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# Mechanism of Dihydrouridine Synthase 2 from Yeast and the Importance of Modifications for Efficient tRNA Reduction*

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**Dihydrouridine synthases (DUSs) are flavin-dependent enzymes that catalyze site-specific reduction of uracils in tRNAs. The mechanism of DUS 2 from *Saccharomyces cerevisiae* was studied. Previously published turnover rates for this DUS were very low. Our studies show that the catalytic cycle consists of reductive and oxidative half-reactions. The enzyme is reduced by NADPH rapidly but has a very slow oxidative half-reaction using *in vitro* transcribed tRNA substrates. Using $tRNA^{Leu}$ purified from a DUS 2 knockout strain of yeast we obtained reaction rate enhancements of 600-fold over *in vitro* transcribed substrates, indicating that other RNA modifications are required for rapid uracil reduction. This demonstrates a previously unknown ordering of modifications and indicates that dihydrouridine formation is a later step in tRNA maturation. We also show that an active site cysteine is important for catalysis, likely in the protonation of uracil during tRNA reduction. Dihydrouridine of modified tRNA from *Escherichia coli* was also oxidized to uridine showing the reaction to be reversible.**

Dihydrouridine is one of the most common modified nucleosides present in the tRNAs of eubacteria, eukaryotes, and some archea (1). This modified nucleoside is formed by the reduction of the carbon-carbon double bond in uridine at specific sites in tRNAs, most commonly in the D-loop. Our understanding of the physiological role of dihydrouridine is still in its infancy, but one possibility is a structural role. NMR studies have shown that dihydrouridine increases the flexibility of the RNA backbone to allow alternate conformations because its non-planar structure disrupts stacking (2). In addition, a role for dihydrouridine in conformational flexibility is also suggested by its distribution among different species. The dihydrouridine content of species adapted to cold environments (psychrophiles) is the highest found in nature, whereas thermophilic species tend to have much less or no dihydrouridine (3). It has been suggested that the chemical stability of RNA structures is enhanced by the absence of dihydrouridine, perhaps explaining why thermophilic species often lack dihydrouridine, to avoid hydrolytic ring opening at high temperatures (4).

Recent studies have hinted at further physiological roles for dihydrouridine. tRNA lacking dihydrouridine, along with other modifications, degrades at a substantially increased rate, approaching that seen for mRNA (5). The main function of dihydrouridine may therefore be to protect tRNAs from degradation. It has long been known that dihydrouridine levels are increased in cancerous tissues (6). Recent studies have shown that the human dihydrouridine synthase 2 is responsible for the increased dihydrouridine levels formed in pulmonary carcinogenesis (7). This information suggests that dihydrouridine plays an important role in preventing tRNA turnover.

The family of enzymes catalyzing the reduction of uridine in tRNA, dihydrouridine synthases (DUS),[3] has recently been identified in *Saccharomyces cerevisiae* and *Escherichia coli.* DUSs from *S. cerevisiae* were identified by biochemical assays of pooled yeast proteins (8), whereas the enzymes from *E. coli* were identified by a bioinformatics approach (9). Sequencing and annotation of many microbial genomes suggests the presence of DUSs in a wide number of species. The enzymes from *S. cerevisiae* are NADPH-dependent with non-overlapping RNA specificity (10).

From recent structural and homology models, and from analogies to other flavoenzymes that act on pyrimidines, putative active site residues can be assigned. The crystal structure of the enzyme from *Thermotoga maritima* (11) has a conserved cysteine residue positioned within a suitable distance to protonate the uracil ring as it is reduced. Although some aspects of dihydrouridine formation are becoming better understood, there have been no studies on the kinetics of these enzymes. To better understand how these enzymes function we have investigated the catalytic mechanism of DUS 2 from *S. cerevisiae* and an active site cysteine mutant. We find that the enzyme is able to reduce tRNA and, surprisingly, prefers modified tRNA substrates to *in vitro* transcribed tRNAs. We also show that the active site cysteine is important for catalysis, likely acting to protonate the uracil ring.

## EXPERIMENTAL PROCEDURES

*Materials*—NADP and NADPH were from Research Products International. ATP, GTP, CTP, and UTP were from Amersham Biosciences. 1-D-Glucose was from Cambridge Isotopes Laboratories. All other chemicals were purchased from Sigma-Aldrich.

*Construction of the S. cerevisiae C117A Mutant Plasmid*—The C117A mutant plasmid was constructed from the pEfx-4b

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[3] The abbreviations used are: DUS, dihydrouridine synthase; DTT, dithiothreitol.

plasmid and mutated using the QuikChange site-directed mutagenesis kit (Stratagene) using the following forward primer ATCAATGCAGGCGCCCCCAAACAGTTCT and reverse primer AGAGAAGTGTTTGGGGGCGCCTGCA.

*Expression and Purification of DUS*—BL21(DE3) cells were transformed with the plasmid for the *S. cerevisiae* enzyme by heat shock and plated on LB agar plates containing 0.1 g/liter ampicillin and incubated overnight at 37 °C. Single colonies were used to inoculate 5 ml of LB, which was shaken at 400 rpm overnight in a 37 °C incubator. From these cultures 1 ml was added to 1 liter of LB containing 0.1 g/liter ampicillin. The cells were grown to an $A_{600}$ of 0.8 and induced using 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (final concentration) and grown for 5–6 h at 37 °C.

The DUS-containing cells were pelleted by centrifugation (16,000 × *g* for 20 min) and resuspended in 100 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol. The cells were sonicated for 15 min on ice. Cell debris was removed by centrifugation (20,000 × *g* for 30 min). The clarified supernatant was then passed over a 3.8-cm × 10-cm TALON metal affinity column from Clontech, which had been loaded with cobalt(II) acetate and pre-equilibrated with 100 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol. The column was then washed with 3 column-volumes of 100 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, and the protein was eluted from the column with the same buffer containing 200 mM imidazole. The eluted enzyme was then washed extensively with 100 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol in an Amicon ultrafiltration device (10,000 molecular weight cut-off). Wild-type DUS 2 was stored at 4 °C with 1 mM DTT after elution from the metal affinity column. The DTT was removed by a PD-10 desalting column immediately prior to experiments.

The enzyme obtained by metal affinity purification showed a single band on SDS-PAGE at ~43 kDa. However, the absorbance spectrum of the enzyme purified in this way had an unusual peak at 340 nm in addition to the expected flavin absorbance. Therefore the enzyme was purified further to remove this contaminant by applying it to a 3.8-cm × 14-cm DEAE-Sepharose column at 25 °C, which was pre-equilibrated in 100 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol. The column was washed extensively with 100 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol. The yellow fractions containing protein free of the contaminating chromophore were collected. Protein that remained bound to the column was eluted with 100 mM HEPES, pH 7.5, 1 M NaCl, 10 mM $MgCl_2$, 10% glycerol and showed a proportionately higher 340 nm peak. Enzyme fractions free of the chromophore were pooled, concentrated, washed with 100 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, and stored at 4 °C.

*Purification of Mature $tRNA^{Leu}$*—Mature $tRNA^{Leu}$ was purified from a *S. cerevisiae* YNR015w knockout strain. A total of 80 liters of yeast cultured in YPD (10 g of yeast extract, 20 g of peptone, 20 g of glucose, 1 liter of $H_2O$) were grown to saturation by shaking at 30 °C in 1-liter flasks. The yeast was pelleted at ~5000 × *g* for 15 min and frozen until use. The frozen cell pellet was resuspended in 50 mM LiCl, 10 mM EDTA, 10 mM Tris-HCl, pH 6.5, 0.1% SDS and thawed to room temperature. Soluble RNA was then extracted with phenol-chloroform buffered to pH 4.7 and centrifuged (4200 × *g* for 30 min). The RNA in the supernatant was precipitated with 2 volumes of ethanol and 0.2 M LiCl. The RNA was washed with 70% ethanol, frozen, and lyophilized. The tRNA was purified further on an oligonucleotide column made by treating NHS-Sepharose (Amersham Biosciences) with an aminated oligonucleotide (IDT Technologies, Coralville, IA) complementary to the 3′-end of $tRNA^{Leu}$-CCA. The tRNA was heated to 50 °C in 1 M NaCl, 10 mM Tris, pH 8.5, and loaded on the column. The column was washed with 3 column volumes of 1 M NaCl, 10 mM Tris, pH 8.5, heated to 50 °C, and eluted in 10 mM Tris, pH 8.5.

