Dehalogenating and NADPH-modifying Activities of Dihydropyrimidine Dehydrogenase*

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Dihydropyrimidine dehydrogenase (DPDase) catalyzed the debromination of 5-bromo-5,6-dihydropyrimidinuracil (BrUH₂) to uracil at pH 7.7 and 37 °C. The debrominating activity of DPDase was increased 5-fold by treatment with H₂O₂, whereas the dehydrogenating activity was inhibited by this treatment. The time course for increasing the debrominating activity by H₂O₂ was similar to that for decreasing the dehydrogenating activity. Thus, the relative amounts of dehydrogenase and debrominating activities of DPDase were reciprocally related. H₂O₂ treatment of DPDase decreased the number of thiol groups reactive with 5,5′-dithiobis(2-nitrobenzoate) from eight/subunit to less than one. The kcat for debronnination of BrUH₂ by H₂O₂-treated DPDase (OxDPDase) was 1.9 s⁻¹, which was comparable with kcat for reduction of thymine (2.1 s⁻¹) by DPDase. Even though the debromination of BrUH₂ to uracil does not involve a net reduction of BrUH₂, NADPH was required for this activity. The reaction of OxDPDase with 5-iodo-5,6-dihydropyrimidinuracil (I₄UH₂) was more complicated than that with BrUH₂. Aerobically, OxDPDase catalyzed the deiodination of IUH₂ to uracil and the iodination of NADPH to 5-iodo-6-hydroxy-1,4,6-trihydropyrimidinuracil adenine dinucleotide phosphate. The turnover number for the iodination reaction was enhanced by NaI and had a value of 3.5 s⁻¹ in the presence of 4 mM IUH₂ and 50 mM NaI. Anaerobically, OxDPDase catalyzed the above reactions, the deiodination of IUH₂ to 5,6-dihydropyrimidinuracil, and the hydridation of NADPH to 6-hydroxy-1,2,3,4-tetrahydropyrimidinuracil adenine dinucleotide phosphate. The turnover number for the anaerobic hydration of NADPH was similar to that for the aerobic iodination of NADPH.

Although the prosthetic groups of dihydropyrimidine dehydrogenase (DPDase, EC 1.3.1.2) have been identified as FAD, FMN, and nonheme iron (1–3), their roles in the reversible reduction of pyrimidines to 5,6-dihydropyrimidinuracil and in the oxidation of NADPH by O₂ is unclear (1–6). In addition to these prosthetic groups, protein thiol groups have been implicated in catalysis. For instance, reductants such as β-mercaptoethanol or dithiobiotreitol are required for maximal dehydrogenation activity (1), and DPDase is inactivated by covalent modification of a single cysteinyi residue with 5-ethyluracil or 5-iodouracil (5, 6). The recent interest in DPDase as a modulator of 5-fluorouracil toxicity (7–12) has prompted investigations into the kinetic and chemical mechanisms of DPDase to understand the roles of these prosthetic groups in catalysis and to develop potent inhibitors for the enzyme (13–16). Elucidation and characterization of the activities associated with DPDase or modified forms of DPDase is essential for understanding the catalytic mechanism of this complex enzyme. Thus, it is reported herein that OxDPDase, which was formed by oxidation of DPDase by H₂O₂, catalyzed the dehalogenation of BrUH₂ and IUH₂, and the modification of NADPH to redox inactive forms.

EXPERIMENTAL PROCEDURES

Materials—Tris, DTT, NADPH, NADP⁺, 5,5′-dithiobis(2-nitrobenzoic acid), N-ethylmaleimide, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, uracil, IUH₂, thymine, bovine liver catalase (C-30), bovine erythrocyte superoxide dismutase, and glucose oxidase (type V.S) were from Sigma; H₂O₂, Br₂, and I₂ were from Aldrich. 5-Ethynyluracil was synthesized at Wellcome Research Laboratories (Research Triangle Park, NC). IUH₂ and BrUH₂ were synthesized and purified as described previously (17).

