Summer Scholar Report

In vitro Study of Human Ribonucleotide Reductase Enzymatic Activity and Assembly of Diferric-Tyrosyl Radical Cofactor

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0Introduction

Ribonucleotide reductases (RNRs) catalyze the conversion of nucleotides to deoxynucleotides in all organisms and play an essential role in DNA replication and DNA repair.[1] Because of their central role, RNRs are also successful targets of several drugs used clinically in the treatment of a number of malignancies. Structurally, human RNR (hRNR) consists of two subunits. The H1 subunit binds nucleoside diphosphates (NDPs) and the dNTP/ATP allosteric effectors. The H2 subunit houses the $Fe^{III}Fe^{III}$-tyrosyl radical cofactor required to initiate inter-subunit radical propagation (>35Å) that leads to thiyl radical generation at the active site on H1 to catalyze NDP reduction.[1,2] Mechanism-based inhibitors (MBIs), such as Gemcitabine (Gemzar®, F_2C, Figure 1a) have been utilized to successfully probe RNR catalytic activity.[3] Clofarabine (Clolar®, ClF, Figure 1b), a prodrug indicated for treatment of leukemia, is a nucleoside analog proposed to target RNR.[4] Elucidating the chemical inactivation mechanism of human RNR by Clolar® is of critical interest to further understand both the chemistry of RNR and the clinical efficacy of Clolar®. Prior to studying Clolar®, it was first necessary to purify and characterize active hRNR H1 and H2 subunits. Unlike *E. coli* RNR, much remains unknown about hRNR; specifically the stability of the diferric-tyrosyl radical cofactor of human H2 versus *E. coli* β is not well understood and presents a challenge to conducting *in vitro* studies. Efforts were made to improve protocols for both the purification of hRNR and the *in vitro* reassembly of H2 active cofactor. Reported here are (1) improvements to the purification of H2, (2) a reproducible method for *in vitro* assembly of the $Fe^{III}Fe^{III}$-tyrosyl radical (Y•) cofactor, (3) preliminary stability studies of the Y• under physiological conditions (pH 7.6, 37°C), and (4) an alternative synthesis of Clolar 5'–monophosphate from Clolar®.

Results and Discussion

Purification of H1 and H2 subunits of Human RNR: Recombinant human $(His)_6$–H1 and $(His)_6$–H2 were expressed in *E. coli*, yielding 1.2 mg/L culture and 3.8 mg/L culture respectively. [5] Poor protein yield and purity prompted purification optimization efforts, which resulted in improved yield, specific activity, and purity.[6] Use of Talon column allowed the removal of Arna, a 74 kDa *E. coli* protein, that previously co-purified with H1.[6] Talon was thus used for the purification of $(His)_6$–H2 to >90% homogeneity, as judged by 10% SDS–PAGE (Figure 2). As–isolated H2 lacks fully active diferric-Y• cofactor required for nucleotide reduction. This cofactor must be assembled *in vitro* following purification. H1 was purified by a similar procedure. The specific activity (S.A.) of H1 (590–700

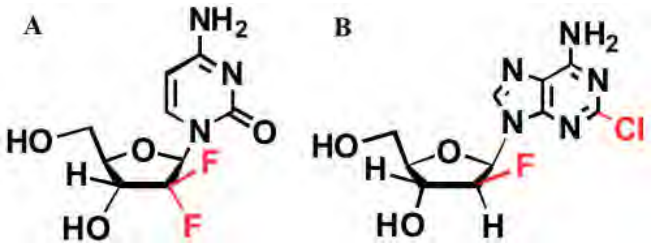

Figure 1. (a) Gemzar® (F_2C) (b) Clolar® (ClF).