*Enzyme Concentrations*—The extinction coefficient of the enzyme was determined by recording the absorbance spectrum of the enzyme, adding SDS (1% final concentration), and recording the spectrum of the FMN liberated upon protein unfolding. The extinction coefficient was calculated from the ratio of the absorbance of the free FMN (12) to that of the native enzyme. The extinction coefficient at 457 nm was 12,800 $M^{-1}$ $cm^{-1}$ in 50% glycerol, 20 mM Tris, pH 8.0, 55 mM NaCl, 1 mM DTT. Enzyme concentrations were routinely determined spectrophotometrically using this extinction coefficient and are given in terms of active site concentrations.

*Reaction Kinetics*—Enzyme solutions for kinetics were made anaerobic in glass cuvettes or tonometers by multiple cycles of evacuation followed by equilibration with an atmosphere of purified argon (13). When reduced enzyme was needed, a gas-tight syringe containing a dithionite solution (~6 mM) was attached to the cuvette, and the anaerobic enzyme was titrated to complete reduction with one equivalent of dithionite, as judged by absorbance spectroscopy. Reactions were performed at 4 °C using 100 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol unless stated otherwise. Absorbance spectra were obtained over time using a Shimadzu UV-2501PC scanning spectrophotometer for slow oxidative half-reactions; rapid reactions were studied anaerobically using a Hi-Tech Scientific SF-61DX2 stopped-flow spectrophotometer using published techniques (13).

*Binding Titrations*—Binding titrations were carried out in quartz fluorescence cuvettes at 25 °C in 100 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$,10% glycerol in a Shimadzu RF-5301 PC spectrofluorophotometer. Enzyme (~20 μM) was titrated with increasing concentrations of ligands. Excitation was at 450 nm, and emission was scanned from 460 to 650 nm, with a maximum fluorescence change observed at 517 nm. For weak or moderate binding, the change in fluorescence was plotted against the concentration of ligand and fit to Equation 1.

$$\Delta F = \frac{\Delta F_{max} L}{K_D + L} \quad \text{(Eq. 1)}$$

Tight binding was fit to Equation 2.

$$\Delta F = \Delta F_{max}\left[\frac{E_0 + L_0 + K_D - \sqrt{(E_0 + L_0 + K_D)^2 - 4E_0L_0}}{2}\right] \quad \text{(Eq. 2)}$$

*tRNA Synthesis*—tRNAs were produced in reactions containing 50 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM spermidine, 20 mM of each NTP, T7 RNA polymerase,

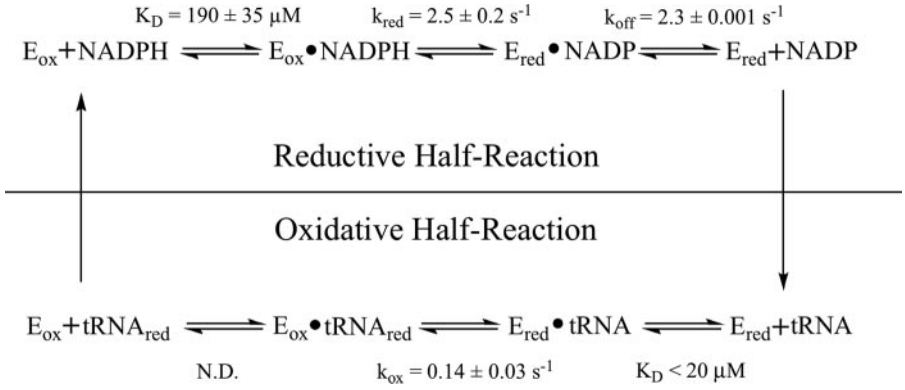



FIGURE 1. **Scheme for the catalytic cycle of DUS 2.**

and DNA template (10 ng/$\mu$l). The templates for each reaction were synthesized by PCR using two complementary primers (Invitrogen) comprising the entire tRNA gene to be transcribed, and a T7 RNA polymerase promoter upstream. The sequence of the *S. cerevisiae* pre-tRNA$^{Leu}$ substrate and intronless variant used were GGTTGTTTGGCCGAGCGGTCTAAGGCGCCTGATTCAAGCTCAGGTATCGTAAGATGCAAGAGTTCGAATCTCTTAGCAACCA and GAAATTAATACGACTCACTATAGGTTGTTTGGCCGAGCGGTCTAAGGCGCCTGATTCAAGCTCAGGTATCGTAAGATGCAAGAGTTCGAATCTCTTAGCAACCA, respectively. Synthetic tRNA was purified by phenol-chloroform extraction to remove RNA polymerase, and the RNA was precipitated with 2 volumes of ethanol. The RNA concentration was calculated from the absorbance at 256 nm using extinction coefficients predicted from the base composition tRNA using the nearest neighbor method (14). Extinction coefficients of 1,019,100 $M^{-1}$ $cm^{-1}$ was used for the *S. cerevisiae* pre-tRNA$^{Leu}$ and an extinction of 764,800 $M^{-1}$ $cm^{-1}$ was used for the intronless and modified tRNA$^{Leu}$ purified from *S. cerevisiae*. Before use tRNA was heated to 65 °C for 10 min and cooled on ice for 5 min to ensure proper folding of the tRNA.

*Dihydrouridine Assay*—Dihydrouridine produced by the reduction of tRNA was determined using a colorimetric assay (15). tRNA (125 $\mu$M in 0.3 ml) was treated with 0.03 ml of 1 M KOH and incubated for 30 min at 37 °C, hydrolyzing the dihydrouridine ring. The sample was then acidified with 0.15 ml of 1 M $H_2SO_4$, and 0.3 ml of a solution of 10 mM 2,3-butanedione-2-oxime and 5 mM *N*-phenyl-p-phenylenediamine was added. The sample was heated for 5 min at 95 °C in a heating block and cooled to 50 °C for 5 min, 0.3 ml of 10 mM $FeCl_3$ was added, and the absorbance at 550 nm was read. Concentrations were determined by comparison with a standard curve determined from known concentrations of dihydrouracil with a linear range between 20 and 250 $\mu$M.

*Preparation of [4-$^2$H]NADPH*—[4*R*-$^2$H]NADPH was prepared as described by Ottolina *et al.* (16). A 10-ml solution of 50 mM sodium bicarbonate, pH 8.5, 0.5 M 2-D-isopropanol, 13 mM NADP, and 20 units of *Thermoanaerobacter brockii* alcohol dehydrogenase (Sigma) was incubated at 30 °C for 4 h. The solution was diluted to 20 ml with $H_2O$ and chromatographed on a 2.8-cm × 40-cm DE-53 column (carbonate form). The [4*R*-$^2$H]NADPH was eluted with a sodium bicarbonate gradient (0–0.5 M, 500 ml). Fractions with a ratio of $A_{254}/A_{340}$ of 2.5 or less were pooled and lyophilized. The lyophilized product was chromatographed with water on a 2-cm × 50-cm Sephadex G-10 column to remove salt (17). Fractions containing [4*R*-$^2$H]NADPH were pooled, and the product was precipitated with 70% acetone at 4 °C to concentrate, dissolved in a minimal amount of water, and lyophilized.