Purification of DPDase and Preparation of OxDPDase—DPDase was purified from bovine liver to a specific activity of approximately 20–25 pmol/h/µg (an A₄₅₀ of 1 corresponded to a protein concentration of 0.37 mg/ml) by a modification of the method of Sihotan and Weber (1) (see also Ref. 5). This preparation of enzyme was further purified by anion-exchange chromatography on a Mono-Q anion-exchange column (Pharmacia Biotech, Inc.) with a 20-ml gradient from 0.0 to 0.15 M sodium phosphate and 5 mM β-mercaptoethanol at pH 7.4. Most of the DPDase activity (~80%) eluted from the column at 40 mM KCl. Although the specific activity (25 umol/A₄₅₀ of the enzyme was not significantly changed by this purification step, several minor contaminating proteins were removed, as judged by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. DPDase was stored at 5 °C in 0.035 M sodium phosphate and 5 mM β-mercaptoethanol and 40 mM KCl at pH 7.4. DPDase was used within 1 week of preparation. Prior to use, the enzyme was treated with 30 mM DT for 15 min at 25 °C to ensure maximal dehydrogenation activity. DT and β-mercaptoethanol were separated from DPDase by size-exclusion chromatography at 25 °C on a column of P-6 resin (1.5 cm x 8 cm) equilibrated with 0.05 M Tris-HCl (pH 7.4). This solution of DPDase was stored at 5 °C and used within 4 h of preparation. OxDPDase was prepared by oxidation of enzymic thiols of DPDase (6 µM) with 2 mM H₂O₂ at 37 °C for 15 min. Excess peroxide was removed with 2 µg/ml catalase. This procedure resulted in enzyme that had less than 10% of the dehydrogenating activity of DPDase. The catalase activity in these preparations of OxDPDase was such that 100 µM H₂O₂ was disproportionated within 15 s.

Preparation of iodinated NADPH (NADPI) and the Hydrolysis Products of NADPH (NADPHx)—NADPI was prepared by adding 0.03 ml of 10 mM I₂ in 95% ethanol to 3 ml of 100 µM NADPH in 0.05 M Tris-HCl (pH 7.7) at 37 °C. The absorbance of NADPH at 340 nm was bleached immediately (<5 s). By analogy with the reaction of I₂ with NADH, the
product (NADP) is 5-ido-6-hydroxy-1,4,6-trihydroroticinamid e adename dinucleotide phosphate (18). NADPH was stored at 5 °C and was used within 1 h of preparation.

NADPH, the epimers of 6-hydroxy-1,4,5,6-tetracydroroticinamid e adename dinucleotide phosphate (19, 20), was synthesized by incubating 10 mg NADPH in 0.5 mM sodium phosphate (pH 6) for 1 h at 37 °C (21) or for 8.25 h with sodium arsenate (pH 7.0) for 18 h (22). Similar products were made by both methods. In addition to NADPH, cyclo tetracydoroticinamid e adename dinucleotide phosphate, the "primary acid product" of NADPH hydration, was a major product of these reactions (19–25).

**Assay of Enzymes**—Dehydrogenating activity of DPDase was assayed spectrophotometrically by monitoring the absorbance decrease at 340 nm associated with the oxidation of NADPH by thymine (5). Reference and sample cuvettes contained DPDase, 100 μM NADPH, and 3 mM DTT in 0.05 M Tris-HCl at pH 7.7 at 37 °C. The reaction in the sample cuvette was initiated with 100 μM thymine. The active site concentration of DPDase was determined by stoichiometric titration of the enzymatic site concentration, the turnover number of DPDase with thymine was 2.1 s⁻¹.

The debroniating activity was assayed by monitoring the absorbance increase at 260 nm associated with the conversion of BrUH₃ to uracil. The Δ_{abs} for this reaction was determined to be 7.5 μm⁻¹ cm⁻¹ in 0.05 M Tris-HCl at pH 7.7 and 37 °C. Reference and sample cuvettes contained 500 μM BrUH₈, 10 μM NADPH, and the NADPH-regenerating system (5 units/ml glucose-6-phosphate dehydrogenase and 2 mM glucose-6-phosphate) in 0.05 M Tris-HCl at pH 7.7 and 37 °C. The reaction was initiated by addition of OxDPDase to the sample cuvette.

