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Stereospecificity of Hydride Transfer For the Catalytically Recycled NAD^+ in CDP-D-glucose 4,6-Dehydratase

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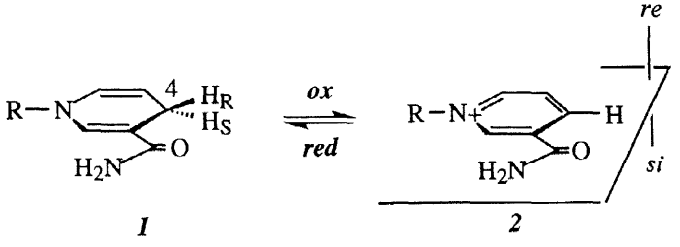
Abstract: CDP-D-glucose 4,6-dehydratase (E_{od}), found in the biosynthetic pathway of 3,6-dideoxysugars, contains a tightly bound NAD^+ that is recycled during catalysis. The stereochemical preference of the hydride transfer to and from the coenzyme in E_{od} was determined to be *pro-S* by analyzing the NAD^+ produced when the apoenzyme was incubated with stereospecifically labeled NADH and its product, CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose.

Introduction

The ability of enzymes to catalyze transformations with strict stereospecificity has fascinated scientists for decades. One area where this is most evident is with the pyridine nucleotide coenzymes NAD and NADP. The methylene hydrogens at C-4 of the dihydronicotinamide ring of NAD(P) are diastereotopic and can be differentiated by enzymes that are NAD(P)-dependent. A catalyst from this class that mediates the transfer of the 4-H to or from the *re* face of the nicotinamide ring is commonly referred to as *pro-R* stereospecific, while transfer to or from the *si* face is designated *pro-S* stereospecific. Ever since this stereochemical stringency was recognized by Fisher *et al.* in 1953,[1] immense efforts have been devoted to the determination of the stereospecificities of numerous enzymes. As a result, hydride transfer mediated by the NAD(P)-dependent oxidoreductases represents the best studied example of the amazing ability of enzymes to differentiate prochiral atoms and/or functional groups, leading to a specific stereochemical outcome for each catalysis. Information on the stereochemical preference for a given enzyme is essential for gaining insights into its reaction mechanism and its evolutionary correlation with other related catalysts.[2a] This information is also critical for deducing the substrate/coenzyme binding orientation in the enzyme active site.[2a] Interestingly, the preference for *pro-R* or *pro-S* stereospecificity is generally evenly distributed among the enzymes examined thus far.[2a]



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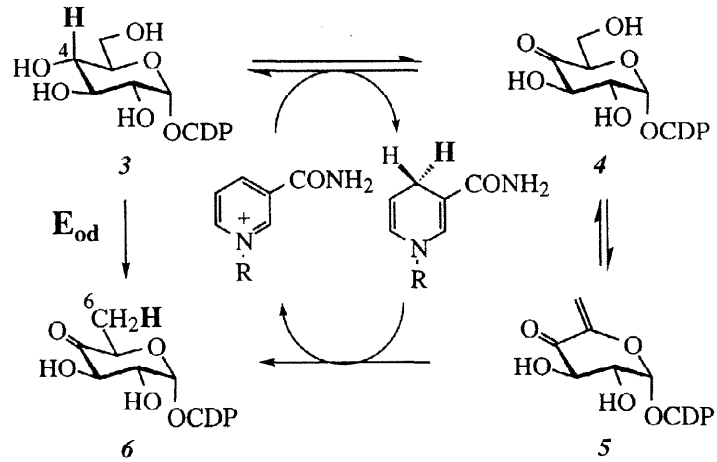
Methods for determining the stereospecificity of the hydride transfer in NAD(P)-dependent enzymes have been well established. Since the pyridine nucleotide coenzyme is in fact acting as a co-substrate in most of these reactions, isolation and characterization of the resulting NAD(P) product is relatively straightforward. For example, in the reduction direction, NAD(P)H (**1**) isotopically labeled at either the *pro-R* or *pro-S* position at C-4 can be incubated with the substrate and enzyme, so that the presence of the isotope at C-4 in the resulting $NAD(P)^+$ (**2**) may be determined by either ^1H NMR, mass spectrometry, or scintillation counting, depending on the isotope used. Alternatively, in the oxidation direction, the stereospecificity of hydride transfer can be analyzed by using NAD^+ labeled at C-4 or by using the appropriately labeled substrate in the incubation. The position of the isotope in the NADH product can then be located by oxidizing it with an enzyme of known stereospecificity and analyzing the resulting NAD^+ for the presence of the isotope.