[4*S*-$^2$H]NADPH was prepared in a 6-ml solution of 50 mM HEPES, pH 7.5, 15 mM 1-D-glucose, 15 mM ATP, 15 mM NADP, 15 mM $MgCl_2$, 100 units of hexokinase, and 100 units of glucose-6-phosphate dehydrogenase. The reaction was incubated at 25 °C for 5 h, and the [4*S*-$^2$H]NADPH was purified as described above.

*Stereochemistry*—The stereochemistry of oxidation of NADPH by the enzyme was determined by incubating 1.5 ml of an aerobic solution containing 2 mM [4*R*-$^2$H]NADPH, 100 $\mu$M of DUS, 100 mM potassium phosphate, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$ in$D_2O$ at 35 °C. After [4*R*-$^2$H]NADPH was completely oxidized (no absorbance at 340 nm; ~2 h), enzyme was removed by ultrafiltration (Amicon Ultra 10,000 molecular weight cut-off), and a 300-MHz $^1$H NMR spectrum was recorded using the water peak as a chemical shift reference (4.2 ppm). An identical procedure was used for [4*S*-$^2$H]NADPH.

*Identification of tRNAs Bound to DUS*—tRNA bound to the enzyme after purification by the metal affinity column was extracted with phenol-chloroform to remove the protein and precipitated with ethanol to yield the pure tRNA. Mixtures of ~3.5 $\mu$mol of tRNA were incubated in a 10-ml reaction at pH 7.4 in 100 mM potassium $P_i$ with 0.8 mM of each amino acid, 10 mM ATP, and 2880 units of aminoacyl-tRNA synthetases from *E. coli* (Sigma A3646-10KU) to ensure complete aminoacylation of the tRNAs. The reactions were incubated for 18 h and extracted with phenol-chloroform, and the tRNA was precipitated with 2 volumes of ethanol. The tRNA was dissolved in 200 $\mu$l of 100 $\mu$M potassium $P_i$, pH 9.0, and incubated for 5 h to hydrolyze the aminoacyl-esters. The tRNA was separated from the amino acids by ultrafiltration with 10,000 molecular weight cut-off Microcon centrifugal filtration devices (Amicon) previously washed with $H_2O$. The flow-through was collected and analyzed by the Protein Facility of the Office of Biotechnology, Iowa State University, on an automated Beckman System Gold HPLC amino acid analyzer.

## RESULTS

The catalytic cycles of flavoenzymes frequently have a reductive half-reaction, in which one substrate reduces the flavin, and an oxidative half-reaction, in which another substrate oxidizes the flavin (Fig. 1). However in many flavoenzymes, the second substrate binds and reacts before the first product is released, deviating from the typical ping-pong mechanism. Transient kinetic experiments were performed to determine the mechanism followed by the flavoenzyme DUS 2 from *S. cerevisiae*.

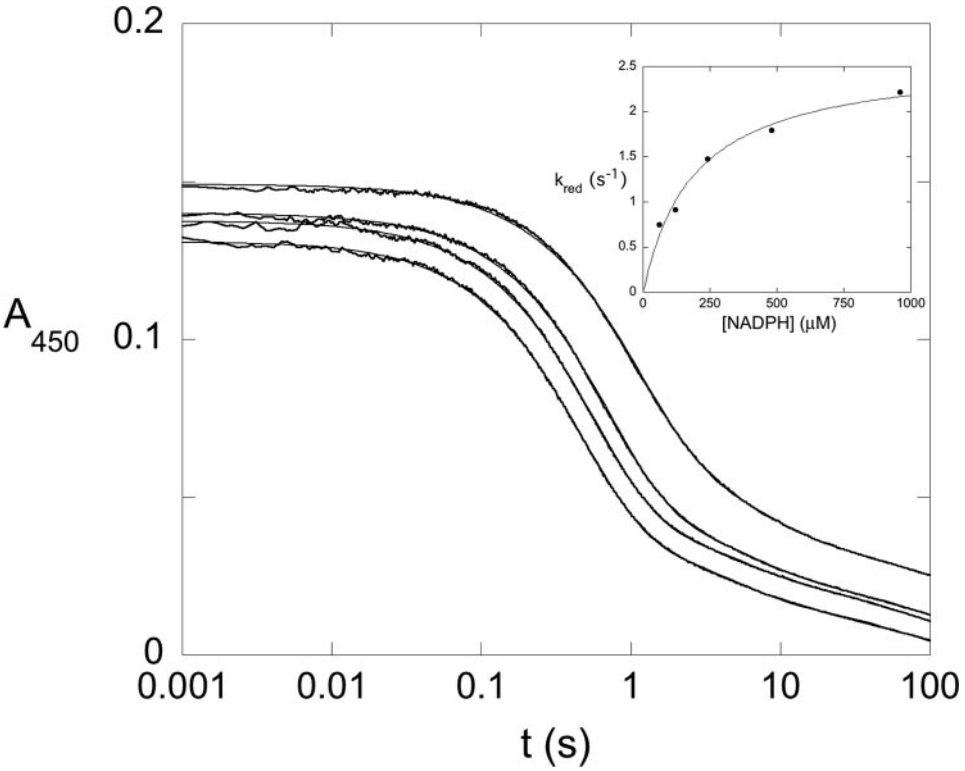



FIGURE 2. **Anaerobic DUS 2 (15 $\mu$M) in 100 mM HEPES, pH 7. 5, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, was mixed with anaerobic solutions of NADPH in a stopped-flow spectrophotometer at 4 °C.** Reaction traces at 450 nm are shown for a series of increasing concentrations of NADPH. Note the logarithmic time scale. The *inset* shows the observed rate constant for the first phase of reduction obtained from fits of each trace *versus* concentration, giving a rate constant for reduction of 2.5 ± 0.2 $s^{-1}$ and a $K_D$ of 190 $\mu$M.

$$k_{obs} = \frac{k_{red}[NADPH]}{K_D + [NADPH]} \quad \text{(Eq. 3)}$$

The *y*-intercept of the plot of the observed rate constant did not differ significantly from zero, indicating that the reverse rate constant is very small and the maximum of 2.5 $s^{-1}$ is the rate constant for flavin reduction. A $K_D$ of 190 ± 35 $\mu$M was determined from the dependence of the observed rate constant on the concentration of NADPH (18). Two other exponentials, together accounting for ~30% of the absorbance decrease, were seen in these traces, with rate constants of 0.21 ± 0.005 $s^{-1}$ and 0.015 ± 0.0007 $s^{-1}$. These did not vary with NADPH concentration and were possibly due to damaged enzyme. This damage might be due to the oxidation of enzyme thiols; the slower phases of enzyme prepared without DTT became more prominent as these preparations aged. A weak, broad charge-transfer absorbance, observed at 580 nm, corresponded to an NADP-reduced flavin charge-transfer complex seen in many NAD(P)-dependent flavoenzymes (19). A trace at 580 nm was fit to two exponentials by fixing the rate constant for the formation of the charge-transfer absorbance to 2.5 $s^{-1}$, the rate constant of flavin reduction. A rate constant of 2.29 ± 0.01 $s^{-1}$ for the second phase was obtained. This could be the rate constant for NADP dissociation from the reduced enzyme, if the equilibrium lies completely toward dissociated reduced enzyme and product, but our data do not directly address this. In a separate experiment, the rate constants for flavin reduction were also measured using [4*R*-$^2$H]NADPH and NADPH giving 0.42 ± .05 $s^{-1}$ and 1.47 ± 0.3 $s^{-1}$, respectively. A kinetic isotope effect value of 3.5 ± 0.2 was calculated, indicating that hydride transfer is at least partly rate-limiting.

