\(^+\) was trapped by reduction to NADPH with glucose-6-phosphate dehydrogenase and glucose 6-phosphate. In the absence of DPDase, 1 \( \mu \text{M} \) NADP\(^+\) was reduced by excess glucose-6-phosphate dehydrogenase (40 \( \mu \text{M} \)) and 1 mM glucose 6-phosphate with a rate constant of 155 ± 2 s\(^{-1}\). In the presence of DPDase, NADPH would be bound to glucose-6-phosphate dehydrogenase or DPDase. Although the fluorescence of NADPH bound to DPDase (i.e. E-NADPH and E\(^{-}\)-NADP\(^+\)) would be reduced relative to that of free NADPH (see Fig. 6 for the emission spectrum of NADPH bound to DPDase), the net fluorescence of the solution would increase upon formation of NADPH. When
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E-NADP+ prepared from 1.3 μM DPDase and 1 μM NADP+ was mixed with 400 units/ml glucose-6-phosphate dehydrogenase (10 μM), the fluorescence of the solution increased in a first-order process with a rate constant of 14.7 ± 0.2 s⁻¹. A similar value for this rate constant (17.5 ± 0.3 s⁻¹) was determined after mixing E-NADP+ with 200 units/ml glucose-6-phosphate. Since reduction of free NADP+ at these concentrations of glucose-6-phosphate dehydrogenase (150 s⁻¹) was not rate-limiting, the limiting rate constant for reduction of E-NADP+ by glucose-6-phosphate must be the dissociation rate constant of E-NADP+.

**DISCUSSION**

Steady-state kinetic data for bovine liver DPDase were consistent with a random rapid-equilibrium mechanism with a Kᵣ for uracil of 0.8 μM, a Kₙ for NADPH of 0.12 μM, and a kₗ for 1.6 s⁻¹. Several experimental results, in addition to the steady-state kinetic data, supported a random rapid-equilibrium mechanism for the bovine liver DPDase. The NADPH-dependent spectral changes in the visible spectrum of DPDase suggested that NADPH reduced the enzyme-bound flavin (Fig. 5). A random rapid-equilibrium kinetic mechanism requires that NADP+ is not released from E-NADP+. Furthermore, the 4-H of NADPH is typically transferred to the N-1 or N-5 position of enzyme-bound flavin during reduction of flavoproteins. Protons at these positions on the flavin are potentially exchangeable with solvent. Thus, DPDase could catalyze the exchange of the 4-H of NADPH with solvent. Exchange of the 4-H of NADPH has been observed with flavoproteins such as xanthine oxidase, NADH-cytochrome c reductase (26, 27), and diaphorase (28). We found that DPDase catalyzed the exchange of tritium in [4S-3H,4R-1H]NADPH with solvent protons in the absence of uracil. The first-order rate constant for exchange of tritium was 1 s⁻¹.