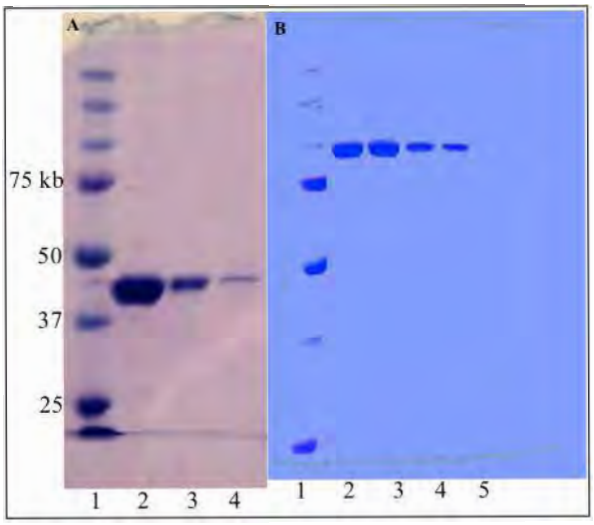

Figure 2. *10% SDS PAGE Analysis of Purified H2 (A) and H1 (B) Following Talon Affinity Chromatography Purification. The numbered lanes correspond to (1) Molecular Weight Markers, (2) 2.0 µg purified protein, (3) 1.0 µg purified protein, (4) 0.5 µg purified protein.*

nmol/min/mg) was measured using [5–^{3}H] CDP for the formation of 2'–deoxycytidine 5'–diphosphate (dCDP) over time in the presence of the ATP allosteric effector and thioredoxin/thioredoxin reductase/NADPH reducing system. *In vitro Assembly (Reconstitution) of H2 Diferric-Y• Cofactor:* The inherent instability of as–isolated mammalian subunits (hRNR H2 and mouse RNR M2) compared to *E. coli* β present a significant challenge to all *in vitro* experiments. The *in vitro* Y• half–lives for *E. coli* RNR β subunit and mouse RNR M2 subunit have previously reported to be on the order of several days and 10 min, respectively.[7] A purification and reconstitution protocol has previously been developed for *E. coli* RNR β subunit that allows study of the stoichiometry and time–scale of the tyrosine oxidation reaction.[8] Using this protocol, addition of Fe^{2+} in the presence of O_2 to the purified apo *E. coli* β subunit spontaneously leads to assembly of the diferric center and oxidation of Y122 (Equation 1). A modified protocol has recently devel-

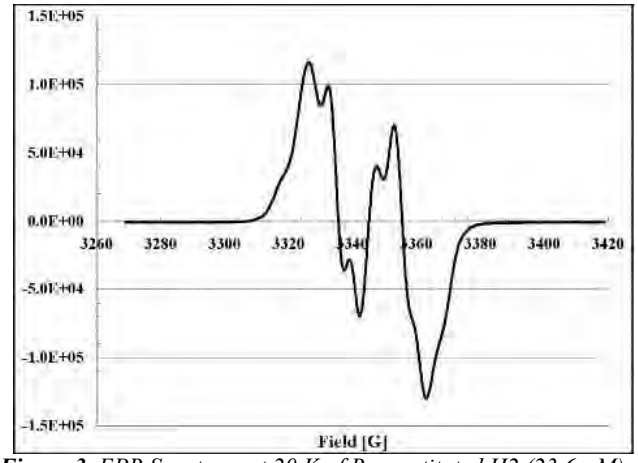

Figure 3. *EPR Spectrum at 20 K of Reconstituted H2 (23.6 μM). The diferric-tyrosyl radical cofactor of H2 was assembled in vitro. EPR spin-quantitation was used to quantify the tyrosyl radical content per H2 dimer. EPR parameters: 9.37 GHz microwave frequency, 0.5 mW modulation amplitude, 100 kHz modulation frequency, 15 scans, DI/N 79.5, DI/Nc=1.85, Y•/H2=$[Y•]_{std}$* $(DI/Nc)_{sample}/(DI/Nc)_{std}$/[H2, dimer]=1.0 Y•/H2 dimer.*