Even though most NAD(P)-dependent enzymes use the pyridine nucleotide coenzyme as a co-substrate, there does exist a special group of enzymes that contain a tightly bound NAD^+ in which the pyridine nucleotide coenzyme is recycled during each catalytic cycle.[2] An example of such an enzyme is CDP-D-glucose 4,6-dehydratase (E_{od}) of *Yersinia pseudotuberculosis,* a homodimeric enzyme from the biosynthetic pathway of 3,6-dideoxysugars.[3] These unusual sugars are found almost exclusively in the lipopolysaccharide of Gram-negative bacteria and are well known antigenic determinants that contribute to the serological specificity of many immunologically active polysaccharides.[4] The C-6 deoxygenation catalyzed by the E_{od} class of enzymes is one of the best characterized biological C-O cleavage events[5] and is necessary for the biosynthesis of all 6-deoxyhexoses.[3]



Various experiments have shown that this conversion consists of three discrete steps as illustrated above: oxidation of CDP-D-glucose (**3**) to the corresponding 4-ketohexose **4**, C5/C6 dehydration to a 4-keto-$\Delta^{5,6}$-glucoseen intermediate **5**, and reduction at C-6 to give the 4-keto-6-deoxyhexose product **6**.[5] This elegant transformation is in fact an intramolecular oxidation-reduction that involves an internal hydrogen transfer from C-4 of the substrate **3** to C-6 of the resulting product **6**. An enzyme-bound NAD^+ is essential for the E_{od} transformation. This cofactor serves as a hydride carrier receiving the 4-H of **3** as a hydride in the oxidative half-reaction and passing the reducing equivalents to C-6 of the dehydration product **5** in the reductive half-reaction. The fact that NAD^+ is regenerated after each catalytic cycle indicates that the pyridine nucleotide coenzyme is

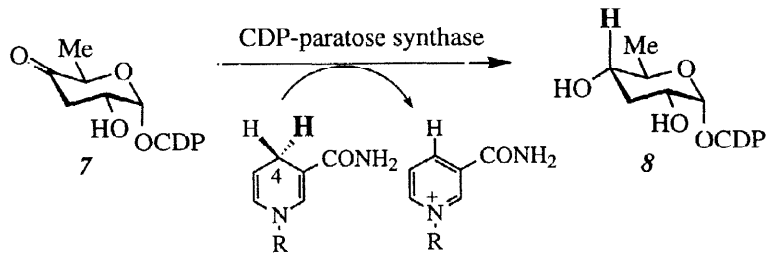
indeed a catalytic prosthetic group. By using stereospecifically labeled substrates, the overall displacement of the C-6 hydroxyl group by the 4-H has been demonstrated to occur with inversion of configuration.[5]

To complete the stereochemical analysis of E_{od}, the stereospecificity of the hydride transfer with regard to NAD^+ must also be defined. Knowing the stereochemical course of hydride transfer will shed light on the structure of the active site, especially the orientation of the nicotinamide ring relative to the substrate, and will also be helpful in designing strategies to control/regulate this important reaction. Since the formation of NADH during E_{od} catalysis is only transient, analysis of the stereochemical preference is much more difficult than for most NAD(P)-dependent enzymes that use NAD(P) as a co-substrate. The fortuitous discovery that E_{od} can use NADH to reduce its own product, CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose (**6**), permitted the stereochemical course of the hydride transfer to and from NAD^+ to be determined in this case. Reported herein is the summary of our attempts to resolve this challenging problem, and the mechanistic implications deduced from the *pro-S* stereospecific outcome.

