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Role of glutamate 243 in the active site of 2-deoxy-*scyllo*-inosose synthase from *Bacillus circulans*

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Abstract—2-Deoxy-*scyllo*-inosose (DOI) synthase is involved in the biosynthesis of 2-deoxystreptamine-containing aminoglycoside antibiotics and catalyzes the carbocyclic formation from D-glucose-6-phosphate (G-6-P) into DOI. The reaction mechanism is proposed to be similar to that of dehydroquinate (DHQ) synthase in the shikimate pathway, and includes oxidation of C-4, β-elimination of phosphate, reduction of C-4, ring opening, and intramolecular aldol cyclization. To investigate the reaction mechanism of DOI synthase, site-directed mutational analysis of three presumable catalytically important amino acids of DOI synthase derived from the butirosin producer *Bacillus circulans* (BtrC) was carried out. Steady state and pre-steady state kinetic analysis suggested that E243 of BtrC is catalytically involved in the phosphate elimination step. Further analysis of the mutant E243Q of BtrC using substrate analogue, glucose-6-phosphonate, clearly confirmed that E243 was responsible to abstract a proton at C-5 in G-6-P and set off phosphate elimination. This glutamate residue is completely conserved in all DOI synthases identified so far and the corresponding amino acid of DHQ synthase is completely conserved as asparagine. Therefore, this characteristic glutamate residue of DOI synthase is a key determinant to distinguish the reaction mechanism between DOI synthase and DHQ synthase as well as primary sequence.

1. Introduction

2-Deoxy-*scyllo*-inosose (DOI) synthase is a crucial enzyme in the biosynthesis of 2-deoxystreptamine (DOS)-containing aminoglycoside antibiotics such as kanamycin and neomycin. DOI synthase constructs the carbocyclic structure of DOS from D-glucose-6-phosphate (G-6-P) by a similar manner to dehydroquinate (DHQ) synthase in the shikimate pathway. DOI synthase was originally isolated from the butirosin producer *Bacillus circulans* and its gene '*btrC*' was reverse genetically cloned.[1,2] Following functional confirmation of the *btrC* gene product as a DOI synthase, a whole butirosin biosynthetic (*btr*) gene cluster consisting of 29 Kb and 25 ORFs was identified.[3,4] Further, the *btrS* gene in the *btr* gene cluster was functionally characterized to encode L-glutamate:DOI aminotransferase, which catalyzes two transamination steps of DOS biosynthesis from G-6-P.[5–7] Based on the sequence information of characteristic BtrC and BtrS in the DOS biosynthesis, many biosynthetic gene clusters for DOS-containing antibiotics including neomycin,[8,9] kanamycin,[10,11] tobramycin,[12,13] gentamicin,[14,15] etc., have been identified so far. A gene for DOI synthase exists in all gene clusters, and several of them were expressed in *Escherichia coli* showing the DOI synthase activity.[9,11–13,15]

The six-membered carbocyclic ring structure of DOI can be conveniently converted into important industrial materials such as catechol and other benzenoids.[16,17] Furthermore, DOI is a versatile material for fine chemicals such as carbaglucose.[18] Thus, a highly efficient enzyme is desirable to provide sufficient amount of DOI from sustainable and environmentally acceptable carbohydrate. To explore the potential of DOI synthase, more precise and detailed features of the chemistry are indispensable.

As we previously reported, the reaction mechanism of DOI synthase is similar to DHQ synthase as well as the protein sequence (Figs. 1 and 2).[2] DOI synthase first catalyzes oxidation of C-4 position in G-6-P using NAD^+, followed by β-elimination of a phosphate group

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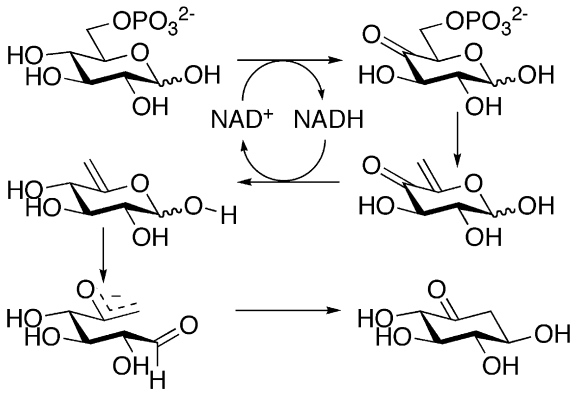


Figure 1. Reaction mechanism of DOI synthase.