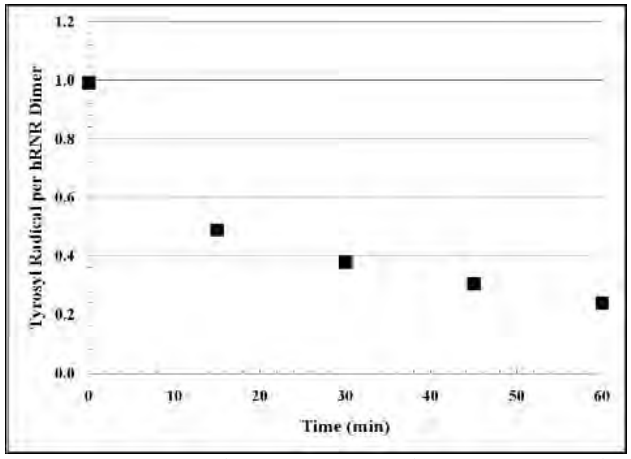

Figure 4. *Time–Dependent in vitro H2 Y• decay. Reconstituted H2 (1.0 Y•/H2 dimer) was incubated at 37°C. At 0, 15, 30, 45, and 60 minute time points 200 μL aliquots were transferred to EPR tubes and immediately flash frozen in liquid N_2 for EPR spin-quantitation of Y•/dimer.*

oped for the reconstitution of the H2 active cofactor. However, the difficulties in reproducibly generating active cofactor were noted in initial studies. Prior to studying putative mechanism based inhibitors (MBI) of hRNR, it is critical to quantify the assembly and stability of the H2 subunit. Here characterizations of the as–isolated and reconstituted hRNR H2 subunit are reported.

Equation 1: $Y\text{-}OH + 2Fe^{2+} + 3/2O_2 + e^- + 3H^+ \rightarrow \bullet O\text{-}Y + Fe^{3+}\text{-}O\text{-}Fe^{3+} + 2\,H_2O$

Previous reports of the activity of the reconstituted diferric-Y• cofactor of H2 *in vitro* yielded 0.8 Y•/H2 dimer and S.A of 1089 nmol/min/mg and 75 nmol/min/mg, respectively. [5,9] I sought to provide a reproducible method for regenerating $(His)_6$–H2 cofactor to the theoretical 1.0 Y•/H2 dimer by first characterizing the as–isolated H2's iron loading, tyrosyl radical content, and specific activity. The iron content of the hRNR H2 subunit[5] as–isolated was measured using a standard ferrozine–based colorimetric assay. Ferrozine binds ferrous iron, but not ferric iron, and forms a complex that absorbs strongly at 562 nm (ε_{562}=27870 $M^{-1}cm^{-1}$). [10] The specific activity of H2 was measured by radioactive assay and Y• content per H2 dimer was measured using EPR spin–quantitation and gave 1.4-1.6 iron equivalents (equiv)/dimer, 0.2 Y•/H2 dimer, and S.A. of 900–1250 nmol/min/mg. With this knowledge in hand, *in vitro* reconstitution was systematically investigated. One Y•/H2 dimer (Figure 3) following *in vitro* reconstitution was achieved reproducibly by reducing the amount of iron equivalents incubated with the H2 in the glove–box at 4°C and alteration of the addition rate of Fe^{2+} to adjust for the as–isolated protein not being in apo form, and to account for potential obligatory conformation changes that regulate iron binding. The reconstituted $(His)_6$–H2 subunit had 3.4 iron equiv/dimer, 1.0 Y•/H2 dimer, and S. A. of 2100–2400 nmol/min/mg.

While working towards developing a reproducible *in vitro* reconstitution method, a publication came out reporting the isolation of H2 (3.1 iron equiv/dimer, 1.23 Y•/H2 dimer, and S.A. of 6000 nmol/ming/mg) without the *in vitro* assembly of cofactor.[11] These results were obtained by overexpression of $(His)_6$–H2 subunit in *E. coli*, harvesting the cell pellet, cell lysate preparation with Bug-buster and Benonase incubation, purification with Ni-NTA resin with elution of protein from the resin by gravity, dialysis of eluate overnight, followed by concentration and activity measurements. Since the half life of the Y• is 25 min, dialysis overnight should leave little radical. In my hands, the report from this group was irreproducible; the $(His)_6$–H2 subunit with 0.6 iron equiv/dimer, no detectable Y• and S.A. of 158 nmol/min/mg. Thus, we used our optimized protocol.

I conducted a preliminary study of the *in vitro* half-life of the H2 Y• 1.0 Y•/dimer, SA 2100 nmol/min/mg) by monitoring its time–dependent decay at 37°C, and pH 7.6. The half–life was 25 minutes (Figure 4). The instability of the human H2 subunit *in vitro* requires that the decay of Y• and the specific activity be monitored during all *in vitro* inhibition experiments to correct for the spontaneous enzyme decay. This *in vitro* half–life of human H2 contrasts with reported *in vitro* half–lives of *E. coli* and mouse β.