The reductive half-reaction of the C117A mutant of DUS 2 was studied in the same way. Traces were fit to three exponentials. A first phase (12.7 ± 2 $s^{-1}$) corresponded to a small initial absorbance decrease independent of NADPH concentration. This was followed by a second phase, which accounted for the majority of the absorbance decrease, giving a reduction rate constant of 5.55 ± 0.01 $s^{-1}$ and a $K_D$ of 53 ± 3 $\mu$M. A band was seen at 675 nm corresponding to an NADP-reduced flavin charge-transfer complex. A trace at 675 nm was fit to two exponentials by fixing the rate constant for the formation of the charge-transfer absorbance to 5.55 $s^{-1}$, giving the rate constant for the second phase of 7 ± 2 $s^{-1}$, and likely corresponds to the rate constant for NADP dissociation from the reduced enzyme.

### *Reductive Half-reaction*

NADPH has been reported to be the reducing substrate for the DUS 2 enzyme from *S. cerevisiae* (10). The reductive half-reaction of DUS 2 is proposed to start with NADPH binding to the enzyme (Fig. 1). The NADPH then transfers a hydride to the active site flavin prosthetic group, reducing the enzyme. Finally NADP dissociates, yielding free reduced enzyme.

In attempting to further understand the mechanism of reduction we investigated the stereofacial selectivity of NADPH oxidation. [4*R*-$^2$H]NADPH and [4*S*-$^2$H]NADPH were oxidized, and the NADP produced was analyzed by $^1$H NMR. When [4*R*-$^2$H]NADPH was the substrate, a proton signal was observed at 8.4 ppm for the proton at the 4-position of the nicotinamide ring of NADP, the product, indicating that the *R*-deuteron was transferred (data not shown). When [4*S*-$^2$H]NADPH was used, the NADP produced lacked the signal at 8.4 ppm, indicating retention of the *S*-deuteron and transfer of the *R*-hydrogen. Therefore, DUS is specific for the *proR* hydrogen of NADPH.

The kinetics of the reductive half-reaction were studied at 4 °C by mixing 15 $\mu$M anaerobic oxidized enzyme with anaerobic solutions of NADPH in a stopped-flow spectrophotometer. The absorbance of the flavin, monitored at 450 nm, decreased in three phases (Fig. 2). The first phase, which contributed the majority of the absorbance decrease and represents flavin reduction, had an observed rate constant that increased hyperbolically with NADPH concentration. Fitting the observed rate constant to Equation 3 give a maximum rate constant of 2.5 ± 0.2 $s^{-1}$ at saturating NADPH.

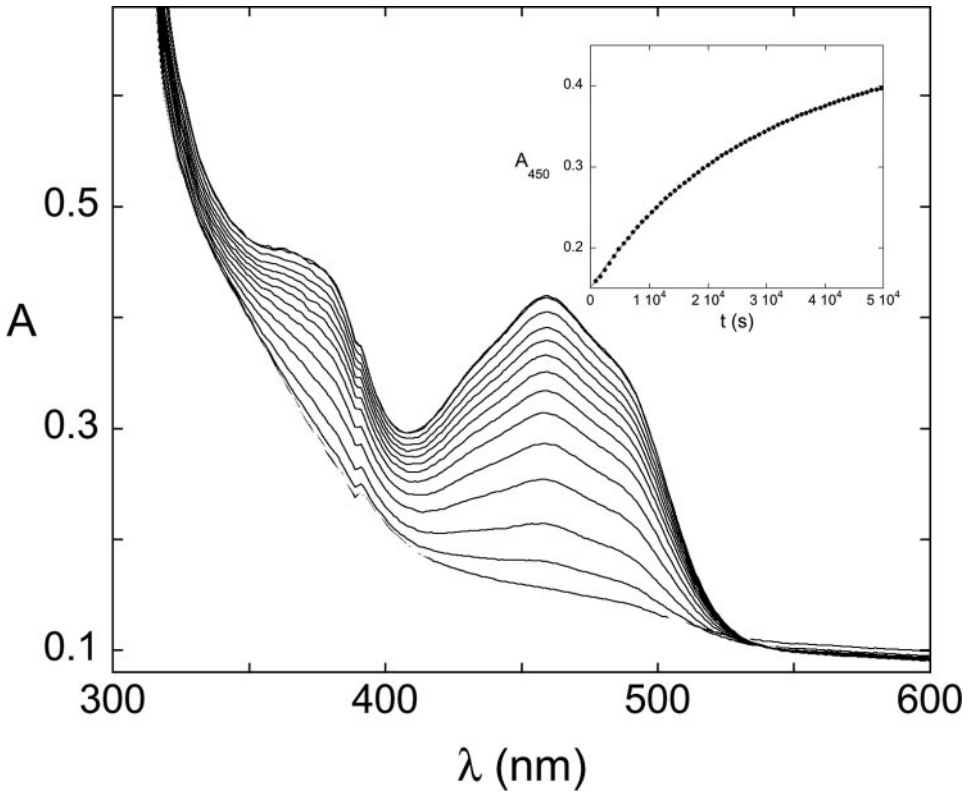



FIGURE 3. **Oxidation of DUS 2 by pre-tRNA$^{Leu}$.** Anaerobic DUS 2 (35 $\mu$M) in 100 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol was reduced by titrating with one equivalent of dithionite. The reduced enzyme was reoxidized at 25 °C by mixing with anaerobic tRNA$^{Leu}$. The *inset* shows the time dependence of flavin oxidation, detected at 450 nm. The *trace* was fit to a single exponential, giving a rate constant of $3.5 \times 10^{-5} \pm 3.6 \times 10^{-7}$ s$^{-1}$.

### *Oxidative Half-reaction*

A general mechanism for the oxidative half-reaction is seen in Fig. 1. The reduced enzyme first binds to tRNA. A specific uracil in the tRNA is then reduced by the enzyme to dihydrouracil. Finally the reduced tRNA leaves the enzyme, completing the oxidative half-reaction. We investigated the oxidative half-reaction of *S. cerevisiae* DUS 2 using synthetic pre-tRNA$^{Leu}$, which was reported to be a substrate (8). The oxidative half-reaction was analyzed by mixing 60 $\mu$M reduced enzyme at 25 °C with saturating concentrations of synthetic yeast pre-tRNA$^{Leu}$ (700 $\mu$M). Surprisingly, the reaction was extremely slow. Spectra were recorded over 16 h as the flavin was oxidized. Reaction traces at 457 nm fit to one exponential yielding a rate constant of $3.5 \times 10^{-5} \pm 4 \times 10^{-7}$ s$^{-1}$ for oxidation (Fig. 3). Product analysis showed that 0.9 ± 0.08 mol of dihydrouridine was formed per mole of flavin oxidized. The same experiment, carried out with the C117A mutant enzyme, gave a similar rate constant for flavin oxidation, $4.8 \times 10^{-5} \pm 3 \times 10^{-7}$ s$^{-1}$. The ratio of dihydrouridine produced per flavin molecule oxidized was 0.84 ± 0.1. The very slow reaction of the pre-tRNA$^{Leu}$ could conceivably be caused by the intron, which is excised during normal maturation. Therefore, the oxidative half-reaction was performed using synthetic tRNA$^{Leu}$ without the intron. Reduction of the intronless RNA was faster by a factor of ~6, with a rate constant of $2.2 \times 10^{-4} \pm 5 \times 10^{-6}$ s$^{-1}$, still a very slow reaction.