Modification of DPDase by OxDPDase was measured spectrophotometrically by the absorbance decrease at 340 nm. The sample and reference cuvettes contained 3.5 μM IUH₃, 100 μM NADPH, 50 mM NaI, and the NADPH-regenerating system. The reaction was initiated by addition of OxDPDase to the sample cuvette. The regenerating system reduced 100 μM NADP⁺ to NADPH within 10 s in the presence of these reactants. Thus, a decrease in absorbance at 340 nm was detected if NADPH was modified to a form that could not be reduced to NADPH by the NADPH-regenerating system.

Oxygen consumption was monitored polarographically with a Yellow Springs Oxygen Biological Monitor Model 5300. The concentration of oxygen in an air-saturated solution at 37 °C was assumed to be 210 μM.

**O₂-scavenging System**—Oxygen was removed from solution with 10 units/ml glucose oxidase, 10 mM glucose, and 7.5 μg/ml catalase. This O₂-scavenging system reduced the oxygen concentration of an air-saturated solution to less than 20 μM within 1 min and did not catalyze any of the reactions discussed herein.

**Product Analysis**—Products of the dehalogenating activity and of the NADPH-modifying activities of OxDPDase were identified and quantified by HPLC analysis on a LichroCart 250-4 100 RP-18 reverse phase column (Merck, Darmstadt, Germany). Prior to HPLC analysis, the samples were deglycosylated by ultrafiltration through a Centrifree-30 membrane (Amicon). Chromatograms were recorded at 210 and 260 nm, and spectra of the products were recorded with a Kontron 440 Diode Array Detector (Everett, MA). Products were identified by comparison of retention times and ultraviolet spectra with known compounds. The products from the debroniating of BrUH₃ were eluted from the column isocratically with 0.1% trifluoroacetic acid (Buffer I) at a flow rate of 1 ml/min. Under these conditions, the retention time (min) of the substrate and products was as follows: BrUH₃ (8.4), IUH₃ (11.2), UH (4.3), and uracil (4.8). The products from the reaction of NADPH with IUH₃ were isocratically eluted from the column with 20 mM ammonium phosphate adjusted to pH 7.3 with NH₄OH (Buffer II) at a flow rate of 1 ml/min. Under these conditions, the retention time (min) of the substrate and products was as follows: IUH₃ (13.3), NADPH (11.2), iodide (2.6), UH (4.9), uracil (5.3), NADPH (16.8), and the epimers of NADPHX (5.7, 7.9). The amount of product in a sample was quantified by comparison of its peak area with a chromatogram with that for a known amount of product.

**DTNB-reactive Thiol Groups in DPDase and OxDPDase**—DTNB-reactive thiol groups of DPDase or OxDPDase were determined by the absorbance increase at 408 nm upon addition of 30 μM DTNB to ~1 μM DPDase or OxDPDase. The product, 5-thio-2-nitrobenzoate, was quantitated with a ε₁ₓ₅ = 13.6 μM⁻¹ cm⁻¹ (26).

**Steady-state Kinetic Data Analysis**—Equation 1 was fitted to the steady-state kinetic data with thymine as substrate and products from the debromination of BrUH₃, were eluted from the column isocratically with 0.1% trifluoroacetic acid (Buffer I) at a flow rate of 1 ml/min and did not catalyze any of the reactions discussed herein.

