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J. Am. Chem. Soc., **2003**, 125 (21), 6348-6349• DOI: 10.1021/ja021487+ • Publication Date (Web): 06 May 2003

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Published on Web 05/06/2003

Reversal of the Apparent Regiospecificity of NAD(P)H-Dependent Hydride Transfer: The Properties of the Difluoromethylene Group, A Carbonyl Mimic

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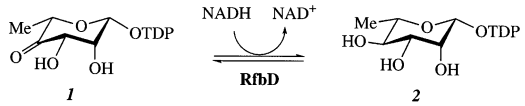
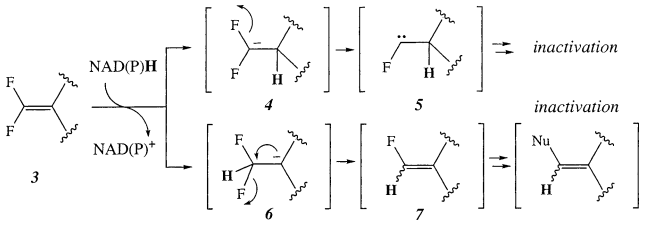
Pyridine nucleotide-dependent oxidoreduction reactions are among the most extensively studied enzymatic reactions. A great number of dehydrogenases of this class, which catalyze the interconversion of a carbonyl and a hydroxyl group, are known with a broad structural range of substrate specificities and diverse catalytic roles.[1] Factors that determine the stereoselectivity of this class of enzymes have been extensively studied.[2] However, little consideration has been given to the related issues that govern the regioselectivity of catalysis. This lack of attention may stem from the fact that hydride addition always occurs at the carbonyl carbon when a keto-substrate is reduced with NAD(P)H. This strict conservation of regiospecificity is predicated by the chemical nature of the keto group and the close proximity of the hydride donor and acceptor.[3] As a part of our ongoing efforts to develop inhibitors for TDP-L-rhamnose synthase, which catalyzes the conversion of TDP-6-deoxy-L-*lyxo*-4-hexulose (**1**) to TDP-L-rhamnose (**2**), we examined the properties of an analogue of **1** in which the difluoromethylene group replaces the carbonyl group. Interestingly, we noted that the enzyme catalyzes a hydride transfer to the difluoromethylene appendage in this analogue (**8**) at the difluorinated end, opposite from the site predicted on the basis of the reduction of a normal keto functional group. This observation is significant because it represents the first example showing that the regiospecificity of hydride transfer catalyzed by a pyridine nucleotide-dependent enzyme can be changed by altering the electrochemical properties of the reaction center. The results are reported herein.



1 *2*

The initial rationale for our inhibitor design is based on the premise that a difluoromethylene group (**3**) is a potential isostere of the carbonyl group due to the comparable electronegativity of fluorine and oxygen, and the capability of both atoms to serve as hydrogen-bond acceptors.[4] As illustrated in Scheme 1, the enzyme-catalyzed hydride reduction of **3**, if it follows the regiospecificity of normal catalysis, would lead to a carbanion intermediate (**4**). However, beause of the repulsion (I_π) between the electron pair of the anionic center and those of the fluorines,[4,5] formation of this α-fluoro-substituted carbanion (**4**) is not favored. Such a repulsive interaction can be alleviated by releasing a fluoride ion from **4**. This α-elimination may be facilitated by the H-bond networking in the active site, and the nascent reactive carbene species (**5**) could covalently modify a proximal residue and inactivate the enzyme. In contrast, hydride addition to the terminal carbon of **3** would generate a β-fluorinated carbanion **6** which can be stabilized inductively by the fluorine electron-withdrawing effect,[4,5] and also by the negative hyperconjugation.[6] In fact, it is well established

Scheme 1



that nucleophilic addition to fluoroalkenes prefers a route in which the number of fluorines β to the electron-rich carbon in the transition state is maximized.[7] Thus, an opposite regioselective addition based solidly on chemical precedence may occur for the hydride reduction of **3**. As shown in Scheme 1, elimination of a fluoride ion from **6** would yield an electrophilic intermediate **7** which could trap an active site nucleophilic residue, resulting in enzyme inactivation. To test this idea, we synthesized TDP-4,6-dideoxy-4-difluoromethylene-L-*lyxo*-hex-4-enopyranose (**8**) and examined its effect on the catalysis of the purified TDP-L-rhamnose synthase (RfbD).[8] This dehydrogenase is NADH-dependent, and its catalysis involves the hydride transfer of the 4′-H_S of NADH to reduce the 4-keto group of **1** to form TDP-L-rhamnose (**2**).

