CHEMPHYSCHEM

Supporting Information

Supplementary Information

Tuning of the H-Transfer Coordinate in Primitive vs. Well-Evolved Enzymes

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MATERIALS AND METHODS

Materials

Reagent-grade chemicals were used as received unless specified otherwise. β-Nicotinamide [U-^{14}C]adenine dinucleotide (50 μCi) was purchased from Amersham Pharmacia (specific radioactivity >220 mCi/mmol), and Congo Red was obtained from Aldrich. Alcohol dehydrogenase from *Thermoanaerobium brockii* (*tb*ADH), and 2-propanol-d_8 were purchased from Sigma. 7,8-Dihydrofolate (DHF) was prepared by dithionite reduction of folic acid as described by Blakely.[1]

Enzyme Preparation. R67 DHFR was expressed, purified, lyophilized as described elsewhere.[2]

Methods

Synthesis of Labeled Cofactors for KIEs.

(*R*)-[4-^{3}H]-NADPH, (*S*)-[4-^{3}H]-NADPH and [Ad- ^{14}C]NADPH were synthesized as described elsewhere.[3-5] (*R*)- [Ad- ^{14}C] -[4-^{2}H]-NADPH was prepared by stereospecific reduction of $NADP^+$ with 2-propanol-d_8 (> 99.8% D at C2 as determined by ^{1}H NMR) using *tb*ADH. All synthesized cofactors were purified by reverse-phase HPLC on a

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Supelco Discovery C18 column as described previously and lyophilized for long-term storage at -80 °C.

Determination of stereospecificity of R67 by stereospecifically labeled NADPH

Being a primitive enzyme, R67 has presented binding promiscuity, and utilizes α-NADP and thio-NADPH as cofactors.[6] Consequently, it was not clear at first whether R67 is as highly stereospecific as highly evolved enzymes. Low stereospecificity would require special treatment and would make accurate determination of KIEs more difficult, as the stereospecificity together with the 1° and 2° KIEs would have to be solved simultaneously.

We stereospecifically labeled the hydrogens at the 4 position of NADPH with ^{3}H, (T), and conducted the R67 DHFR reaction to determine stereospecificity by examination of the radioactivity in the products. For (*S*)-[4-^{3}H]-NADPH, the *pro-R* hydrogen on NADPH was transferred to the reaction product, tetrahydrofolate (H_4F), and all radioactivity remains on $NADP^+$. While using (*R*)-[4-^{3}H]-NADPH, T on *pro-R* position was transferred, and all radioactivity was found at THF. The radioactivity at $NADP^+$ was undetectable within experimental error (<0.1%) and the same as for cDHFR, which was used as control with the same labeled substrates. As a conclusion, R67 DHFR is a type A DHFR that stereospecifically transfer *pro-R* hydrogen from NADPH.

Experimental procedure: The R67 DHFR enzymatic reaction was conducted with 200 μM of DHF and 4 μM of NADPH containing cold NADPH and trace amount of 4*R* [4-^{3}H] NADPH or 4*S* [4-^{3}H] NADPH in MTEN buffer (50 mM 2-morpholinoethanesulfonic

acid, 25 mM Tris, 25 mM ethanolamine, and 100 mM NaCl) at pH 8.0. All reactions were carried under steady state conditions to complete the NADPH consumption. After completed enzyme reaction, aliquots were immediately frozen at -80 °C on dry ice. Prior to the HPLC analysis, the sample was thawed, and oxygen was gently bubbled into the reaction mixture for 15 min to convert THF into its oxidized form.[5,7] Aliquots were then injected onto an HPLC equipped with a Supelco reverse phase C18 column, and the eluent was analyzed by Packard flo scintillation analyzer, or Liquid scintillation counter (LSC).[5]

Co-purification of isotopically labeled NADPH for Competitive KIE Experiments.

[Ad-^{14}C]NADPH and (*R*)-[4-^{3}H]- NADPH (H/T experiments) or (*R*)- [Ad-^{14}C, 4-^{2}H]NADPH and (*R*)-[4-^{3}H]- NADPH (D/T experiments) were combined at a radioactivity in DPM ratio close to 1:6 (^{14}C/^{3}H), compensating for the lower efficiency of tritium scintillation counting. Each of the mixtures was copurified by reverse-phase HPLC on a Supelco Discovery C18 column (25 cm x 4.6 mm, 5 *m*L), divided into aliquots containing 360,000 DPM of ^{14}C, and flash frozen for short-term storage (up to 3 weeks) at -80 °C.

Competitive Kinetic Isotope Effects (KIEs). One aliquot of the copurified NADPH was thawed just before use. DHF was added to the reaction mixture to a final concentration of 200 μM (approximately 50-fold excess over the 4 μM total NADPH). The final volume was brought to 990 μL by adding MTEN, and the pH was readjusted to 8.0 at the experimental temperature. Before starting the enzyme reaction, two 80 μL of samples (t_0) were withdrawn for quality control. The reaction was initiated by addition of R67 DHFR

enzyme solution, and reacted for up to 40 min. At various time points, 80 μL of aliquots which have varied fractional conversions (*f*) ranging from 15 to 85% as determined from the distribution of ^{14}C between NADPH and $NADP^+$, were withdrawn, and quenched with 20 μl of Congo Red (final concentration of 5 mM). To ascertain complete fractional conversion, *c*DHFR (approximately 0.2 unit) was added to the residue of the mixture, and two aliquots for t_∞ samples were quenched after 20 min additional incubation. All quenched samples were immediately frozen and stored at -80 °C.

HPLC Analysis

The detailed HPLC analysis followed ref. 5. In short, prior to HPLC-LSC analysis, the samples were thawed and oxidized through bubbling of oxygen for 15 min at room temperature. The samples were then injected into the reverse-phase HPLC system. Fractions (0.8 mL) were collected, mixed with 10 mL of Ultima Gold liquid scintillation cocktail (PerkinElmer), and stored in the dark for 24 h before radioactivity determination in LSC analysis (Packard Tricarb Tr2900 LSC).

The fractional conversion (*f*) of NADPH was determined from the ratio of ^{14}C in the product to the total amount of ^{14}C.

$$f = \frac{[\text{Ad-}^{14}\text{C}]\text{NADP}^+}{[\text{Ad-}^{14}\text{C}]\text{NADP}^+ + [\text{Ad-}^{14}\text{C}]\text{NADPH}}$$

The observed KIEs were calculated according to the following equation.[8]

$$KIE = \frac{\ln(1-f)}{\ln\left[1-f\left(\frac{Rt}{R\infty}\right)\right]}$$

where R_t is the ratio of ^3H to ^{14}C in products at various fractional conversions, and $R_¥$ is ratio at complete conversion from infinite time samples. Experiments at one temperature point were performed at least in triplicate. The isotope effects for the observed KIEs were plotted with Arrhenius plot for KIEs and fitted with exponential regression using KaleidaGraph.

$$\frac{kl}{kh} = \frac{Al}{Ah} e^{\Delta Ea_{h-l} / RT}$$

where *l* and *h* are the light and heavy isotopes.

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