CDP-6-deoxy-6,6-difluoro-D-glucose: A Mechanism-Based Inhibitor for CDP-D-glucose 4,6-Dehydratase

Cheng-Wei T. Chang, Xuemei H. Chen, and Hung-wen Liu*

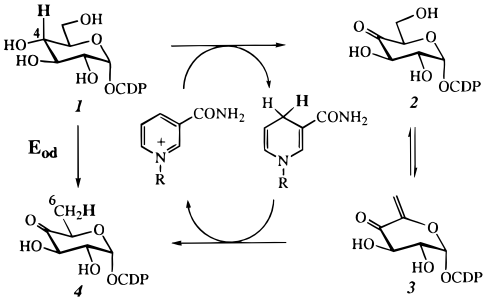
Department of Chemistry
University of Minnesota
Minneapolis, Minnesota 55455

Received June 24, 1998

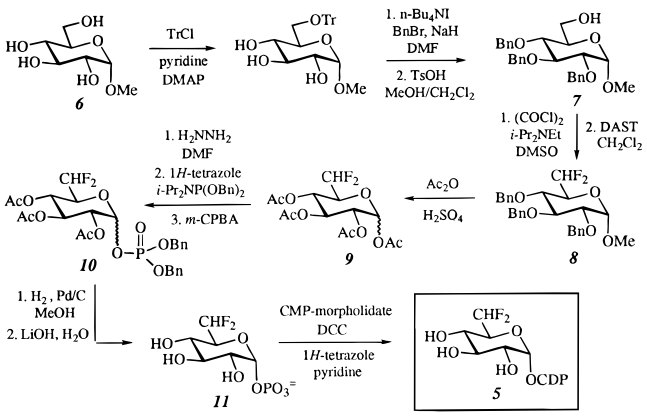
CDP-D-glucose 4,6-dehydratase (E_{od}),[1] isolated from *Yersinia pseudotuberculosis*, is a homo-dimeric enzyme that catalyzes the transformation of CDP-D-glucose (**1**) to CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose (**4**).[2] The catalysis, shown in Scheme 1, has been established to proceed via three discrete steps, oxidation of CDP-D-glucose to 4-keto-glucose **2**, C-5/C-6 dehydration to a 4-keto-$\Delta^{5,6}$-glucoseen intermediate **3**, and reduction at C-6 of **3** to give the final product **4**.[3] This intramolecular oxidation−reduction is characteristic for an internal hydrogen transfer from C-4 of the substrate **1** to C-6 of the resulting 4-keto-6-deoxyhexose product **4**, and the hydride carrier is an enzyme-bound NAD^+. Since NAD^+ is regenerated at the end of each catalytic cycle, it is in essence a prosthetic group, contrary to most of the other nicotinamide dinucleotide-dependent enzymes in which $NAD(P)^+$ functions merely as a cosubstrate.[4] To develop methods to control and/or regulate this intriguing enzymatic conversion, we decided to prepare substrate analogues that, upon incubation with E_{od}, would lead to either inhibition or turnover, depending on the mode of catalysis. Reported herein are the synthesis and characterization of a CDP-difluoroglucose derivative **5**, which has been shown to be the first mechanism-based inhibitor for E_{od}.

The designed inhibitor **5** was synthesized from methyl α-D-glucoside (**6**) according to the reactions delineated in Scheme 2. Selective tritylation of the 6-OH, followed by perbenzylation and removal of the 6-trityl group, afforded **7** in 37% combined yield. The exposed C-6 hydroxyl group was oxidized under Swern conditions, and the crude product was fluorinated with diethylaminosulfur trifluoride (DAST)[5] to give 6-deoxy-6,6-difluoroglucoside **8** in nearly quantitative yield. Conversion of **8** to **9** involved acid treatment and peracetylation (74% yield). Subsequent treatment with hydrazine in DMF[6] selectively removed the 1-*O*-acetyl group. Phosphorylation was then carried out in the presence of *N*,*N*-diisopropyl dibenzylphosphamidite and 1*H*-tetrazole.[7] After oxidation of the resulting phosphite with *m*-chloroperbenzoic acid (*m*-CPBA), the phosphate product **10** was purified by triethylamine-treated silica gel chromatography (ether/hexane gradient) to separate the α and β anomers (α:β = 3:1). The desired α-anomer, isolated in 31% yield (from **9**), was subjected to hydrogenation and basic hydrolysis to remove the acetyl groups to form **11** (90% combined yield). The final step involved coupling of **11** with cytidine 5′-monophospho-morpholidate in pyridine to give **5**,[6] which, after purification by Dowex 1X-8 (formate form) ion-exchange chromatography (NH_4HCO_3 gradient), was isolated in 25% yield.[8]