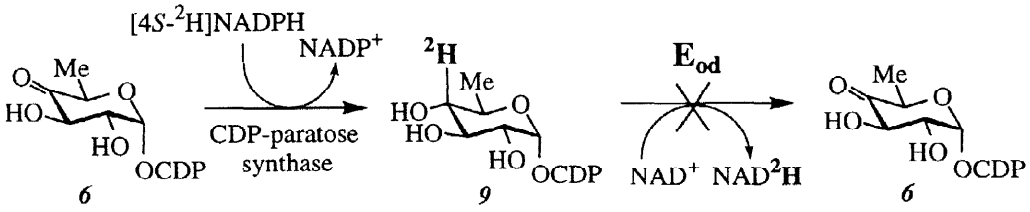
Results and Discussion

The stereochemical preference for the hydride transfer to and from the NAD^+ coenzyme has been determined in only a few enzymes that use $NAD(P)^+$ catalytically.[2a] One such example is TDP-D-glucose 4,6-dehydratase of *Esherichia coli*,[6] which catalyzes the formation of TDP-6-deoxy-D-*glycero*-L-*threo*- 4-hexulose from TDP-D-glucose using NAD^+ in an oxidation-reduction process via a mechanism virtually identical to that of E_{od}. The stereospecificity of the hydride transfer step of this enzyme was cleverly determined by Wang and Gabriel using [4-^{3}H]-TDP-6-deoxy-D-glucose as the substrate in a single turnover event. Since C-6 was already deoxygenated, the product generated after C-4 oxidation in their experiment was identical to the final product of a normal E_{od} reaction. Thus, the reaction was terminated after the initial oxidation, and the resulting NADH remained reduced at the end of the incubation. As a result, the isotopic label of the substrate was transferred to C-4 of the accumulated NADH, which, upon release from the active site, allowed the facial selectivity of isotope incorporation to be determined in TDP- D-glucose 4,6-dehydratase as "*si* face" specific.

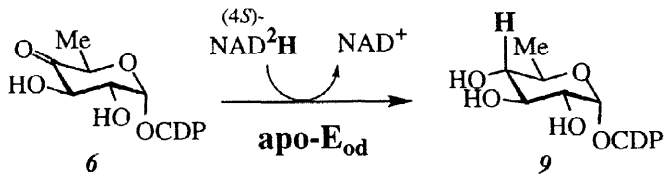
In light of the close resemblance of E_{od} to TDP-D-glucose 4,6-dehydratase of *E. coli*, a similar approach was attempted to determine the facial selectivity of hydride transfer in E_{od}. Synthesis of CDP-6-deoxy-D-glucose labeled with deuterium at C-4 was performed enzymatically by paratose synthase from *Salmonella typhi*, which has been cloned, overexpressed, and purified previously in our lab.[7] Even though this enzyme normally catalyzes



the reduction of CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**7**) to CDP-paratose (**8**), our earlier work showed that it can also act on the E_{od} product, CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose (**6**) to generate CDP-6-deoxy-D-glucose (**9**).[7] The use of [4*S*-^{2}H]NADPH in the enzyme reaction provided the desired labeled compound **9**. It is worth mentioning that this enzymatic preparation of labeled **9** is a much more convenient and cleaner reaction than the procedure used by Wang and Gabriel.[6] However, when **9** was incubated with E_{od}, no NADH could be detected in the reaction mixture by FPLC. Instead, a large amount of starting material was recovered. It should be noted that compound **9** is a competitive inhibitor for E_{od} since the enzyme activity was completely inhibited in the presence of excess **9** (three molar equivalents to substrate concentration). Thus, it appears that, unlike TDP-D-glucose 4,6-dehydratase from *E. coli*, E_{od} from *Y. pseudotuberculosis* either does not process the 6-deoxy substrate analogue, or the equilibrium of this reaction greatly favors **9** and not the accumulation of NADH.