Given these slow reaction rates, as well as slow steady-state turnover numbers previously reported (8), we hypothesized that there are other factors affecting the reaction of DUS 2 with tRNA. One possibility is that *in vitro* transcribed tRNA is not the native DUS 2 substrate. During processing there are multiple modifications of tRNA, and it has been shown that some modification enzymes are dependent upon prior post-transcriptional modifications (20). We therefore attempted to determine if one or more other modifications of the tRNA was necessary for rapid reaction. To accomplish this we purified a single tRNA$^{Leu}$ species from a DUS 2 knockout strain using an oligonucleotide affinity column. This allowed us to purify tRNA, which should have all the modifications normally present in the tRNA not dependent on the reduction step DUS 2 performs.

This partially matured tRNA was reduced by DUS 2 dramatically faster than synthetic tRNA. Reduced DUS 2 (20 $\mu$M) was oxidized at 4 °C by mixing with various concentrations of the modified tRNA$^{Leu}$ lacking dihydrouridine in anaerobic stopped flow experiments. The absorbance increases at 450 nm were fit to single exponentials. The observed rate constants did not vary with tRNA concentration, indicating a $K_D$ much lower than 20 $\mu$M (Fig. 4), in stark contrast to synthetic tRNA. The observed rate constant for reaction of the modified tRNA$^{Leu}$ substrate was 0.14 ± 0.03 s$^{-1}$, >600-fold faster than the reaction of the *in vitro* transcribed intronless substrate at 25 °C. Because the reduction was saturated at all accessible tRNA concentrations, it was not possible to detect whether, as tRNA concentration approaches zero, there was a finite intercept for the observed rate constant; this value would be the rate constant for the reverse of flavin oxidation. The saturating value of the observed rate constant (obtained at all concentrations) is the sum of the forward and reverse rate constants (18), but it is not yet possible to parse the contributions of each. Nonetheless, the fast reaction and tight binding indicate that at least one prior modification of the tRNA is very important for substrate recognition by the enzyme.

The same experiment was carried out using the C117A mutant of DUS 2. Reduced enzyme (15 $\mu$M) was mixed with 50 $\mu$M and 100 $\mu$M of partially matured tRNA in anaerobic cuvettes at 25 °C. Both reactions gave the same observed rate constant, indicating saturation with tRNA. The reaction was very slow; spectra were taken over several hours. The increase in absorbance at 450 nm with time (Fig. 5), was fit to an exponential, giving a rate constant of $8.7 \times 10^{-5}$ s$^{-1}$ ± $3 \times 10^{-6}$ s$^{-1}$, 1600-fold slower than the wild-type despite the 21° higher reaction temperature. This indicates that Cys-

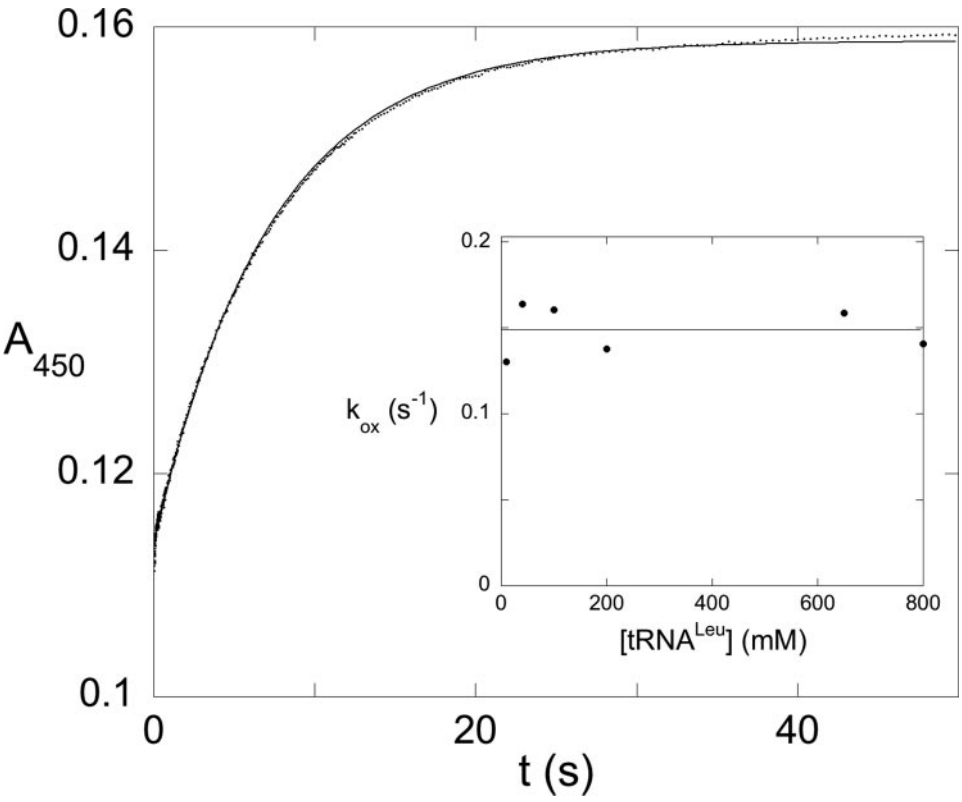



FIGURE 4. **Oxidation of DUS 2 by modified tRNA$^{Leu}$.** Anaerobic DUS 2 (20 $\mu$M) in 100 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol was reduced by titrating with dithionite. The reduced enzyme was reoxidized at 4 °C by mixing with increasing amounts of tRNA$^{Leu}$, up to 800 $\mu$M, in a stopped-flow spectrophotometer. The absorbance was monitored at 450 nm, and *traces* were fit to single exponentials. The *inset* shows concentration dependence for flavin oxidation by modified tRNA$^{Leu}$ yielding a rate constant of 0.14 ± 0.03 $s^{-1}$.

117 is very important for the reduction of tRNA, consistent with its proposed role as an active site acid. Interestingly, the rate constant is only enhanced 2-fold over that obtained with the *in vitro* transcribed pre-tRNA$^{Leu}$ and is ~2.5-fold slower than the intronless tRNA. This may indicate that the reason for the slow reaction rate in the *in vitro* transcribed substrate is improper positioning of the tRNA relative to Cys-117.

The importance of modifications in increasing the affinity of tRNA was corroborated by an observation from the purification of DUS 2 by affinity chromatography. The enzyme expressed in *E. coli*, purified by metal affinity chromatography, gave a single protein band on an SDS-PAGE. The absorbance spectrum of this preparation had peaks characteristic of a flavoenzyme at 370 nm and 450 nm, but also had a peak at 340 nm. This unusual chromophore was separated from the enzyme either by DEAE chromatography or by phenol-chloroform extraction. The compound isolated by phenol-chloroform extraction has the spectrum of a nucleic acid, with absorbance maxima at 260 nm and also at 340 nm, where 4-thiouridine absorbs. 4-Thiouridine is a minor constituent of tRNAs, indicating that tRNA is tightly bound to both the wild-type and C117A enzymes when purified by metal affinity chromatography. Similar results were obtained with the dihydrouridine synthase from *T. maritima*, and a chemical analysis in that case confirmed the presence of 4-thiouridine.[4]

[4] L. W. Rider, M. B. Ottosen, S. G. Gattis, and B. A. Palfey, unpublished results.

To identify which of the one or more tRNAs were bound to wild-type DUS, tRNA isolated from the enzyme was treated with a mixture of amino acids and aminoacyl-tRNA synthetases. The charged tRNA was purified, amino acids were hydrolyzed at basic, pH at 25 °C or 60 °C, and the free amino acids were determined by amino acid analysis (Fig. 6). Glycine constituted over 40% of the total. All other amino acids were detected, except for tryptophan, which is degraded by the amino acid analysis procedure. These results suggest either a strong preference for *E. coli* tRNA$^{Gly}$ over the other *E. coli* tRNAs or that there is a higher proportion of tRNA$^{Gly}$ in *E. coli*-expressing DUS 2.

