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COMMUNICATIONS

Published on Web 10/19/2005

Activation of Orotidine 5′-Monophosphate Decarboxylase by Phosphite Dianion: The Whole Substrate is the Sum of Two Parts

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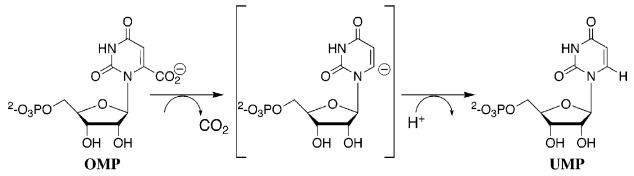
We report that the binding of exogenous phosphite dianion to orotidine 5′-monophosphate decarboxylase results in an 80 000-fold increase in k_{cat}/K_m for enzyme-catalyzed decarboxylation of a truncated substrate lacking a 5′-phosphodianion moiety, which corresponds to a transition state stabilization of 6.6 kcal/mol.

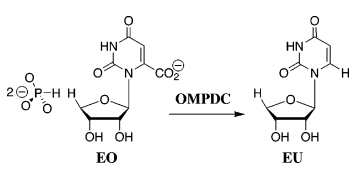
Orotidine 5′-monophosphate decarboxylase (OMPDC) is a remarkable enzyme because it employs no metal ions or other cofactors but yet effects an enormous 10^{17}-fold acceleration of the chemically very difficult decarboxylation of orotidine 5′-monophosphate (**OMP**) to give uridine 5′-monophosphate (**UMP**).[1,2] Recent X-ray structures of OMPDC,[3−7] along with isotope effect studies,[8,9] support the decarboxylation of **OMP** to give a putative vinylic carbanion intermediate which undergoes protonation by the enzyme to give **UMP** (Scheme 1). The crystallographic structure of yeast OMPDC complexed with the transition state analogue 6-hydroxyuridine 5′-phosphate shows that ligand binding results in substantial motions of loops to "close" the active site and the formation of numerous protein−ligand contacts, including five hydrogen bonds to the phosphodianion group.[4] The importance of the latter interactions in catalysis has been demonstrated by site-directed mutagenesis[10] and by the greatly reduced activity of OMPDC toward decarboxylation of orotidine, which lacks the 5′-phosphodianion group.[11]

Scheme 1



The phosphodianion group at **OMP** may act simply as an "anchor" that allows for efficient substrate recognition. Alternatively, this small binding determinant, which lies remote from the reacting center of the substrate, may serve to effect an enzyme conformational change leading to formation of an active site that is optimized for transition state stabilization. To distinguish these possibilities, we aimed to establish whether the *covalent connection* between the reacting portion of the substrate and the nonreacting remote phosphodianion group is necessary for efficient catalysis by OMPDC. Thus, we designed a truncated substrate at which the $CH_2OPO_3^{2-}$ group of **OMP** is replaced by a single hydrogen to give 1-(β-D-erythrofuranosyl)orotic acid (**EO**) and have determined whether exogenous phosphite dianion can serve as the excised phosphodianion "piece" (Scheme 2).[12]

Scheme 2



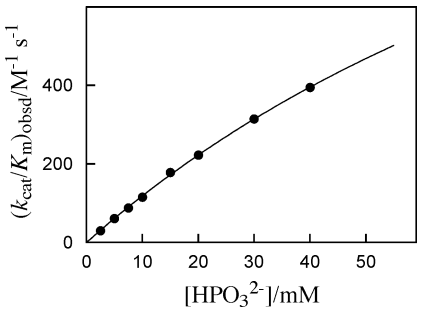


Figure 1. Dependence of $(k_{cat}/K_m)_{obsd}$ for the decarboxylation of 0.1 mM **EO**, monitored spectrophotometrically at 283 nm, by C155S mutant yeast OMPDC on the concentration of added phosphite dianion at pH 7.0, 25 °C, and $I = 0.14$ (NaCl).