Scheme 1



Scheme 2



When compound **5** was incubated with E_{od}[9] at 25 °C in 20 mM Tris·HCl buffer (pH 7.5), time-dependent inactivation occurred.[10] The k_{inact} of 2.4×10^{-2} min^{-1} and K_I of 0.94 mM of this inactivation were deduced from a plot of $t_{1/2}$ versus $[I]^{-1}$. Since extensive dialysis failed to regenerate the enzyme activity, the observed inactivation is clearly irreversible. Considering the fact that E_{od} was fully protected from inhibition by **5** (0.8 mM) in the presence of substrate (0.8 mM), we deduced that the effect of **5** on E_{od} must be active-site directed. Similar to the catalytic situation, we could detect no apparent accumulation of NADH spectrofluorometrically during the course of inactivation. However, a fluorine peak at δ −119.6, which is due to the

(1) Abbreviations: CDP, cytidine 5′-diphosphate; NAD^+, β-nicotinamide adenine dinucleotide; NADH, β-nicotinamide adenine dinucleotide, reduced form; Tris, tris(hydroxymethyl)amino-methane; GC, gas chromatography; MS, mass spectroscopy.

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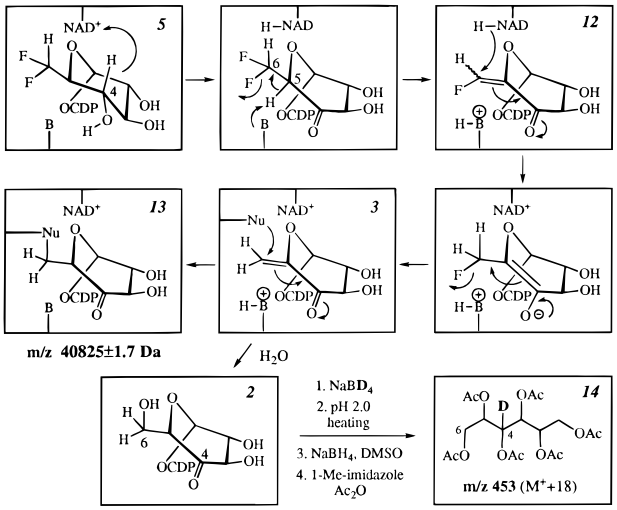
(8) Spectral data of **5**: ^1H NMR (D_2O) δ 7.71 (1 H, d, J = 7.5 Hz, cytidine H-6), 5.97 (1H, t, J_{HF} = 52.6, 6-H), 5.93 (1 H, d, J = 7.5, cytidine H-5), 5.79 (1 H, d, J = 3.0, cytidine H- 1′), 5.44 (1H, dd, J_{HP} = 6.9, J_{HH} = 3.5, H-1), 3.8−3.4 (6 H, m, cytidine H-2′, 3′, 4′, 5′), 3.60 (1 H, t, J = 9.4, H-3), 3.45 (1 H, t, J = 9.4, H-4), 3.40 (1 H, ddd, J = 9.4, 3.5, 2.0, H-2). ^{13}C NMR (D_2O) δ 159.6, 149.1, 144.0, 114.3 (t, J_{CF} = 242.2 Hz, 6-C), 95.4 (d, J_{CP} = 6.1, 1-C), 89.7, 83.3 (d, J_{CP} = 9.0, 4′-C), 74.5, 72.5, 71.2 (d, J_{CP} = 8.2, 2-C), 70.7 (t, J_{CF} = 10.2, C-5), 69.1, 68.6, 64.4 (d, J_{CP} = 5.7, 5′-C). ^{31}P NMR (D_2O) δ −11.1 (d, J = 19.2 Hz), −13.0 (d, J = 19.2). ^{19}F NMR (D_2O) δ −133.0 (dd, J = 53.6, 10.9 Hz), −133.3 (dd, J = 53.6, 16.4). High-resolution MALDI-MS calcd for $C_{15}H_{21}F_2N_3O_{15}P_2$ $(M + H)^+$ 583.0416, found 583.0488.