**Product Analysis**—Products of the dehalogenating activity and of the NADPH-modifying activities of OxDPDase were identified and quantified by HPLC analysis on a LichroCart 250-4 100 RP-18 reverse phase column (Merck, Darmstadt, Germany). Prior to HPLC analysis, the samples were deglycosylated by ultrafiltration through a Centrifree-30 membrane (Amicon). Chromatograms were recorded at 210 and 260 nm, and spectra of the products were recorded with a Kontron 440 Diode Array Detector (Everett, MA). Products were identified by comparison of retention times and ultraviolet spectra with known compounds. The products from the debroniating of BrUH₃ were eluted from the column isocratically with 0.1% trifluoroacetic acid (Buffer I) at a flow rate of 1 ml/min. Under these conditions, the retention time (min) of the substrate and products was as follows: BrUH₃ (8.4), IUH₃ (11.2), UH (4.3), and uracil (4.8). The products from the reaction of NADPH with IUH₃ were isocratically eluted from the column with 20 mM ammonium phosphate adjusted to pH 7.3 with NH₄OH (Buffer II) at a flow rate of 1 ml/min. Under these conditions, the retention time (min) of the substrate and products was as follows: IUH₃ (13.3), NADPH (11.2), iodide (2.6), UH (4.9), uracil (5.3), NADPH (16.8), and the epimers of NADPHX (5.7, 7.9). The amount of product in a sample was quantified by comparison of its peak area with a chromatogram with that for a known amount of product.

**Results**

Copurification of the Dehydrogenating and the Debroniating Activities of DPDase—DPDase has been purified previously from bovine liver to near homogeneity by a modification of the method of Shiotani and Weber (1, 5). DPDase from this preparation was purified to homogeneity by anion-exchange chromatography on a Mono-Q anion-exchange column. The dehydrogenating specific activity with thymine as substrate enhanced the debroniating activity by over 95% and en-
were analyzed by reverse phase HPLC using Buffer I. Uracil and UH, other products were detected in the column eluant.

bond formation, such as occurs for inactivation of glutathione by H2O2 were accompanied by a loss of thiol groups reactive with H2O2, and not to DTNB-reactive thiol groups/subunit, whereas DPDase treated with H2O2 had less than one DTNB-reactive thiol group/subunit. Whereas DPDase treated with 5,5'-dithiobis(2-nitrobenzoic acid). DPDase had eight DTNB-reactive thiol groups/subunit, whereas DPDase treated with H2O2 had less than one DTNB-reactive thiol/subunit.

The dehydrogenating activity was restored to enzyme oxidized by H2O2 by treatment with 10 mM DTT for 20 min at 37 °C. This result suggested that the catalytically important thiol groups were oxidized to disulfides by H2O2 and not to higher oxidation states. The possibility that oxidation of thiol groups on DPDase by H2O2 involved intersubunit disulfide bond formation, such as occurs for inactivation of glutathione transferase-P form by H2O2 (28), was eliminated by the finding that the subunit molecular weights of DPDase and OxDPDase were the same by nonreducing sodium dodecyl sulfate acrylamide gel electrophoresis.

Products of the Degradation Reaction—The major product from the reaction of BrUH2 with OxDPDase was uracil (Fig. 2). Product concentrations at the end of a reaction of 100 μM BrUH2 with OxDPDase were 38 μM uracil, 4 μM UH3, and an unidentified product with a retention time of 3.8 min (Fig. 2). NADPH was required for this reaction (Fig. 3). Because OxDPDase catalyzed the conversion of approximately 50% of racemic BrUH2 to products (Fig. 2) and because the enzyme was not inhibited during the debromination of BrUH2 (Fig. 3), OxDPDase appeared to be catalyzing the stereospecific debromination of racemic BrUH2. OxDPDase deiodinated IUH2 to uracil and catalyzed the formation of products not observed with BrUH2 as substrate (see below).

Steady-state Kinetics for the Debromination of BrUH2—The steady-state kinetic parameters for debromination of BrUH2 catalyzed by OxDPDase were determined from initial velocity data at varying concentrations of BrUH2 and NADPH. Equation 1 described these data with $k_{\text{cat}} = 1.9 \pm 0.1$ s$^{-1}$, $K_{\text{BrUH2}} = 260 \pm 40$ μM, $K_{\text{NADPH}} = 1.3 \pm 0.2$ μM, and $K_{\text{NADPH/BrUH2}} = 230 \pm 40$ μM. The debromination activity (100 μM BrUH2) was stimulated 1.5-fold by 1 μM UH3 or 50 μM uracil but was inhibited 15% by 300 μM uracil, indicating that UH3 and uracil were weak effectors of the debrominating activity of OxDPDase. In contrast to the time course for debromination of BrUH2, the time courses for deiodination of IUH2 by OxDPDase were markedly nonlinear (data not shown). This was probably due to the modification of NADPH that occurred during the reaction (see below). Consequently, the steady-state kinetic parameters for deiodination of IUH2 by OxDPDase were not determined.