Alternative Preparation of ClFMP from Clolar®: Recent work noted difficulty purifying ClFMP which was generated enzymatically from Clolar® and ATP with HdCK. [6] This procedure yielded an equilibrium mixture of starting material and products: ClFMP and ADP. A multi–step DEAE anion exchange chromatography was utilized to isolate ClFMP. I investigated an alternative two–step method for the purification of ClFMP from ADP, which, as found previously, coeluted on anion exchange chromatography at 350 mM triethylammonium bicarbonate (TEAB). A periodate cleavage step was introduced to destroy ADP, using a protocol previously reported. [12] Sodium periodate selec-

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tively reacts with the cis–diol of the sugar of ADP to cleave the 2'–C-3'–C bond, generating a dialdehyde. The ClFMP is unreactive. After removal of excess periodate, excess methyl amine (pH 7.5) is added to form iminium ions, leading to the elimination of pyrophosphate. Inorganic pyrophosphatase irreversibly converts the inorganic pyrophosphate to inorganic phosphate. Anion exchange chromatography using a linear gradient from 0–600 mM TEAB (pH 6.8) allowed recovery of homogenous ClFMP (eluted at 350 mM TEAB).
Study of Inhibition of E. coli α Subunit by Clofarabine 5'–diphosphate in Presence of 10 Fold Molar Excess β: Preliminary progress curves for dCDP formation showed possible biphasic time-dependent inhibition. These studies suggested that ClFDP may be a slow-binding, reversible inhibitor of *E. coli α* RNR subunit (Figure 5).

Materials and Methods: *General:* Clofarabine was purchased from AK Scientific. The pET–9d expression vector for human deoxycytidine kinase (His_6–HdCK) was obtained in *E. coli* BL21 (DE3) pLysS strain as a gift from Dr. Staffan Eriksson. The purification of *E. coli* thioredoxin (TR, 40 units/mg) [13] and *E. coli* thioredoxin reductase (TRR, 1400 units/mg), [14] HdCK (S.A. 150 nmol/min/mg measured by spectrophotometric assay using pyruvate kinase and lactate dehydrogenase) and UMP–CMP kinase (4.8 μmol/mg/ min by the [γ-^{32} P]ATP phosphate transfer assay) have previously been described. [15] UV–vis absorption spectra were obtained and spectrophotometric assays were carried out using a Cary 3 UV–vis spectrophotometer (Varian, Walnut Creek, CA). X–band EPR spectra were acquired using a Bruker EMX spectrometer (Bruker, Madison, WI).
Isolation and Characterization of E. coli RNR α and β Subunits: Wild type *E. coli* RNR $(His)_6$–α subunit (S.A. 1600-2000 nmol/min/mg) was purified and pre–reduced as previously described; [16] protein concentration was determined using ε_{280nm} = 189 $mM^{-1}cm^{-1}$(6). Wild type *E. coli* $(His)_6$–β subunit was over-expressed in *E. coli* using recombinant technology and purified from cellular extracts by affinity chromatography, Ni^{2+}–NTA resin, as previously reported. [17,18] The diferric-tyrosyl-Y• cofactor was assembled *in vitro* as previously described. [17] Protein concentration was determined using ε_{280nm} = 131 $mM^{-1}cm^{-1}$. [19] Specific activity (6000–7000 nmol/min/mg) was measured by radioactive and NADPH coupled spectrophotometric assay. Y• content (1–1.2 radicals per dimer) was measured both by the drop-line correction spectroscopy method and by EPR spectroscopy, as previously reported. [8] EPR spectra were acquired using a Bruker EMX X–band spectrometer at 77 K equipped with a quartz finger dewar and at 20 K using an Oxford Instruments liquid helium cryostat (9.38 GHz Microwave Frequency, 1 mW Microwave Power, 1 Gauss Modulation Amplitude). Radical content was quantified against a standard solution of 1 mM $CuSO_4$ in 50 mM EDTA by double integration of spectra registered at non-saturating microwave levels by standard Bruker software.