Compound **8** was prepared starting from methyl L-rhamnose and was isolated as a mixture of α and β anomers (**8α**:**8*β*** = 3:1).[9] Because of the instability of **8** upon further purification and lyophilization, this mixture was used directly in the subsequent experiments.[10] When TDP-L-rhamnose synthase (1 μM) was incubated with excess **8** (6.1 mM of the α/β mixture), no irreversible inactivation was detected. On the contrary, the enzyme recognizes **8** as a substrate, and catalysis follows Michaelis−Menten kinetics with a $k_{cat} = 12.7 \pm 3.8$ min^{-1} and a $K_m = 40.1 \pm 17.0$ mM in 50 mM potassium phosphate buffer (pH 7.5) at 20 °C.[11] Comparing the $k_{cat} = 258.3 \pm 25.0$ min^{-1} and $K_m = 0.30 \pm 0.03$ mM for the natural substrate (**1**) under identical conditions, this difluoromethylene analogue (**8**) is clearly a kinetically competent, albeit very poor, substrate.

The ^{19}F NMR of **8*β*** exhibits two doublets at δ −87.7 and −92.5 ($J = 41.5$ Hz). The intensity of these two doublets decreased with the concomitant appearance of a singlet at δ −119.9 when **8** (7.6 mM) was incubated with TDP-L-rhamnose synthase (1 μM) and NADH (0.28 mM) in 50 mM potassium phosphate buffer (pH 7.5) at room temperature. Because this new signal coincides with the resonance of fluoride ion, the enzymatic processing of **8*β*** must involve C−F bond cleavage. The turnover product(s) was identified by a previously developed GC/MS method[12] in which the sugar products, generated in the incubation with [4′*S*-^{2}H]NADH,[13] were subjected to acid hydrolysis, sodium borohydride reduction, and acetylation in sequence to yield glycidol tetraacetates whose MS fragmentation patterns could be readily analyzed.[12] On the basis of the GC/MS (both EI and CI) results, we concluded that these

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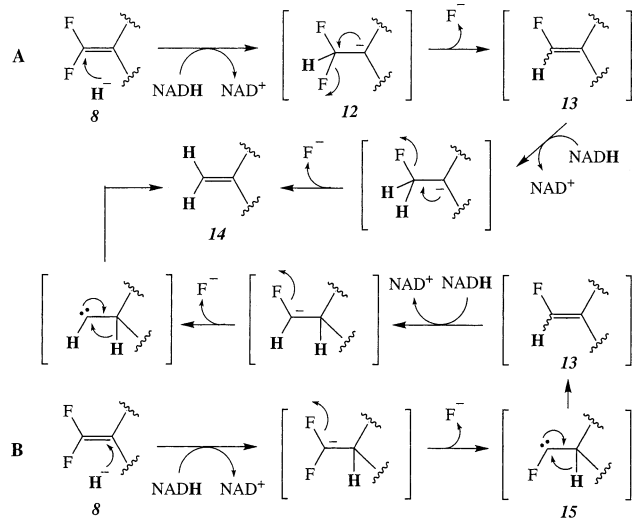
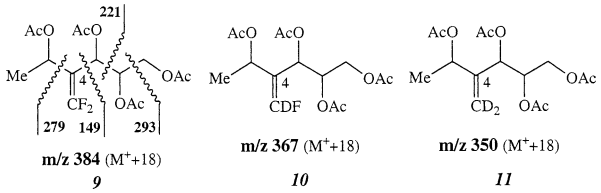
Scheme 2



glycidol derivatives are a mixture of **9**, **10**, and **11**.[14] Interestingly,



the mass spectra for the C-4 containing fragments in **10** and **11** are uniformly shifted by one mass unit, and each of those fragments in **11** also contains a M + 2 peak. Such an increment of one and/or two mass unit(s) of these C-4 bearing fragments is indicative of deuterium incorporation from [4′*S*-^{2}H]-NADH into the turnover products at C-4′ of the exocyclic difluoromethylene group. Such a reversal of regioselectivity for hydride transfer has not been reported for pyridine nucleotide-dependent enzymes.