The very slow decarboxylation of **EO** in the presence of a large quantity of OMPDC was followed in a discontinuous assay in which the initial velocity of formation of the product 1-(β-D-erythrofuranosyl)uridine (**EU**) was monitored by HPLC. The reactions of 5.6 or 10.5 mM **EO** ($\ll K_m$, vide infra) in the presence of 0.11 or 0.16 mM yeast OMPDC,[13] respectively, in 50 mM MOPS buffer (pH 7.1) at 25 °C and $I = 0.14$ (NaCl) gave the same second-order rate constant $(k_{cat}/K_m)_E = 2.1 \times 10^{-2}\ M^{-1}\ s^{-1}$ for decarboxylation of **EO** catalyzed by OMPDC.[14]

First-order rate constants k_{obsd} (s^{-1}) for the *complete* reaction of 0.1 mM **EO** ($\ll K_m$) to give **EU** in the presence of 13.8 μM yeast OMPDC[13] and various concentrations of phosphite buffer (80% dianion, pH 7.0) at 25 °C and $I = 0.14$ (NaCl) were determined spectrophotometrically by monitoring the decrease in absorbance at 283 nm. These reactions were followed for up to 180 min and obeyed excellent first-order kinetics with stable endpoints.[15] Figure 1 shows the dependence of the observed second-order rate constant $(k_{cat}/K_m)_{obsd} = k_{obsd}/[OMPDC]$ for the OMPDC-catalyzed decarboxylation of **EO** on the concentration of phosphite dianion. The data were fit to eq 1, derived for Scheme 3,[16] to give $K_d = 140 \pm 13$ mM[17] for binding of phosphite dianion to OMPDC. The slope of the linear correlation at $[HPO_3^{2-}] \le 10$ mM gives the third-order rate constant $(k_{cat}/K_m)_{E\cdot HPi}/K_d = 11\,700 \pm 600\ M^{-2}\ s^{-1}$. This was combined with $K_d = 140$ mM to give $(k_{cat}/K_m)_{E\cdot HPi} = 1600 \pm 200\ M^{-1}\ s^{-1}$ for decarboxylation of **EO** catalyzed by OMPDC *saturated with phosphite dianion* (Scheme 3, E = OMPDC).

Scheme 3

$$\mathbf{E + EO} \underset{K_d/[HPO_3^{2-}]}{\rightleftharpoons} \mathbf{E{\cdot}HPO_3^{2-} + EO} \xrightarrow{(k_{cat}/K_m)_{E\cdot HPi}} \mathbf{E + EU}$$

$$(k_{cat}/K_m)_{obsd} = \frac{(k_{cat}/K_m)_{E\cdot HPi}}{1 + K_d/[HPO_3^{2-}]} \qquad (1)$$

The ratio of second-order rate constants for the decarboxylation of **EO** catalyzed by OMPDC saturated with phosphite dianion and by free OMPDC, $(k_{cat}/K_m)_{E\cdot HPi}/(k_{cat}/K_m)_E = 1600/(2.1 \times 10^{-2}) = 7.6 \times 10^4$, shows that phosphite dianion binds more tightly to the

10.1021/ja055493s CCC: $30.25

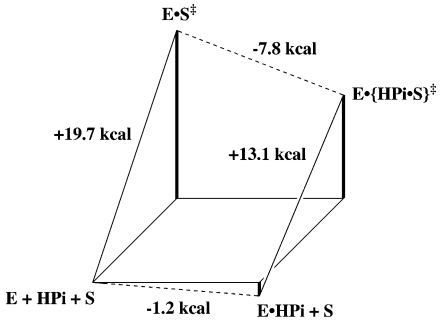


Figure 2. Free energy diagram for the reactions of **EO** (**S**) catalyzed by OMPDC (**E**) in the presence and absence of phosphite dianion (**HPi**).

transition state for the OMPDC-catalyzed decarboxylation of **EO** than to free OMPDC in the ground state by 6.6 kcal/mol. This is illustrated in the free energy diagram in Figure 2, which also shows that the total intrinsic binding energy of phosphite dianion in the transition state is 7.8 kcal/mol. The intrinsic binding energy of the phosphodianion group of **OMP**, calculated from the ratio of second-order rate constants k_{cat}/K_m for the OMPDC-catalyzed reactions of **OMP** (9.4×10^6 M^{-1} s^{-1})[18] and **EO** (2.1×10^{-2} M^{-1} s^{-1}), is 11.8 kcal/mol. Thus, a very large fraction of the intrinsic binding energy of the phosphodianion group of **OMP** can be "reconstituted" by binding the small ligand phosphite dianion to OMPDC. The remaining 4 kcal/mol largely represents the entropic price of binding two entities rather than one.[19]