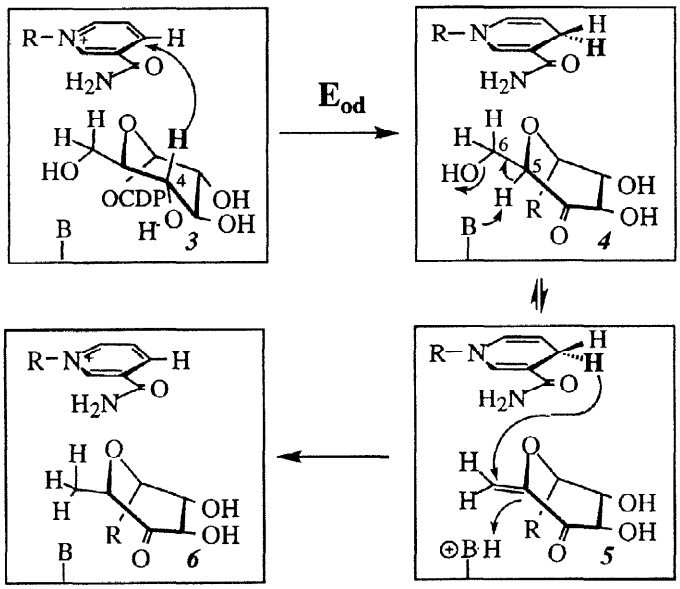


Meanwhile, it was discovered in a separate experiment that E_{od} can use NADH to reduce its own product, CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose (**6**), to CDP-6-deoxyglucose (**9**). It has been shown earlier that E_{od} contains not only bound NAD^+ needed for catalysis, but also a significant amount of tightly bound NADH.[8] The co-existence of NADH- and NAD^+-containing species complicates the kinetic characterization of E_{od} catalysis; however, the presence of NADH also inadvertently allowed the observation of the bleaching of NADH at 340 nm in the presence of E_{od} product. This finding enabled the facial selectivity of hydride transfer from NAD^+ in E_{od} to be determined by an entirely different strategy. Crucial to this approach was the availability of an established method to prepare apoenzyme[8] that could later be reconstituted with NADH specifically labeled at either the *pro-R* or *pro-S* position. In the actual experiment, apo-E_{od} and E_{od} product were incubated with either [4*S*-^{2}H] or [4*R*-^{2}H]NADH. The reaction was boiled to release the resulting NAD^+, which was purified by FPLC and analyzed by ^{1}H NMR. The signal for H-4 at δ 8.65 was retained in the NAD^+ sample derived from the incubation with [4*S*-^{2}H]NADH, indicating the transfer of the *pro-S* deuterium during turnover. On the contrary, incubation with the [4*R*-^{2}H]NADH resulted in the retention of the deuterium on the nicotinamide ring, since the δ 8.65 signal was absent, thus confirming the hydride transfer is *pro-S* stereospecific.