The affinities of synthetic tRNAs were measured in fluorometric titrations. Binding to the oxidized enzyme caused a significant quenching of flavin fluorescence (Fig. 7*A*). Pre-tRNA$^{Leu}$ has a $K_D$ of 40 ± 1 $\mu$M, and the intronless Pre-tRNA$^{Leu}$ has a $K_d$ of 160 ± 40 $\mu$M, much higher than that of modified tRNA purified from *S. cerevisiae* (which was too low to be measured accurately) (Fig. 7*B*), indicating that modification of tRNA is important for the binding the DUS 2.

*Reverse Reaction with E. coli tRNA*—To investigate whether reduction of the tRNA could be reversed, bulk *E. coli* tRNA (90 $\mu$M) was treated with 40 $\mu$M DUS 2 aerobically for 18 h. Analysis of the tRNA showed a 78% decrease in dihydrouridine, indicating that the enzyme can oxidize dihydrouridine in tRNA to uridine, and the enzyme can be oxidized by oxygen. The presence of some dihydrouridine in tRNA after extended incubation with dihydrouridine synthases suggests a preference for certain tRNA substrates. *E. coli* tRNA has dihydrouridine modifications in several positions, so it is likely that the enzyme from yeast used to oxidize the tRNA mixture has a preference for only a subset of these modified positions.

The kinetics of enzyme reduction by dihydrouridine-containing tRNA from *E. coli* was studied at 4 °C, pH 7.5, with 20 $\mu$M enzyme (after mixing) in anaerobic stopped-flow experiments that recorded the flavin absorbance decrease at 450 nm (Fig. 8). Traces were fit to two exponentials. The first phase, which contributed the major absorbance decrease, had an observed rate constant that increased hyperbolically to a maximum rate constant for flavin reduction of 0.28 ± 0.03 $s^{-1}$. The second phase, which contributed a small absorbance change, gave a rate constant of 0.05 ± 0.001 $s^{-1}$. These data indicate reducing uracil on tRNA to dihydrouracil is reversible; *i.e.* the a redox potential of the bulk *E. coli* tRNA is close to or lower than

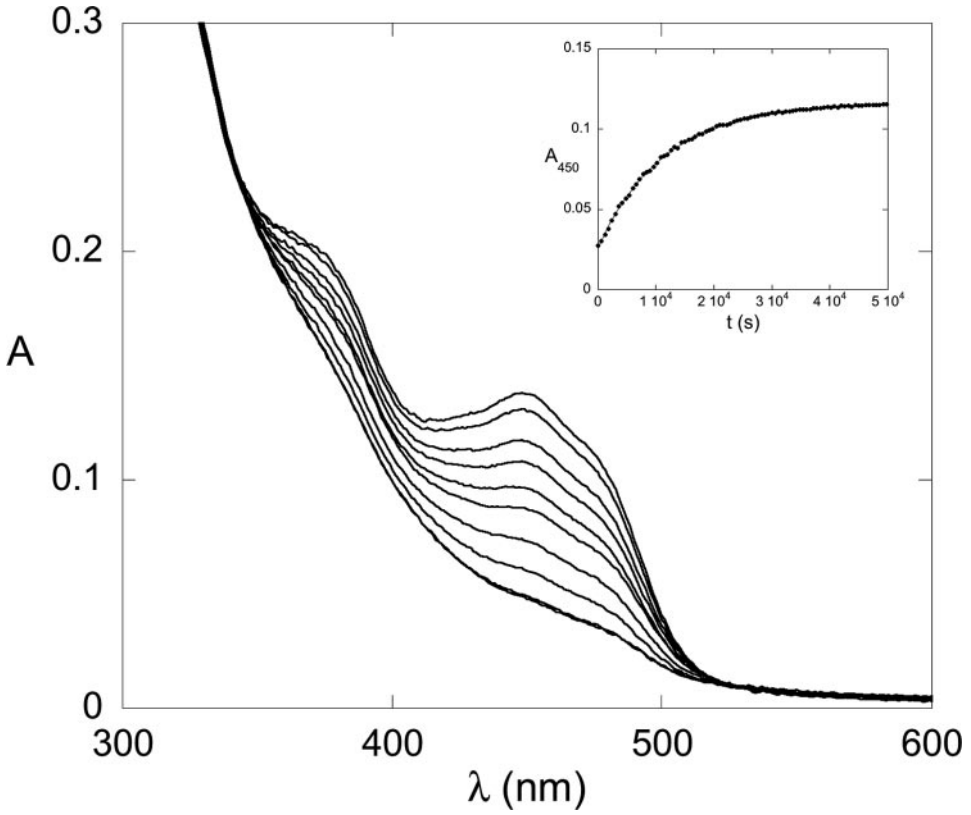



FIGURE 5. **Oxidation of the C117A mutant by modified tRNA$^{Leu}$.** Anaerobic enzyme (15 $\mu$M) in 100 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol was reduced by titrating with one equivalent of dithionite. The reduced enzyme was reoxidized by mixing with anaerobic modified tRNA$^{Leu}$. The *inset* shows the time dependence of flavin oxidation, detected at 450 nm. The *trace* was fit to a single exponential, giving a rate constant of $8.7 \times 10^{-5} \pm 3 \times 10^{-6}$ s$^{-1}$.

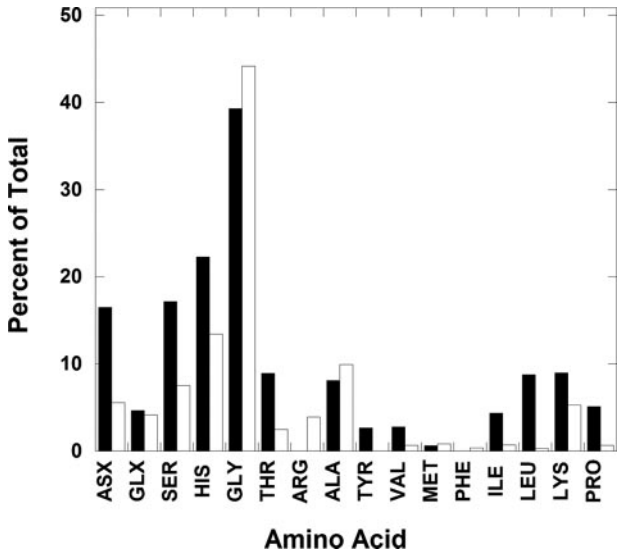



FIGURE 6. **Identification of tRNAs bound to enzyme.** tRNA was extracted with phenol-chloroform from the enzyme purified by metal-affinity chromatography and aminoacylated by *E. coli* tRNA synthetases. After isolating the charged tRNA from the small molecules, the amino acids were hydrolyzed from the tRNA at 25 °C (*white*) or 65 °C (*black*). The percentage of each was determined by amino acid analysis. The *plot* shows the percentage of each amino acid except for tryptophan and cysteine, which were not analyzable. Aspartate and asparagine are indistinguishable from one another by this method as are glutamine and glutamate. Almost all amino acids are present, with glycine being the major component at ~40%.

that of the enzyme. Note that it is important not to over-interpret these observed rate constants; they were obtained from an unfractionated mixture of mature *E. coli* tRNA and do not quantitatively represent the reaction of a pure yeast system.

*Oxidation of DUS 2 by Molecular Oxygen*—Oxidation of the enzyme by molecular oxygen was studied out in stopped-flow experiments at 4 °C by mixing reduced enzyme with buffer bubbled with various concentrations of oxygen (0–100%). Flavin oxidation was followed at 450 nm, and traces were fit to three exponentials. The second phase constituted the majority of the absorbance change and is attributed to oxidation, whereas the other rate constants had much smaller amplitudes. These are attributed to damaged enzyme or free FMN. The observed rate constant of the large phase showed a linear dependence on oxygen concentration, indicating a simple bimolecular reaction having a rate constant of $14\ \text{M}^{-1}\ \text{s}^{-1}$.