A value of $K_m = 100 \pm 40$ mM (range of uncertainty) for binding of **EO** to OMPDC complexed with phosphite dianion was determined from progress curve analysis of the reaction of 19.0 mM **EO** in the presence of 15 μM OMPDC, 250 mM MOPS buffer (pH 7.1), and 40 mM phosphite dianion at 25 °C and $I = 0.29$ (NaCl), monitored spectrophotometrically at 308 nm.[20,21] This can be combined with $(k_{cat}/K_m)_{E\cdot HPi} = 1600$ M^{-1} s^{-1} to give $k_{cat} = 160 \pm 70$ s^{-1} for turnover of the truncated substrate **EO** in the active site of OMPDC that also contains phosphite dianion, which is significantly larger than $k_{cat} = 15$ s^{-1} for turnover of the natural phosphorylated substrate **OMP**.[18]

We conclude that, despite the weaker binding to OMPDC of the individual **EO** and HPO_3^{2-} parts ($K_mK_d = 0.014$ M^2) than of **OMP** ($K_m = 1.6 \times 10^{-6}$ M),[18] once bound, OMPDC provides a slightly greater stabilization of the transition state for reaction of the parts than of the whole substrate. This may be because tethering of the parts restricts access to protein and/or ligand conformations that result in maximal transition state stabilization. Another intriguing possibility is that severing the covalent link to the phosphodianion group reduces or alters its interactions with the loop that closes the active site and changes the dynamics of loop opening. This might result in a change in the rate-limiting step from product release for the reaction of **OMP** to a chemical step for the reaction of **EO**.

The potent activation of OMPDC by phosphite dianion implies that a major role of the phosphodianion group of **OMP** is to provide binding interactions that are used to drive an enzyme conformational change resulting in formation of an active site environment that is optimized for transition state stabilization. OMPDC is the titular member of the recently identified OMP decarboxylase "suprafamily", whose members share a $(\beta,\alpha)_8$-barrel fold and highly conserved active sites but catalyze the reactions of substrates bearing a remote phosphodianion group using a variety of mechanisms.[22,23] We suggest that utilization of the intrinsic binding energy of the substrate phosphodianion group to drive a protein conformational change that generates an active site optimized for transition state stabilization is a common mechanistic strategy employed by this family of enzymes, whose members catalyze reactions that appear otherwise to be unrelated.

Acknowledgment. We thank the National Institutes of Health (GM 39754) for financial support, and Professor Dick Wolfenden and Dr. Annette Sievers for a generous gift of C155S mutant yeast OMPDC.

Supporting Information Available: Full details of the error estimations, the determination of K_m for **EO** from progress curve analysis, and the syntheses of **EO** and **EU** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) We use C155S mutant yeast OMPDC, which is more stable than but kinetically and structurally essentially identical with the wild-type enzyme.[11] Standard spectrophotometric assays at 279 nm ($\Delta\epsilon = -2400$ M^{-1} cm^{-1}) were conducted in 10 mM MOPS buffer 50% free base (pH 7.1) at 25 °C and $I = 0.105$ (NaCl), with 40−50 μM **OMP** (25−30K_m) and 10−20 nM OMPDC.[18] Concentrations of OMPDC were calculated from the values of V_{max} using a value of $k_{cat} = 15$ s^{-1}.[18]
(14) Reactions in a total volume of 160 μL were monitored for 7−8 h. Periodic standard assay[13] of the reaction mixture (10 000-fold dilution) showed that there was no significant decrease in enzyme activity during this time. At various times, an aliquot (20 μL) was removed and quenched to pH 3.8 by the addition of 180 μL of ice-cold 5.7 mM formic acid, and the protein was removed by ultrafiltration. The filtrate (150 μL) was analyzed by HPLC using a Waters Nova-Pak C_{18} 4 μm column and a linear gradient of 25 mM NH_4OAc (pH 8.5)/MeOH, with peak detection at 262 nm. The concentration of **EU** was obtained from the peak area by interpolation of a standard curve constructed using authentic **EU**.
(15) Standard assay[13] of the reaction mixture (1000-fold dilution) showed that there was no decrease in enzyme activity during this time.
(16) Under the conditions of these experiments, the contribution of the decarboxylation of **EO** catalyzed by free OMPDC is <0.1%, so that all the observed activity results from catalysis by the OMPDC·HPO_3^{2-} complex.
(17) Unless stated otherwise, the quoted errors are standard errors that were evaluated as described in the Supporting Information.
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(21) The reaction was monitored over a period of 60 min, during which time there is no decrease in enzyme activity.[15] Under these conditions, there is no significant difference in the first-order rate constants for the reaction of 1 mM **EO** in the presence and absence of 19.3 mM **EU**, monitored at 297 and 295 nm, respectively, so that there is negligible product inhibition.
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JA055493S