Inhibition of Debrominating and Dehydrogenating Activities of DPDase by 5-Ethynyluracil—5-Ethynyluracil is a mechanism-based inactivator of DPDase that inactivates the dehydrogenation activity of DPDase by covalent modification of a cysteinyl residue at the uracil binding site (6). Consequently, 5-ethynyluracil was tested as an inactivator of the debrominating activity of DPDase. DPDase was incubated with 0.5 mM DTT, 200 μM, NADPH and 100 μM 5-ethynyluracil for 15 min at 37 °C. Inactivated DPDase was separated from small molecules by size-exclusion chromatography on a column of P-6 resin equilibrated in 0.05 M Tris-HCl at 37 °C and pH 7.7. The enzyme was assayed for deiodinating activity before converting to OxDPDase and was assayed for debrominating activity after converting to OxDPDase by treatment with 0.5 mM H2O2 for 10 min at 37 °C. 5-Ethynyluracil completely (95%) inactivated the deiodinating activity of DPDase but reduced the debrominating activity by only 70%.

Modification of NADPH by IUH2—Because the NADPH-regenerating system rapidly reduced NADP+ to NADPH, any decrease in the amount of NADPH in the presence of OxDPDase or DPDase was the result of the formation of a product other than NADP+. OxDPDase catalyzed the reduction of O2 by NADPH approximately one-third as rapidly as DPDase. During the reduction of O2 by 0.24 μM OxDPDase in the presence of the NADPH-regenerating system, the absorbance at 340 nm of a solution of NADPH was not decreased (Fig. 4). In contrast, the absorbance at 340 nm of an aerobic solution of NADPH with 0.24 μM OxDPDase, 3.3 mM IUH2, and 100 μM NADPH and the NADPH-regenerating system decreased (Fig. 4). This result indicated modification of NADPH to a product other than NADP+, under these conditions. The rate of modification of NADPH by OxDPDase was calculated from the rate of absorbance decrease to be 4.3 μM/min (Fig. 4). OxDPDase catalyzed the modification of NADPH approximately 3-fold faster than DPDase.

The rate of modification of NADPH by OxDPDase was increased by addition of NaI or by removal of O2. The aerobic reaction velocity was increased from 4.3 μM/min to 29 μM/min by 50 mM NaI. In the absence of NaI, the reaction velocity was increased to 43 μM/min by the removal of O2 (Fig. 4).
modification reactions were absolutely dependent on IUHα (Fig. 4). The NADPH-regenerating system was active under these conditions.

Stoichiometric amounts of O2 (120 μM) and NADPH (100 μM) were consumed in the aerobic reaction with 50 mM NaI, 3.3 mM IUHα, 100 μM NADPH, and the NADPH-regenerating system (Fig. 4). The initial rate of O2 consumption by 0.24 μM OxDPDase was 27 μM/min, and the initial rate of NADPH modification was 29 μM/min. The reaction was not inhibited by 15 μg/ml catalase or 170 μg/ml superoxide dismutase. Consequently, enzyme-free peroxide and superoxide were not involved in the modification reaction.