## DISCUSSION

Dihydrouridine, a modified nucleoside found in most forms of life, is synthesized by dihydrouridine synthases. These flavin-dependent enzymes use NADPH to reduce specific uracils in tRNA and are homologous to dihydroorotate dehydrogenases and dihydropyrimidine dehydrogenases (9). All of these enzymes use a flavin prosthetic group to transfer a hydride when either reducing the enone of the pyrimidine or oxidizing the dihydropyrimidine to the enone and have either a cysteine or a serine as an active site acid/base. Dihydroorotate dehydrogenases oxidize the dihydrouracil ring of dihydroorotate using FMN and either cysteine or serine as a base; their FMNs are oxidized by fumarate, quinones, or an iron-sulfur protein. Dihydropyrimidine dehydrogenases reduce uracil using FMN as the hydride donor, and an active site cysteine as an acid after the FMN is first reduced by an iron-sulfur cluster. In both cases, the chemistry of the half-reaction involving the pyrimidine is similar, and these enzymes use $\alpha/\beta$-barrel scaffolds and similar active site architectures to carry out the reactions.

There is significant sequence similarity between dihydroorotate dehydrogenases, dihydropyrimidine dehydrogenases, and known DUSs (9). Furthermore, the structure of an FMN-binding $\alpha/\beta$-barrel from *T. maritima* having significant similarity to dihydroorotate dehydrogenases and dihydropyrimidine dehydrogenases was determined (11). This protein was assigned by sequence homology as a DUS (21), and we have shown that it performs this function.[5] Its structure reinforces the analogy to the other,

[5] L. W. Rider, M. B. Ottosen, S. G. Gattis, and B. A. Palfey, unpublished data.

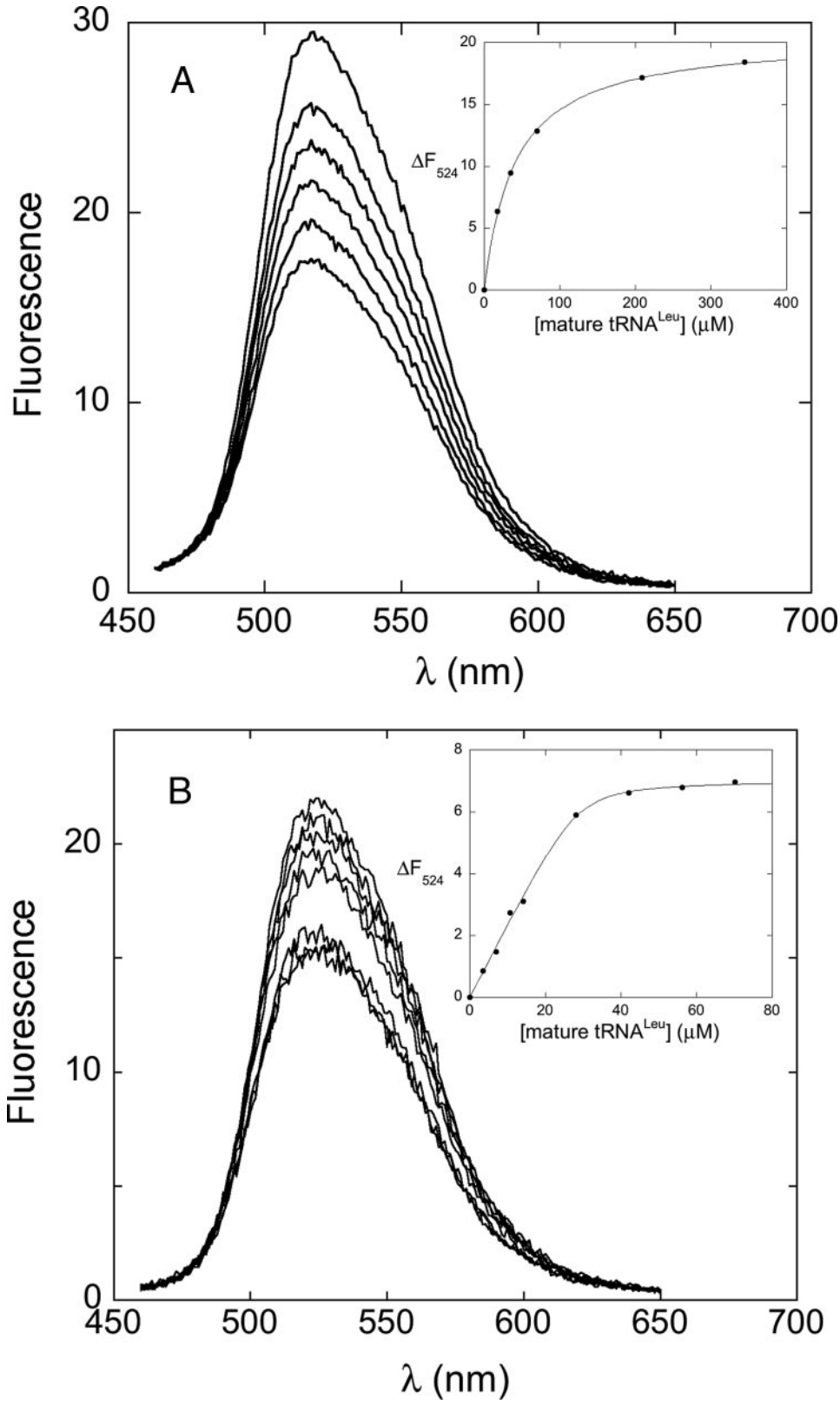



FIGURE 7. **Ligand Binding to DUS 2.** *A*, binding of pre-tRNA$^{Leu}$ to DUS 2. DUS 2 was titrated at 25 °C with increasing amounts of pre-tRNA$^{Leu}$ up to 380 $\mu$M. Upon addition of tRNA the fluorescence emission spectrum of the flavin was recorded (excitation 450 nm). The change in fluorescence *versus* the concentration (*inset*) gave a $K_D$ of 40 $\pm$ 1 $\mu$M. *B*, binding of modified tRNA$^{Leu}$ to DUS 2. DUS 2 was titrated with increasing amounts of modified tRNA$^{Leu}$ up to 68 $\mu$M. Upon addition of tRNA the fluorescence emission spectrum of the flavin was recorded (excitation at 450 nm). The change in fluorescence *versus* the concentration was fit to Equation 2 for tight binding to give a rough $K_D$ of 0.8 $\pm$ 0.5 $\mu$M.

more thoroughly characterized pyrimidine-metabolizing flavoproteins. Therefore it seemed likely that DUSs would use a similar mechanism for catalysis, involving two half-reactions. In the reductive half-reaction, NADPH would bind to the oxidized enzyme, a hydride would be transferred to N5 of FMN, and NADP would then dissociate (Fig. 1). In the oxidative half-reaction, tRNA would bind to the reduced enzyme and the hydride from FMN would reduce C6 of the uracil ring accompanied by a proton transfer from an active site acid to C5. Our results show that DUS 2 from *S. cerevisiae* conformed to this template for the catalytic cycle.

In the reductive half reaction, the enzyme was reduced by the *pro-R* hydrogen of NADPH with a rate constant of 2.5 $s^{-1}$, which is fast enough to be physiologically relevant. The kinetic isotope effect of 3.5 for flavin reduction indicates that the chemistry for this step is at least partially rate-limiting and is similar to the reductive half-reactions of many flavoenzymes.