(9) E_{od} was prepared from *Escherichia coli* strain HB101-pJT8 according to the published procedure.[2c]

(10) Activity of E_{od} was determined spectrophotometrically by measuring the formation of CDP-4-keto-6-deoxy-D-glucose (**4**) as previously described.[2c] The blank was prepared by boiling the enzyme for 5 min prior to the addition of other reagents. One unit of enzyme activity corresponds to the formation of 1 μmol of product per hour under the assay conditions.

S0002-7863(98)02198-2 CCC: $15.00
Published on Web 09/02/1998

Scheme 3



released fluoride ion,[11] was discernible by ^{19}F NMR after incubation at 37 °C.

A sample of inactivated E_{od}, from which the unreacted inhibitor **1** had been removed by thorough dialysis, was subjected to electron spray ionization MS analysis.[12] In addition to the intact E_{od}, which has a molecular mass of 40 280 ± 5.4 Da per monomer, a new species with a molecular mass of 40 825 ± 1.7 Da per monomer was found. The mass increase of 545 Da indicates that the covalently modified E_{od} is likely an adduct (Scheme 3, **13**) with a CDP-4-keto-6-deoxyglucosyl moiety which has a calculated molecular weight of 545. On the basis of these data and the above findings, a plausible mechanism can now be proposed. As depicted in Scheme 3, the inactivation may be initiated by C-4 oxidation followed by C-5/C-6 elimination to give **12**, with the subsequent reductive elimination leading to the regeneration of NAD^+ and the formation of **3** as the nascent product. In normal catalysis, the key intermediate **3** will be reduced by NADH (see Scheme 1). However, in the absence of a hydride source, this conjugated 4-keto-$\Delta^{5,6}$-glucoseen may serve as a Michael acceptor and trap an active site nucleophile to give **13** and subsequently impair the enzyme.[13] The detection of a turnover product (**2**) in the incubation mixture by a previously developed GC−MS assay lent further credence to this model.[2d,14] As summarized in Scheme 3, the inactivated E_{od} was removed by a Centricon-10, and the filtrate, after lyophilization, was subjected to sodium borodeuteride reduction, acid hydrolysis, further reduction, and acetylation. The mass data (*m*/*z* of M + NH_4^+ = 453) of the resulting glycidol peracetate **14** is consistent with the data of a turnover product with a 4-keto-glucose skeleton (**2**), the formation of which may be rationalized by the hydration of **3**.[15]

It is worth mentioning that the important features making the incorporation of fluorine(s) into biological molecules particularly attractive include the favorable steric interactions toward the biological targets, the unique effects of fluoridation on the bond strength, and the susceptibility of fluoride ion as a leaving group.[16] Clearly, compound **5**, as a mechanism-based inhibitor for E_{od}, is a new addition to a great variety of organofluorine compounds that have been developed and established to exhibit potent biological activities.[17] It should be noted that the E_{od} reaction has been distinguished as the common entry in the formation of all 6-deoxyhexoses,[2,3] many of which play key roles as cellular components or are an indispensable part of secondary metabolites.[18] With the development of effective inhibitors for E_{od} and perhaps other enzymes of this class, we may be able to better control and regulate the biosynthesis of many unusual sugars. Further characterization of the modified protein will certainly provide valuable information on the active site of this important class of enzymes, for which a crystal structure is still lacking.

Acknowledgment. This work was supported by a National Institutes of Health grant GM 35906.

JA982198H

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(12) The MS measurements were performed at the MS facility of the Cancer Research Institute, University of Minnesota.

(13) Alternatively, intermediate **12** could also act as a Michael acceptor to trap the active-site nucleophile and the resulting adduct after reduction would lead to an identical final product **13**. While both routes are feasible, the one shown in Scheme 3 is more likely, in view of its close resemblance to the normal catalytic mechanism.

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(15) The assignment was based on the observation that the MS fragments of **14** containing the C-4 oxygen linkage (*m*/*z* 187, 259, 289, and 361) are all uniformly shifted by one mass unit.[2d] The increment of one mass unit of these fragments bearing the C-4 ester linkage is indicative of a deuterium incorporation through the reduction of a 4-keto-glucose derivative **2**.

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