Products from the Modification of NADPH by OxDPDase—The products of the aerobic modification of 100 μM NADPH by OxDPDase in the presence of 3.3 mM IUHα and 50 mM NaI were analyzed by reverse phase HPLC as described under "Experimental Procedures." The chromatogram of a sample of the solution prior to addition of enzyme had peaks corresponding to iodide, NADPH, and IUHα at 2.6 min, 11.2 min, and 13.3 min, respectively (Fig. 5A). The chromatogram of a sample of the solution after incubation with 0.24 μM OxDPDase for 10 min demonstrated that over 95% of the NADPH was consumed and that several products with absorbance at 260 nm were formed (Fig. 5A). Uracil and with a retention time of 5.3 min (λmax = 259 nm), and a species with a retention time of 16.8 min (λmax = 263 nm) were the major products. The latter had the same formation of NADPI as described under "Experimental Procedures." The chromatogram of a sample of the solution prior to addition of enzyme had peaks corresponding to iodide, NADPH, and IUHα at 2.6 min, 11.2 min, and 13.3 min, respectively (Fig. 5A). The chromatogram of a sample of the solution after incubation with 0.24 μM OxDPDase for 10 min demonstrated that over 95% of the NADPH was consumed and that several products with absorbance at 260 nm were formed (Fig. 5A). Uracil and IUHα were estimated to be less than 10 μM from a chromatogram that monitored the column effluent at 210 nm. The product with a retention time of 3.2 min (λmax = 266 nm) was not identified. The product mixture from the anaerobic modification of 100 μM NADPH by 3.3 mM IUHα and 0.24 μM OxDPDase (Fig. 5B) was more complex than that from the aerobic reaction (Fig. 5A). HPLC of a sample from the anaerobic reaction of 0.24 μM OxDPDase with 100 μM NADPH, 3.3 mM IUHα, and the NADPH-regenerating system demonstrated that 130 μM uracil, 240 μM IUHα, and 16 μM NADPI were formed, and 79 μM NADPH was consumed (Fig. 5B). In addition to these products, significant amounts of products with retention times of 5.7, 6.8, 7.9, and 9.4 min and with absorbance maxima at 263 nm were observed. Because less than 20% of the NADPH consumed in the anaerobic reaction was accounted for as NADPI, these products were probably modified forms of NADPH. NADPH is hydrated nonenzymatically under acidic conditions to a mixture of products (22). The analogous reaction with NADH has been studied extensively (18, 19, 23–25). The initial products from the nonenzymatic hydration of NADH are epimers of 6-hydroxy-1,4,5,6-tetrahydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX) that subsequently cyclize to cyclohexatrihydronicotinamide adenine dinucleotide phosphate (NADPHX).
the products of the enzymatic reaction with retention times of 5.7, 6.8, 7.9, and 9.4 min were all hydration products of NADPH with extinction coefficients similar to that for NADP, then 85% of the NADPH consumed could be accounted for. The relative amounts of the products from the anaerobic reaction of OxDPDase with NADPH and IUH₂ were not changed significantly by 50 mM NaI.

**Steady-state Kinetic Parameters for the Modification of NADPH by OxDPDase**—Aerobically, the major product from the OxDPDase-catalyzed modification of NADPH by IUH₂ was NADPI (Fig. 5A). The steady-state kinetic parameters for aerobic iodination of NADPH by OxDPDase and IUH₂ were determined from the initial velocity of NADPH disappearance (Δε₈₅₀ = 6.22 mm⁻¹ cm⁻¹) in the presence of the NADPH-regenerating system. The turnover number determined with 230 μM NADPH and 50 mM NaI had a biphasic dependence on IUH₂ concentration that was analyzed by Equation 2 (data not shown). The kinetic parameters for the high affinity catalytic site were a Kᵦ for IUH₂ of 180 ± 30 μM and a kₐₗₖ for 1.2 ± 0.1 s⁻¹. The turnover number of the low affinity site was linearly dependent on IUH₂ concentration (kₐₗₖ/Kᵦ = 0.39 ± 0.02 mm⁻¹ s⁻¹). The kₐₗₖ for NADPH with 0.8 mM IUH₂ and 50 mM NaI was 34 ± 4 μM·s⁻¹. NaI increased the turnover number for iodination of NADPH from 0.22 ± 0.04 s⁻¹ to 2.37 ± 0.05 s⁻¹ with 3.7 ± 0.4 mM NaI yielding one-half of the maximal effect. The iodination of 210 μM NADPH by 0.08 mM OxDPDase and 500 μM IUH₂ was inhibited 60% by 330 μM uracil and less than 10% by 330 μM UH₂.