In the oxidative half-reaction, tRNA oxidizes the reduced flavin, accomplishing the presumed physiological role of the enzyme. Synthetic tRNA, used in most studies of tRNA-modifying enzymes, is a poor substrate for this enzyme. The $K_D$ value was higher than that found for many tRNA-modifying enzymes, and, even at saturation, the reaction was extremely slow. Interestingly, tRNA co-purifies with the enzyme, which would require a very low $K_D$ value, strongly suggesting that modifications of the tRNA strengthen binding.

An estimate of the minimum rate DUS 2 that must sustain normal cellular dihydrouridine levels can be made from published data. *S. cerevisiae* has at least $\sim 1.5 \times 10^6$ tRNA molecules per cell (22). This number of tRNA molecules must be made each generation (neglecting turnover). Yeast has a doubling time of 1.5–2 h. This means $1.5 \times 10^6$ tRNAs would need to be modified every 120 min, or ~200 per second. In *S. cerevisiae*, *in vivo* DUS 2 spec-

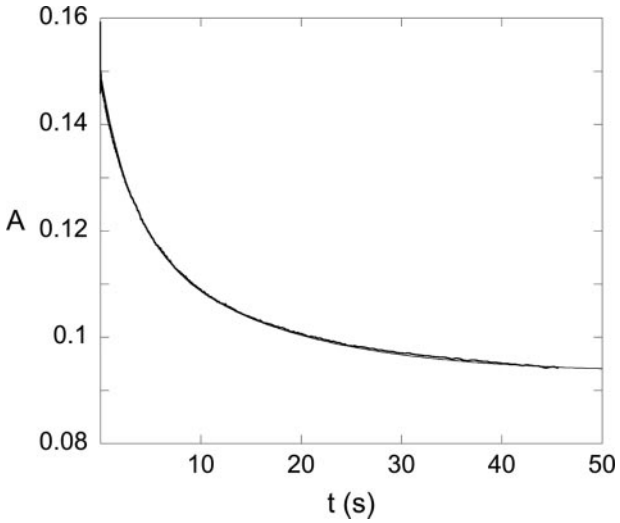
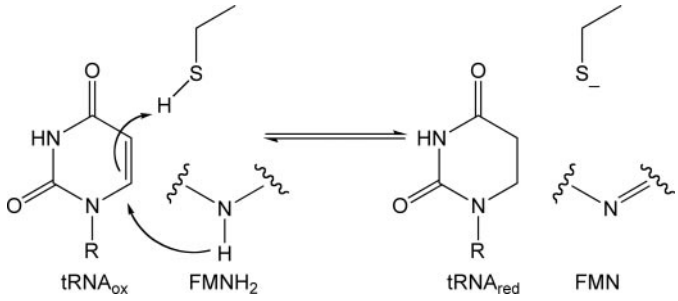



FIGURE 8. **Reduction of *S. cerevisiae* DUS 2 with *E. coli* tRNA.** Anaerobic DUS 2 (20 $\mu$M) in 100 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, was mixed with anaerobic solutions of *E. coli* tRNA in a stopped-flow spectrophotometer at 4 °C. A reaction *trace* at 450 nm is shown. The fit gives an observed rate constant for the first phase of flavin reduction of 0.28 ± 0.03 $s^{-1}$, contributing to the majority of the absorbance change. The second phase shows a much smaller absorbance change and gives a rate constant of 0.05 ± 0.001 $s^{-1}$.




SCHEME 1

ificity studies show DUS 2 should modify all the tRNAs in the cell once. DUS 2 has a copy number of ~2,650 proteins per cell (23), which means it must modify ~0.080 tRNA molecules per second, giving a lower threshold for how fast the overall catalytic cycle, and each of its steps, must be. The reductive half-reaction of *S. cerevisiae* DUS 2 easily meets this physiological requirement. However, the oxidative half-reaction with an *in vitro* transcribed tRNA was not fast enough to meet our estimated physiological requirement. Interestingly, a specific activity was reported for DUS 1 from *S. cerevisiae* with *in vitro* transcribed tRNA (10). When this number (22,200 units/mg) is converted to a turnover number, a very low value, ~$10^{-7}$ $s^{-1}$, is obtained.

All available data, therefore, indicate that purified DUSs reduce synthetic tRNA too slowly to accomplish their demonstrated physiological roles. However, modified tRNA reacts much more rapidly, with an observed rate constant of 0.14 ± 0.03 $s^{-1}$, ~600 times faster (although determined at a temperature 21 °C lower) than the corresponding *in vitro* transcribed tRNA. This is much faster than our estimate for the minimum value necessary to support physiology. The large difference between synthetic and processed tRNA indicates there is a critical need for one or more modifications in order for it to be recognized as a substrate and is consistent with the observation that tRNA is tightly bound when the enzyme is purified from *E. coli*. Co-purification requires a very low $K_D$, yet *in vitro* transcribed tRNAs have dissociation constants of tens of micromolar or higher.

The identity of the necessary modification is not yet known. If this modification causes tight RNA binding, then it apparently is in both the partially mature $tRNA^{Leu}$ from *S. cerevisiae* and the mature *E. coli* tRNA that co-purifies with overexpressed DUS 2. Six modifications have been reported (1) that are common to tRNA from *S. cerevisiae* and *E. coli*: N4-acetylcytidine, 5-methylcytidine, 1-methylguanosine, 2′-*O*-methylguanosine, pseudouridine, and ribothymine. Interestingly, knockouts of the Trm3 enzyme lack not only methylated guanosine position 18, but also lack dihydrouridine at position 20 (24), making it perhaps the best candidate for the critical tRNA modification. In contrast, the ~6-fold increase in the RNA reduction rate in the absence of the intron in synthetic substrates suggests that the intron plays only a small role in substrate recognition.

It is also possible that DUSs might act *in vivo* in a complex with one or more protein partners. The recent findings that the human DUS 2 interacts with a multifunctional glutamyl-prolyl-tRNA-synthetase complex (7) supports this hypothesis. However, no protein partners for the *E. coli* or *S. cerevisiae* DUSs have yet been identified, and our data present no reason to invoke such a complex.

All DUSs have a conserved cysteine. Sequence and structural similarities to dihydroorotate dehydrogenases and dihydropyrimidine dehydrogenases imply that this cysteine will act as the acid during the reduction of the uracil ring. The importance of this cysteine was demonstrated for the *E. coli* DUS ybjN (20) by transforming a knockout strain with the C117A mutant, causing a clear loss in dihydrouridine content in tRNA isolated from this strain. However, the oxidation of the C117A DUS 2 mutant with synthetic tRNA gave rates similar to those for the wild-type enzyme, indicating that the active site cysteine is not important in this half-reaction when reducing synthetic substrates. In contrast, when using modified tRNA from *S. cerevisiae*, a dramatic difference (~1600×) in the rate constants for the reactions of the wild-type and C117A mutants is seen. These reactions were carried out at different temperatures, with the reactions for the C117A mutant being 21 °C higher than those used for the wild-type enzyme; the reaction rate with the C117A mutant enzyme would be even slower at 4 °C, where wild-type was studied. These data are therefore consistent with Cys-117 protonating the uracil ring during reduction of tRNA (Scheme 1). Interestingly, our results indicate that Cys-117 cannot act as a general acid unless tRNA has at least one critical modification, suggesting that the modification is needed to properly orient the reactive uracil in the active site.

## CONCLUSIONS

DUS 2 from *S. cerevisiae* is reduced by the *pro-R* hydride of NADPH. It uses Cys-117 as a general acid in the reduction of tRNA as the hydride from reduced FMN is transferred to the uracil ring. Unlike most tRNA-modifying enzymes studied to

date, rapid reaction requires at least one prior modification of the tRNA substrate. This necessity for modification indicates an ordered sequence of tRNA modifications and suggests that dihydrouridine formation may be one of the later steps in the process.

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