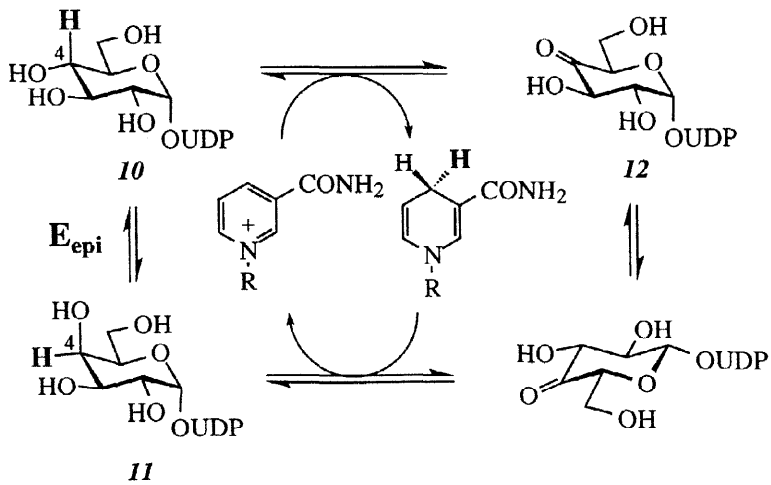


With these results, it can be surmised that the substrate is bound to the *si* face of the nicotinamide ring in the active site and the facial selectivity of hydride transfer is "*si* face" specific. The stereochemical course of the E_{od} reaction can now be summarized as illustrated below. Initially, E_{od} catalyzes the transfer of the C-4 hydrogen

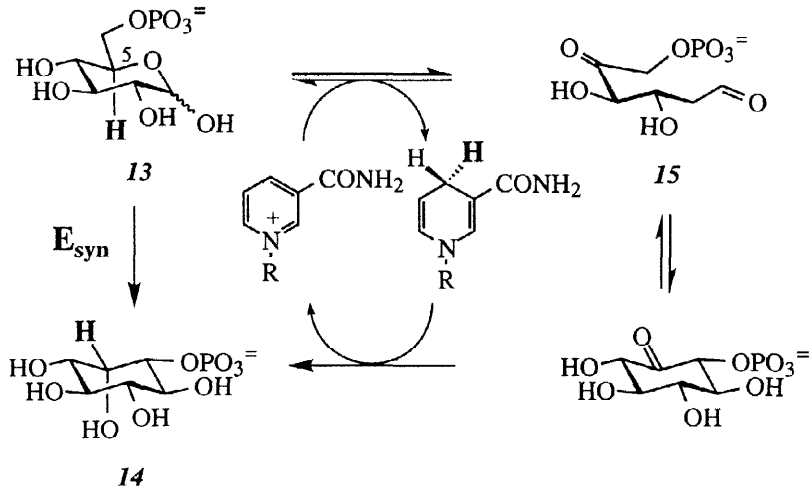
from **3** to the *si* face of the nicotinamide ring, followed by dehydration to give the intermediate **5**. The *pro-S* hydrogen of the dihydronicotinamide is then returned as a hydride to C-6 to give the product **6**. Since the nicotinamide cofactor serves as a hydride carrier in this catalysis, the intramolecular hydrogen transfer from C-4 to C-6 must occur suprafacially. The completed stereochemical analysis of E_{od} provides a more comprehensive picture of the mechanism and active site of this important enzyme.



Other NAD(P)$^+$ regenerating enzymes whose stereochemical preference have been assigned include UDP-D-glucose 4-epimerase. This is an NAD$^+$ dependent enzyme that promotes the interconversion between UDP-D-glucose (**10**) and UDP-D-galactose (**11**) via a 4-ketohexose intermediate (**12**). Nelsestuen and Kirkwood[9] determined that chemical reduction of the tightly bound NAD$^+$ in this enzyme with NaB^3H_4 resulted in NAD^{3}H with the majority of tritium at the *pro-S* position. When this reduced enzyme complex was incubated with the 4-ketohexose intermediate **12**, the isotopic label was quantitatively transferred to the TDP-sugars, thus demonstrating that UDP-D-glucose 4-epimerase is a *pro-S* specific catalyst.



Another interesting example is the study of L-*myo*-inositol-1-phosphate synthase, which catalyzes the isomerization of glucose 6-phosphate (**13**) to L-*myo*-inositol-1-phosphate (**14**) by way of a 5-ketoglucose 6-phosphate intermediate (**15**). Due to the reversibility of the initial oxidation step, it was possible for Byun and Jenness[10] to observe tritium transfer to glucose-6-phosphate when [4*S*-^{3}H]NADH was added to the enzyme reaction. Since very little radioactivity was detected in the substrate when [4*R*-^{3}H]NADH was used, the hydride transfer catalyzed by L-*myo*-inositol-1-phosphate synthase is also *pro-S* stereospecific.