Because of the difficulty in maintaining anaerobic conditions for initial velocity determination and because of the formation of multiple products (Fig. 5B), a detailed kinetic study of the anaerobic modification of NADPH was not made. Nonetheless, the catalytic activity of OxDPDase in the anaerobic modification of NADPH by IUH₂ was comparable with that for the aerobic iodination of NADPH (Fig. 4).

**Nonenzymatic Formation of Uracil from BrUH₂ and IUH₂**—IUH₂ and BrUH₂ are intrinsically reactive molecules that undergo buffer-catalyzed elimination of halide to form uracil under appropriate conditions (30). The dehalogenation of 1 mM IUH₂ or 1 mM BrUH₂ to uracil was monitored by the absorbance change at 260 nm associated with uracil formation. Thus, IUH₂ was deiodinated to uracil with a first-order rate constant of (2 ± 0.2) × 10⁻² mm⁻¹s⁻¹ (0.8 mM Tris (0.8 mM at pH 7.7) at 93 °C). Similarly, BrUH₂ was debrinated to uracil with a first-order rate constant of (1.2 ± 0.1) × 10⁻⁲ mm⁻¹s⁻¹ in 0.9 M Tris-HCl (pH 7.7) at 80 °C.

**DISCUSSION**

DPDase catalyzes the reversible reduction of pyrimidines to 5,6-dihydropyrimidines with NADPH as an electron donor and the oxidation of NADPH by molecular oxygen (1-6). Results presented herein demonstrated that an oxidized form of DPDase (OxDPDase), which did not catalyze the reversible reduction of pyrimidines, catalyzed the following unique reactions: 1) the dehalogenation of BrUH₂ and IUH₂; 2) the iodination of NADPH by IUH₂; and 3) the modification of NADPH to species with spectral and chromatographic properties similar to hydrated NADPH (NADPH₃). The kₐₗₖ values of OxDPDase for these reactions were similar to that for reduction of thymine (2.1 s⁻¹) by DPDase.

The debrinating activity of OxDPDase was dependent on NADPH. This result was unexpected, because dehalogenation of BrUH₂ to uracil did not involve a net reduction of BrUH₂. Consequently, NADPH presumably served as an effector by maintaining OxDPDase in the catalytically active conformation for debrination of BrUH₂. In contrast to the affinity (Kᵦ/Aff = 1.3 μM) of NADPH for the debrinating activity of DPDase, uracil and UH₂ were low affinity effectors of the reaction. These results and the finding that 5-ethyluracil, which binds covalently to DPDase at the uracil binding site (5), inhibited OxDPDase activity by less than 7% suggested that the debrination reaction was not occurring at the uracil binding site. Because BrUH₂ was easily debrinated to uracil nonenzymatically with 1.10 of 50 min in 0.8 mM Tris at pH 7.7 and 80 °C, enzymatic debrination of BrUH₂ by OxDPDase may only involve the fortuitous, but stereospecific, binding of BrUH₂ to a site on OxDPDase that had a properly positioned enzymatic base in the OxDPDase-NADPH complex to assist the removal of the 6-proton of BrUH₂ (Scheme 1, X = Br). The mechanism for iodination of H₂O₂ with DPDase to give OxDPDase on the relative amounts of dehydrogenase and oxidase activities of the enzyme. H₂O₂ inactivated the dehydrogenase activity of DPDase and enhanced its dehalogenating activity. H₂O₂ inactivated xanthine dehydrogenase activity and enhanced xanthine oxidase activity (31, 32). The treatment of DPDase with H₂O₂ resulted in the oxidation of 8 thiol groups per subunit, whereas the treatment of xanthine oxidase resulted in the oxidation of 14 thiol groups per subunit. Xanthine oxidase (31, 32) and OxDPDase are converted back to their respective dehydrogenase forms by treatment with DTT.