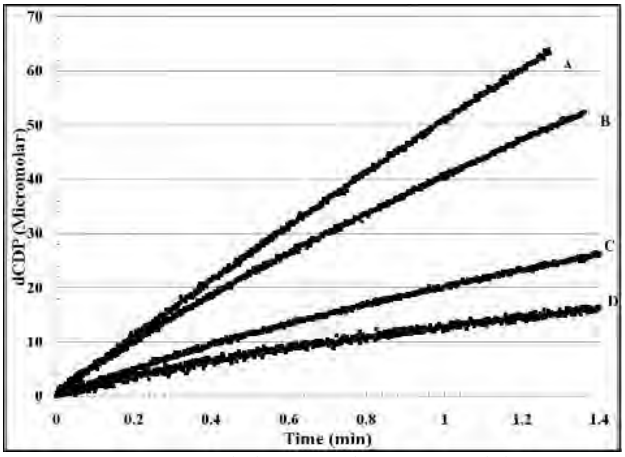

Figure 5. *NADPH Coupled Spectrophotometric Inhibition Assay, Biphasic Time-Dependent Inhibition of E. coli α RNR subunit by ClFDP at 25°C (A: 0 μM ClFDP, B: 20 μM, C: 40 μM, D: 80 μM). The reaction mixture (300 μL: 200 μM NADPH, 1 mM CDP, 3 mM ATP, 30 μM TR, 0.5 μM TRR, 50 mM Hepes (pH 7.6), 15 mM $MgCl_2$, 1 mM EDTA, 0.2μM α , and 2μM β) without CDP (1 mM final concentration, saturating substrate conditions) or ClFDP (0-80 μM final concentration) was pre-incubated at 25°C for 1 min. CDP/CLDP was added and reduction of absorbance at 340 nm was continuously monitored for 1.5 minutes after addition; 1 nmol of NADPH oxidized per minute corresponds to 1 nmol of dCDP formed per minute.*

Isolation and Characterization of Human RNR H1 and H2 Subunits: The $(His)_6$–H1 and $(His)_6$–H2 subunits were purified using a modified protocol reported previously. [5] Talon (Clontech) resin was used instead of Ni-NTA, and a dATP affinity column was used as a second step to achieve higher purity, higher specific activies and reduced purification time.
In vitro Assembly of Human RNR H2 Active Cofactor, Diferric Y•: Human $(His)_6$–H2 subunit (50 μM) in 500 μL of 50 mM Hepes (pH 7.6), 100 mM KCl, 10% glycerol was deoxygenated by six cycles of evaluation (for 3X10 s) followed by argon flushing using standard Schlenk line technique. The deoxygenated $(His)_6$–H2 solution was brought into the glove–box (M. Braun, Stratham, NH) and stored at 4°C. Incrementally over a 15 min period 3 equivalents of Fe (II) (deoxygenated ferrous ammonium sulfate in 50 mM Tris (pH 7.6) and 100 mM KCl were added; the concentration of Fe(II) was determined by ferrozine assay. [10] The resulting mixture was incubated at 4°C for an additional 15 min. The protein was then removed from the glove–box and 170 μL (8-fold excess of 3.5 equiv/dimer required) of O_2(g) saturated buffer was added and O_2(g) was blown over the surface of the protein solution. Excess iron was removed by Sephadex G25 chromatography (40 mL, 2.5 X 30 cm). An activity assay in the presence of seven–fold molar excess human $(His)_6$–H1 subunit was carried out, and 250 μL of the protein solution was transferred to an EPR tube and frozen in liquid N_2 for EPR spin–quantitation of Y•/dimer.
Radioactive and Spectrophotometric Assays: Measurement of E. coli and Human RNR SA: The reduction of CDP by *E. coli* and human RNR was assayed by measuring the oxidation of NADPH coupled to dCDP formation and the forma-