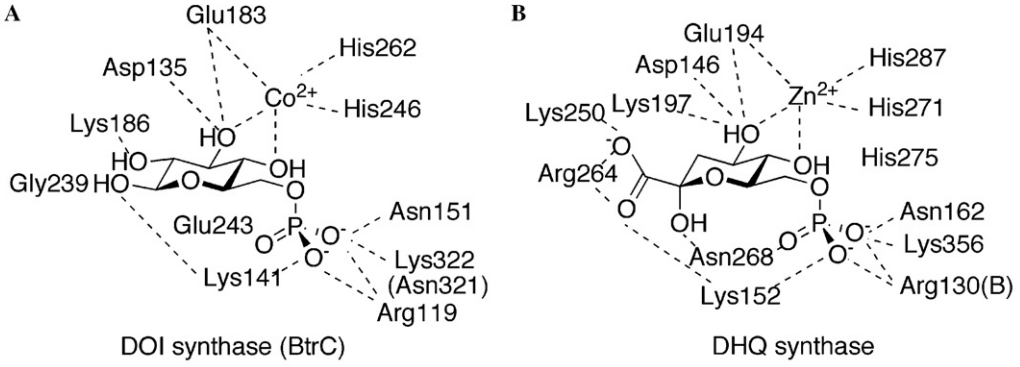
producing an enone intermediate. Subsequent reduction of C-4 using the produced NADH in the active site forms cyclic enol ether, and the last aldol-type intramolecular condensation between C-1 and C-6 stereospecifically leads to DOI. One difference between DOI synthase and DHQ synthase reaction is the substrate, G-6-P and 3-deoxy-D-*arabino*-heputulosonate-7-phosphate (DAHP), respectively (Figs. 2 and 3). Another is the reaction stereochemistry during β-elimination of phosphate and the intramolecular aldol-type condensation (Fig. 3). Enzymatic analysis with chirally deuterium labeled glucose-6-phosphate clearly suggested two possible reaction mechanisms of DOI synthase, (1) *anti*-fashion of β-elimination of phosphate and a *chair*-conformation of hexocyclic intermediate in the aldol condensation, or (2) *syn*-fashion of β-elimination and a *boat*-conformation of hexocyclic intermediate, while DHQ synthase reaction mechanism was reported to be '*syn*-fashion' of β-elimination of the phosphate and 'a *chair*-conformation' of hexocyclic intermediate in the aldol condensation.[19,20] Therefore, the mode of substrate recognition and catalytically important amino acid residues were thought to be slightly different. In fact, two amino acids of DOI synthase (cf. G239 and E243 in BtrC) in the presumed active site were found to be significantly different from those of DHQ synthase (cf. R264 and N268 in DHQ synthase of *Aspergillus nidulans*), although these amino acids are highly conserved in each family of enzymes (Fig. 2C).[2,18] In addition, a carbacyclic analogue of G-6-P was found to be a mechanism-based and irreversible inhibitor binding to K141 of BtrC, where Michael addition of K141 into the α,β-unsaturated intermediate seemed to occur, while the carbacyclic analogue of DAHP was reported to be a reversible inhibitor of DHQ synthase.[18,21] Since the lysine residue (K141 of BtrC) is highly conserved in both family of enzymes, slightly different roles and/or position should be considered.

Under these backgrounds, mutational analysis of DOI synthase, BtrC, was carried out to investigate the functions of these important amino acids to discriminate DOI synthase and DHQ synthase reaction. As a result, E243 of BtrC was clearly found to be a key determinant to distinguish the reaction mechanism between DOI synthase and DHQ synthase.

2. Results and discussion

2.1. Mutational analysis of BtrC

Two amino acids G239 and E243 in the presumed active site of BtrC are significantly different from corresponding



C

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          119   135   141   151   183 186   225   239  243 246 250  262    321
BtrC    ···FRG···SDSVLSIKQ···KNL···CELVKN···AKM···KGLIFEYGHTIGHA···LHG···DNK···
AlloH   ···FRG···LDSVLSLKQ···KNL···CEVVKN···AKA···EGLVLEYGHTVGHA···SHG···DNK···
NeoC    ···FRG···LDSVLSLKQ···KNL···GEVVKN···AKA···SGLVLEYGHTAGHA···AHG···DNK···
TbmA    ···FRG···LDSVLSLKQ···KNL···CEVVKN···AKA···EGLVLEYGHTVGHA···SHG···DNK···
KanA    ···FRG···LDSVLSLKQ···KNL···CEVVKN···AKA···EGLILEYGHTVGHA···PHG···DNK···
GtmA    ···FRG···SDSVLSLKQ···KNL···CEVVKN···AKA···AGLVLEYGHTVGHA···SHG···DNK···
An      ···MRG···VDSSIGGKT···KNL···AEVIKT···HKA···LRNLLNWGHSIGHA···LHG···DKK···
          130b  146   152   162   194 197   250   264 268 271 275  287    356
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Figure 2. Substrate recognition models of DOI synthase and DHQ synthase. Model structure of DOI synthase from *Bacillus circulans* (A) was constructed based on the crystal structure of DHQ synthase from *Aspergillus nidulans* (B, PDB id; 1dqs). Extracted sequence alignment of DOI synthases (BtrC, *Bacillus circulans*; AlloH, *Streptoalloteichus hindustanus*; NeoC, *Streptomyces fradiae*; TbmA, *Streptomyces tenebrarius*; KanA, *Streptomyces kanamyceticus*; GtmA, *Micromonospora echinospora*) and DHQ synthase (An, *Aspergillus nidulans*) is shown in (C). The probable catalytically important amino acids are shown in color. The red amino acid residues of BtrC were mutated in the present study. The amino acid number of BtrC is shown at the top of text and the amino acid number of DHQS from *Aspergillus nidulans* is shown at the bottom of text.