We also monitored the exchange reaction by following the small absorbance change in the 340 nm absorbance band of DPDase (Fig. 3). Spectral shifts have been observed for the exchange of 1H in peptide bonds and for the exchange of ring and amino protons of nucleic acids (29). The first-order rate constant for DPDase-catalyzed exchange of deuterium in [4S-3H,4R-1H]NADPH was determined (150 s⁻¹) was not rate-limiting for uracil of 0.8 μM, a Kᵣ for NADPH of 0.12 μM, and a kₗ for 1.6 s⁻¹. Several experimental results, in addition to the steady-state kinetic data, supported a random rapid-equilibrium mechanism for the bovine liver DPDase. The NADPH-dependent spectral changes in the visible spectrum of DPDase suggested that NADPH reduced the enzyme-bound flavin (Fig. 5). A random rapid-equilibrium kinetic mechanism requires that NADP+ is not released from E-NADP+. Furthermore, the 4-H of NADPH is typically transferred to the N-1 or N-5 position of enzyme-bound flavin during reduction of flavoproteins. Protons at these positions on the flavin are potentially exchangeable with solvent. Thus, DPDase could catalyze the exchange of the 4-H of NADPH with solvent. Exchange of the 4-H of NADPH has been observed with flavoproteins such as xanthine oxidase, NADH-cytochrome c reductase (26, 27), and diaphorase (28). We found that DPDase catalyzed the exchange of tritium in [4S-3H,4R-1H]NADPH with solvent protons in the absence of uracil. The first-order rate constant for exchange of tritium was 1 s⁻¹. We also monitored the exchange reaction by following the small absorbance change in the 340 nm absorbance band of NADPH upon formation of NADPH (Fig. 4). Spectral shifts have been observed for the exchange of 1H in peptide bonds and for the exchange of ring and amino protons of nucleic acids (29). The first-order rate constant for DPDase-catalyzed exchange of deuterium in [4S-3H,4R-1H]NADPH was 5.4 s⁻¹. Because the exchange of deuterium in [4S-3H,4R-1H]NADPH with solvent was faster than the exchange of tritium in [4S-3H,4R-1H]NADPH with solvent, the first-order rate constant for exchange of proton in NADPH would be even larger than that for deuterium. Thus, the first-order rate constant for the exchange of 4-H of NADPH (~5.4 s⁻¹) is significantly greater than kₗ for 1.6 s⁻¹ for reduction of uracil by NADPH. Consequently, the intermediates on the exchange reaction pathway are kinetically competent intermediates for the reduction of uracil by NADPH.

The stereochemistry for exchange of 4-H on NADPH (pro-S hydrogen) by bovine liver DPDase was the same as the stereochemistry for the transfer of the 4-H of NADPH to H₂O by porcine liver DPDase in the presence of uracil (18). Bovine liver DPDase did not require uracil for the exchange reaction. The facile exchange of the 4-H of NADPH could explain the lack of hydrogen transfer from NADPH to uracil (24, 30). Furthermore, DPDase that has been inactivated by 5-ethyluracil, which covalently modifies the uracil binding site, catalyzes the exchange at the same rate as native enzyme. These results support a random mechanism in which binding of uracil and NADPH occur at different sites, and NADP+ does not dissociate from its complex with reduced enzyme (E-NADP+). In contrast to these findings, steady-state data for the porcine liver DPDase were described by a nonclassical ping-pong mechanism (18). One significant kinetic parameter for examining the kinetic parameters of DPDase and that of Podschun et al. (18) was that we used glucose-6-phosphate dehydrogenase to ensure that NADP+ did not accumulate during the reaction. Possibly, NAPD+ was a sufficiently potent product inhibitor that true initial velocities could not be determined spectrophotometrically. Alternatively, these kinetic differences may be the result of differences between the enzymes isolated from bovine and porcine liver.

In summary, the kinetics of bovine liver DPDase-catalyzed reduction of uracil by NADPH followed a random rapid-equilibrium mechanism. This kinetic mechanism was supported by the finding that DPDase catalyzed the exchange of the 4-H of NADPH with solvent in a reaction that was more rapid than turnover and was not affected by inactivation of the enzyme by 5-ethyluracil, which reacts at the uracil binding site (9). Consequently, the NADPH and uracil binding sites were physically distinct. Furthermore, NADPH reduced DPDase with a rate constant that was at least 100-fold larger than kₗ, and the rate constants for dissociation of NADP+ and 5,6-dihydrouracil from the enzyme were at least 10-fold larger than kₗ. Because kₗ, exhibited a large solvent isotope effect (3.6-fold) and because kₗ was not the result of slow reduction of the enzyme by NADPH or the slow dissociation of products, it is reasonable to propose that kₗ is an internal redox process in which reducing equivalents and protons are transferred from a prosthetic group at the NADPH binding site to a prosthetic group at the uracil binding site.

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The value of 20 for the deuterium isotope effect (kₗ/kₕ) suggests that multiple steps are isotopically sensitive or that an unusual hydrogen transfer mechanism is involved such as observed with adenosylcobalamin-dependent enzymes (32).

Similar results were observed when glucose dehydrogenase was used to reduce the NADP+ generated in the DPDase reaction (D. Porter, unpublished observation).
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