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tion of radioactive dCDP from [5–^{3}H] CDP. In the NADPH oxidation method, the disappearance of $A_{340\ nm}$ was followed continuously using a Cary 3 spectrophotometer (Varian). The following were incubated in a final volume of 300 µL: 200 µM NADPH, 1 mM CDP, 3 mM ATP, 30 µM TR, 0.5 µM TRR, 50 mM Hepes (pH 7.6), 15 mM $MgCl_2$, 1 mM EDTA, 2 µM (or 0.2µM) α , and 0.2 µM (or 2µM) β. The reaction mixture was pre-incubated at 25°C for 1 min. The subunit being assayed in 10-fold molar excess of the other subunit was added to initiate the reaction. Initial velocities were measured and used to calculate nmol of NADPH oxidized per min; 1 nmol of NADPH oxidized per min corresponds to 1 nmol of dCDP formed per min. [20] For the radioactive assay method, a reaction mixture contained in a final volume of 210 µL: 50 mM Hepes (pH 7.6), 15 mM $MgCl_2$, 1 mM EDTA, 0.3 µM (or 3 µM) α, 3 µM (or 0.3 µM) β, 3 mM ATP, 1 mM [5–^{3}H] CDP (S.A. 5926 cpm/nmol,ViTrax, Placentia, CA) 30 µM *E. coli* TR, 0.5 µM TRR, and 1 mM NADPH. The assay mixture was pre-incubated at 37°C for 2 min, and the reaction was initiated by the addition of [5–^{3}H] CDP. Aliquots, 30 µL each, were removed over a 10 min time period and quenched in a boiling water bath for 2 min. dC production was analyzed, subsequent to dephosphorylation with alkaline phosphatase as previously described, [21] and analyzed by the method of Steeper and Steuart. [10] The reduction of CDP by hRNR was measured only by the formation of [5–^{3}H] dCDP. The NADPH coupled spectrophotometric assay could not be used to measure the specific activity of hRNR subunits or the holoenzyme, since the reduction of absorbance at 340 nm versus time is not appreciable compared to the background to allow for specific activity quantitation.

Time–Dependent Decay of Human H2 Y· at 37°C and pH 7.6: Reconstituted human H2 (1.0 Y•/dimer, SA 2100 nmol/min/mg) was incubated at 37°C. Aliquots (200 µL) were transferred to EPR tubes at 0, 15, 30, 45, and 60 min and samples were immediately flash frozen in liquid N_2 for EPR spin-quantitation of the Y•/dimer.

Clofarabine to ClFMP: The reaction mixture contained in a final volume of 9 mL: 1 mM Clofarabine, 5 mM ATP, 2 mM DTT, 0.5 mg/mL BSA, 0.1 mg/mL HdCK, 50 mM Tris (pH 7.6), 100 mM KCl, and 10 mM $MgCl_2$. The reaction was initiated by the addition of hdCK and the mixture incubated at 37°C for 45 min. The reaction mixture was loaded on a DEAE-Sephadex A-25 column (20 mL X 20 cm X 1 cm) equilibrated with 5 mM TEAB (pH 6.8) and the column washed with 50 mL of 5 mM TEAB. The product was eluted using a 150 mL X 150 mL linear gradient from 5 to 400 mM TEAB. Fractions (5 mL) were assayed for A_{260nm} and A_{280nm}; the nucleotide containing fractions were combined and the solvent was removed *in vacuo*. ClFMP eluted at 350 mM TEAB. ^{31}P NMR and ^{1}H NMR revealed contaminating ADP in addition to the ClFMP product. ClFMP was purified by oxidative cleavage of ADP with periodate followed by pyrophosphatase treatment to convert liberated pyrophosphate into inorganic phosphate as previously reported.[12] Following this step, a second DEAE anion exchange chromatography step with the same aforementioned gradient was used to purify ClFMP.

ClFMP to ClFDP: This synthesis is reported in a publication under review. [6]

Spectrophotometric Assay to Study Inhibition of E. coli α by ClFDP in Presence of 10 Fold Molar Excess β: The reaction mixture contained in a final volume of 300 µL: 200 µM NADPH, 1 mM CDP, 3 mM ATP, 30 µM TR, 0.5 µM TRR, 50 mM Hepes (pH 7.6), 15 mM $MgCl_2$, 1 mM EDTA, 2 µM (or 0.2µM) α, and 0.2 µM (or 2µM) β. The reaction mixture without CDP (1 mM final concentration) or ClFDP (0-80 µM final concentration) was pre-incubated at 25°C for 1 min. CDP/ClDP was added and the reduction of absorbance at 340 nm was continuously monitored for 1.5 minutes after addition; 1 nmol of NADPH oxidized per minute corresponds to 1 nmol of dCDP formed per minute. [20]

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