It is interesting to note that all of the enzymes discussed above share three common features; they use NAD^+ catalytically, act on sugar substrates, and are "*si* face" specific with respect to the transfer of one of the diastereotopic methylene hydrogens at C-4 of the nicotinamide nucleotide coenzyme. It is possible that these enzymes may have evolved from a common progenitor whose catalytic core has persevered throughout the enzyme's subsequent diversification. However, any conclusions about whether the *pro-S* preference is a general characteristic for this class of enzymes will have to await further results. With the addition of the method used for CDP-glucose 4,6-dehydratase, there have now been at least four different approaches used for defining the facial selectivity for four different enzymes that use NAD^+ catalytically. The fact that each individual enzyme required a separate method of analysis accentuates the complexity of determining the stereospecificity of the hydride transfer for this type of enzyme.

Experimental

General Methods. The NMR spectra were acquired on a Varian Unity 300 spectrometer. CDP-glucose 4,6-dehydratase was purified from *E. coli* HB101-pJT8 according to the previously reported procedure.[11] CDP-paratose synthase was purified from *E. coli* BL21(DE3)-pTHS-3 as previously described.[7] Apo-E_{od} was obtained by a previous method.[8]

Preparation of [4S-^{2}H]NADPH: The 4 mL incubation mixture contained 26 μmol of $NADP^+$, 276 μmol of D-[1-^{2}H]glucose, 8 units of glucose dehydrogenase (Sigma), and 100 mM potassium phosphate buffer, pH 7.4.[12] The reaction was incubated for 1 h at room temperature, and the conversion was monitored by the production of [4*S*-^{2}H]NADPH at 340 nm. The [4*S*-^{2}H]NADPH was used without further purification since earlier attempts at

purification by FPLC with a MonoQ HR (10/10) column resulted in considerable degradation. The purity and chirality were determined by ^{1}H NMR analysis.

Preparation of CDP-6-deoxy-D-glycero-L-threo-4-hexulose (6). The 1 mL incubation mixture contained 49 μmol of CDP-D-glucose (**3**), 300 units of E_{od}, and 20 mM Tris-HCl, pH 7.5. The reaction was incubated at room temperature for 1 h, and the generation of the product, CDP-6-deoxy-D-glycero-L-threo-4-hexulose (**6**), was monitored spectrophotometrically at 320 nm under alkaline conditions.[13] The E_{od} protein was removed with a Centricon 10 microconcentrator.

Preparation of CDP-6-deoxy-D-[4-^{2}H]glucose (9). Initial efforts towards reducing E_{od} product, CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose (**6**), with paratose synthase and the unpurified [4*S*-^{2}H]NADPH were unsuccessful. It was later found that removal of the glucose dehydrogenase with a Centricon 10 microconcentrator (Amicon) is necessary before adding CDP-paratose synthase. The filtrate, containing 14 μmoles of [4*S*-^{2}H]NADPH calculated spectrophotometrically at 340 nm (ε = 6220 $M^{-1}cm^{-1}$), was incubated with 14 μmol of CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose (**6**) and 3.5 mg of paratose synthase for 1 h at room temperature, and the conversion was monitored by following the consumption of [4*S*-^{2}H]NADPH spectrophotometrically at 340 nm. The product was purified by FPLC using a MonoQ HR (10/10) column with a gradient where solvent A is water and solvent B is 0.5 M NH_4HCO_3. The linear gradient ranged from 0 to 35% B over 30 min, followed by a final wash with 100% B for 5 min. Using a flow rate of 3 mL/min, the CDP-6-deoxy-D-[4-^{2}H]glucose (**9**) was eluted at 17.5 min. Purified **9** was lyophilized and stored at -20 °C. ^{1}H NMR (D_2O, 300 MHz) δ 7.76 (1 H, d, *J* = 7.6 Hz, cytidine H-6), 5.93 (1 H, d, *J* = 7.6 Hz, cytidine H-5), 5.79 (1 H, d, *J* = 4.2 Hz, cytidine H-1'), 5.33 (1 H, dd, *J* = 6.6, 3.3 Hz, H-1), 4.17-4.00 (5 H, m, cytidine H-2', 3', 4', 5'), 3.76 (1 H, dq, *J* = 9.6, 6.3 Hz, H-5), 3.51 (1 H, dd, *J* = 9.6, 9.6 Hz, H-3), 3.40 (1 H, m, $J_{2,3}$ = 9.6 Hz, H-2), 2.96 (1 H, dd, *J* = 9.6, 9.6 Hz, H-4), 1.06 (3 H, d, *J* = 6.3 Hz, 5-Me).