Our results may be directly explained by a mechanism in which the addition of hydride from NADH occurs at the difluorinated carbon of the exocyclic methylene double bond of **8** (Scheme 2A). The π bond can be restored when the nascent carbanion (**12**) eliminates one of the β-fluorines to give **13**.[15] A second round of hydride reduction will result in loss of the remaining vinylic fluoride to afford **14**.[16] This mechanistic proposal is unique because the enzyme-catalyzed hydride transfer takes place at the opposite end of the π bond in the reduction of a difluoromethylene group from what is observed in the reduction of a carbonyl group. An alternative route (Scheme 2B), which does not alter the regiochemistry of the initial hydride addition, is also conceivable. This mechanism involves the formation of a carbene intermediate (**15**) followed by a 1,2-H shift to give **13**, which then undergoes a second round of carbene formation and 1,2-H rearrangement to yield the observed product (**14**). The proposed rearrangement is well documented in carbene chemistry[17] and is expected to be extremely facile.[18] However, while proposed, the intermediacy of a carbene species has not been fully substantiated in enzyme catalysis.[19]

Clearly, the difluoromethylene functionality in compound **8** has assumed a role as a carbonyl mimic with an apparently reversed regioselectivity for hydride reduction. The implication of either the reversal of the site of hydride attack or the participation of a carbene intermediate to account for the experimental results is provocative. While the regiospecificity of hydride reduction in enzyme reactions is determined by the effective binding of the substrate with a defined orientation in the enzyme active site, our results indicate that the regioselectivity can also be affected by the electrochemical characteristics of the reaction center. Even though the competence of the difluoromethylene group as a carbonyl equivalent is poor, the alternative outcome in regioselectivity of hydride reduction may find application in enzyme catalysis.

Acknowledgment. This work was supported in part by National Institutes of Health Grants GM35906 and GM54346. C.L. thanks the Singer-Polignac Foundation (France) for a Postdoctoral Fellowship. We would also like to thank the reviewers of this paper for their valuable comments on the mechanisms shown in Scheme 2.

Supporting Information Available: Scheme and synthesis (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(3) On the basis of the theoretical transition structure of hydride transfer involving 1,4-dihydropyridine (Wu, Y.-D.; Houk, K. N. *J. Am. Chem. Soc.* **1987**, *109*, 2226) and the well-defined normal C−H bond length of 1.073 Å for R_2CH_2 (*The Chemist's Companion*; Gordon, A. J., Ford, R. A., Eds.; John-Wiley: New York, 1987), one can estimate the net distance for an effective hydride transfer to be approximately 0.55 Å or less (see: Kreevoy, M. M.; Ostovic, D.; Truhlar, D. G.; Garrett, B. C. *J. Phys. Chem.* **1986**, *90*, 3766).
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(8) The *rfb*D gene (Jiang, X.-M.; Neal, B.; Santiago, F.; Lee, S. J.; Romana, L. K.; Reeves, P. R. *Mol. Microbiol.* **1991**, *5*, 695) was amplified by the polymerase chain reaction (PCR) using the genomic DNA of *Salmonella enterica* LT2 as the template and was cloned into a pUC18 vector. Purification of the RfbD protein (TDP-L-rhamnose synthase) from the *E. coli* HB101-*rfb*D recombinant strain involved two chromatographic steps: DEAE-Sepharose and Mono Q.
(9) The scheme and a brief discussion of the synthesis can be found in the Supporting Information.
(10) Because the natural substrate (**1**) has a β-configuration at the anomeric carbon, only the β-isomer of **8** should be recognized and processed by TDP-L-rhamnose synthase.
(11) A typical assay included 5 mM compound **8** (mixture of α/β isomers), 2.8 mM NADH, 1 μM of enzyme in 50 mM potassium phosphate buffer (pH 7.5). This mixture was incubated at 25 °C for 60 min, and the products were analyzed directly by NMR or derivatized followed by GC/MS analysis.[12]
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(14) Compound **9** was the predominant species derived mainly from the unreacted **8**α, and no **10** and **11** were detected when the incubation was performed in the absence of enzyme.
(15) The fact that **13** could only be detected by MS analysis (to give **10**) but not by ^{19}F NMR indicates that little of this intermediate was accumulated during turnover.
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JA021487+