The products from the OxDPDase-catalyzed reaction of IUH₂ with NADPH were dependent on the reaction conditions. The major product in the presence of Nal and O₂ was uracil and NADPI, with the latter being stoichiometric with O₂ consumption (Fig. 4). Superoxide dismutase and catalase did not inhibit the iodination reaction. Consequently, iodination of NADPH by OxDPDase was the rate-limiting enzyme-bound iodinating species and was not the result of a nonenzymatic reaction with products formed enzymatically from one or more of the substrates H₂O₂, iodide and NADPH. NADPI was also a product of the anaerobic reaction of OxDPDase with IUH₂ and NADPH in the absence of NaI. In this case, IUH₂ was necessarily the source of the iodo group for iodination of NADPH. Thus, NADPI formation occurred by two pathways. Aerobically, NADPH was iodinated by a species such as HOI that could have been generated from enzyme-bound H₂O₂ and iodide. Anaerobically, NADPH could be iodinated by attack of the C-5 position of dihydronicotinamide on the iodo moiety of IUH₂ (Scheme 2).

The mechanism for iodination of NADPH presented in Scheme 2 predicts that equal amounts of NADPI and UH₂ would be made. During the anaerobic iodination of NADPH, however, 10-fold more UH₂ was made than NADPI (Fig. 5B). Because OxDPDase does not catalyze the reduction of uracil to UH₂, a mechanism for generation of UH₂ other than reduction of the uracil formed by the deiodination of IUH₂ must exist. One possibility is the reductive dehalogenation of IUH₂ as shown in Scheme 3. The mechanism of Scheme 3 is written with the hydride from NADPH directly displacing iodide from IUH₂. Alternatively, this hydride could be from a prosthetic group on OxDPDase, such as reduced flavin, or from a thiol group on the enzyme. This reaction is analogous to the nonenzymatic reductive deiodination of IUH₂ by DTT to give UH₂ in quantitative yield (5).
Aerobically, the amount of NADPH modified by OxDPDase and IUH₂ in the presence of NaI could be accounted for by the amount of NADPI made (Fig. 5A). Anaerobically, NADPI accounted for only 20% of the NADPH consumed. The remainder of the NADPH appeared as species that had spectral properties similar to the hydration products of NADPH. The hydration of NADPH and NADH is a relatively facile nonenzymatic reaction (19-25) that readily occurs in 0.5 M potassium phosphate buffer at pH 6.0. Thus, OxDPDase could facilitate hydration of NADPH by juxtaposition of a suitable general acid and the C-5 position of the dihydronicotinamide ring. The function of IUH₂ in the hydration reaction is unclear. Possibly, IUH₂ binding to the OxDPDase-NADPH complex perturbs the environment of the C-3 carboxamide of the dihydronicotinamide ring sufficiently so that the reactivity of the 5,6 double bond is enhanced. Model studies have demonstrated that the reactivity of the 5,6 double bond of dihydronicotinamides is very dependent on the nature of the substituent at C-3 (18).

Numerous dehydrogenases have been shown to catalyze the hydration of NADH (33-38). The hydration reaction of NADH catalyzed by glyceraldehyde-3-phosphate dehydrogenase has been studied extensively (19, 33-36). Glyceraldehyde-3-phosphate dehydrogenase has a $k_{\text{cat}}$ for hydration of NADH under optimal conditions (pH 5.2) of 0.017 s⁻¹ (35), whereas $k_{\text{cat}}$ in the dehydrogenation reaction under optimal conditions (pH 8.4) is over 300 s⁻¹ (36). Thus, glyceraldehyde-3-phosphate dehydrogenase catalyzes its physiological reaction approximately 4 orders of magnitude faster than it catalyzes the hydration of NADH. In contrast, the anaerobic modification of NADPH by OxDPDase in the presence of 4 mM IUH₂ had a turnover number that was similar to that for the physiological reduction of thymine.

In summary, these studies demonstrate that in addition to catalyzing the dehydrogenation and oxidation of physiological substrates, DPDase under appropriate conditions catalyzes the dehalogenation of IUH₂ and BrUH₂, the iodination of NADPH by IUH₂, and the hydration of NADPH in the presence of IUH₂. Any chemical mechanism proposed for DPDase catalysis should also explain the activities of OxDPDase.

REFERENCES