Incubation of E_{od} and CDP-6-deoxy-D-[4-^{2}H]glucose (9): The 7 mL incubation mixture contained 2.2 μmol of CDP-6-deoxy-D-[4-^{2}H]glucose (**9**), 4 μmol of NAD^+, 90 mg of E_{od}, and 100 mM potassium phosphate buffer, pH 7.5. The reaction was incubated for 2 h at room temperature and subsequently boiled for 4 min to denature the E_{od} enzyme. The precipitated protein was removed by centrifugation, and the presence of NADH in the supernatant was analyzed by FPLC using a MonoQ HR (10/10) column with a gradient where solvent A is water and solvent B is 0.5 M NH_4HCO_3. The linear gradient ranged from 20 to 80% B over 30 minutes followed by a final wash with 100% B for 5 min.

Preparation of [4R-^{2}H]NADH: The 5 mL incubation mixture contained 1.2 mg of yeast alcohol dehydrogenase, 47 μmol of NAD^+, 19 μmol of [1,2-$^2H_5$]CH_3CH_2OH, and 100 mM potassium phosphate buffer, pH 8.5.[14,15] The reaction was incubated at 37 °C for 1 h and the progress of the reaction was monitored by the production of [4*R*-^{2}H]NADH at 340 nm. The protein was removed by a Centricon 10 microconcentrator, and the product was purified by FPLC using a MonoQ HR (10/10) column with a gradient where solvent A is water and solvent B is 0.5 M NH_4HCO_3. The gradient ranged from 0 to 50% B over 20 min followed by a 5 min wash with 100% B. Using a flow rate of 3mL/min, the [4*R*-^{2}H]NADH was eluted at 16 min. The purified [4*R*-^{2}H]NADH was lyophilized and the purity was analyzed by ^{1}H NMR.

Preparation of [4S-^{2}H]NADH: The 5 mL incubation mixture contained 43 μmol of NAD^+, 1.8 mg of lipoamide, 18 mg of dithiothreitol, 0.9 mg of lipoamide dehydrogenase, and 50 mM NH_4HCO_3 made with ^2H_2O.[16] The pH was maintained between 8 and 9 by the addition of NaO^2H. The progress of the conversion was monitored by the production of [4S-^{2}H]NADH at 340 nm. The protein was removed by a Centricon 10 microconcentrator and purified by FPLC as described for the [4R-^{2}H]NADH. The purified [4S-^{2}H]NADH was lyophilized and the purity was analyzed by ^{1}H NMR.

Determination of Stereospecificity of Hydride Transfer from NADH Mediated by E_{od}. The stereospecificity of E_{od} was determined by incubating 0.7 μmol of apo-E_{od} with 1.8 μmol of CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose (**6**), 3 μmol of either [4S-^{2}H] or [4R-^{2}H]NADH, and 100 mM potassium phosphate buffer, pH 7.5, for 1 h at room temperature. The progress of the reaction was monitored by the consumption of NAD^2H spectrophotometrically at 340 nm. The reactions were boiled for 4 min to denature the protein and release the NAD^+. The precipitated protein was removed by centrifugation, and the supernatant was purified by FPLC as described for the CDP-6-deoxy-D-[4-^{2}H]glucose (**9**). Using a flow rate of 3 mL/min, the NAD^+ was eluted at 7.5 min. The purified NAD^+ was lyophilized and analyzed by ^{1}H NMR.

Acknowledgment

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