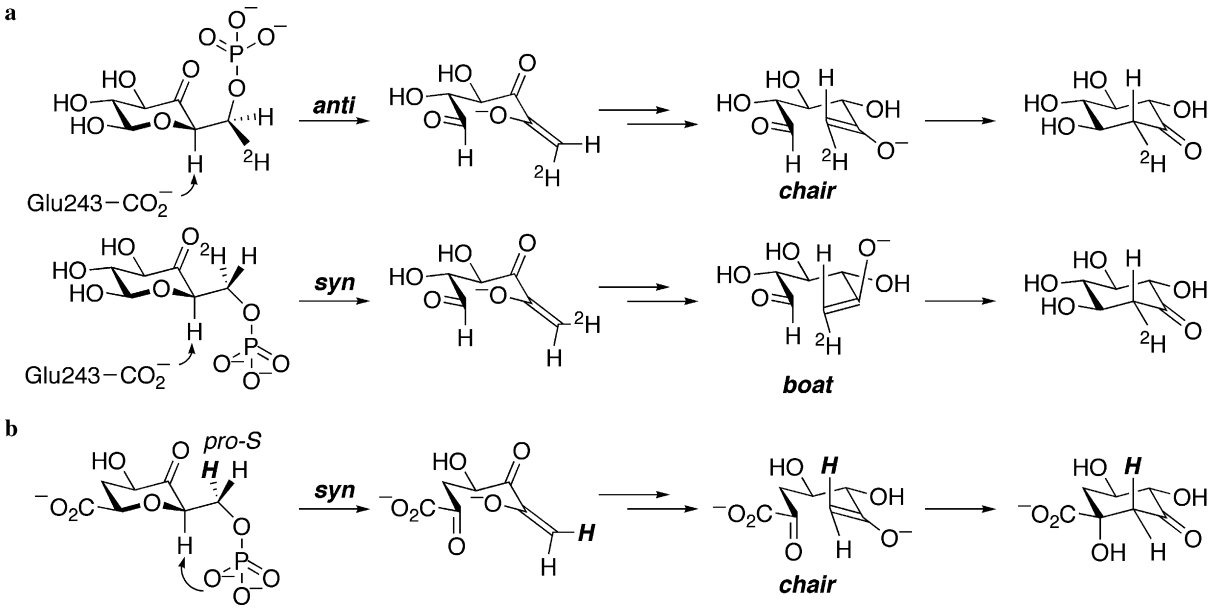

Figure 3. Comparison of the stereochemistry in the DOI synthase reaction (a) and DHQ synthase reaction (b). Two stereochemical courses, *anti-chair* and *syn-boat*, are proposed in the DOI synthase reaction. See details in Ref. 19.

amino acids of DHQ synthase (R264 and N268 of DHQ synthase from *A. nidulans*) by sequence alignment (Fig. 2). Thus, G239 of BtrC was first mutated to an arginine by a standard site-directed mutagenesis. However, G239R was easily precipitated indicating that G239R does not have an ability to form correct quaternary structure. The corresponding arginine residue in DHQ synthase was proposed to have a critical role for domain closure as well as substrate recognition.[22–25]

On the other hand, E243 of BtrC was mutated to the similar size of neutral amino acid glutamine. E243Q was expressed as a soluble protein and was purified by the previously reported procedure for the wild type of BtrC. The K_m value for G-6-P of E243Q (110 μM) was two times lower than the wild type (230 μM, Table 1). This result suggests that the neutral glutamine residue does not electrostatically interfere with the phosphate group of G-6-P with slightly better affinity. On the other hand, the k_{cat} value (0.017 s^{-1}) was significantly lower than the wild type (0.41 s^{-1}, 24 times less). Therefore, this mutation caused significant reduction of the catalytic ability of BtrC indicating that E243 is an important catalytic residue in the reaction of DOI synthase beyond recognition of substrate molecule.

K141 of BtrC was also mutated to the similar size of neutral amino acid glutamine and K141Q was obtained as a soluble protein. However, the activity of K141Q was almost undetectable. In the presence of high concentrations of G-6-P (>5 mM), DOI was somehow produced by K141Q. It is thus obvious that Lys141 plays an important role in recognition of G-6-P. Presumably, the positive charge of the terminal ammonium of K141 could stabilize the negative charge of the phosphate in G-6-P. This result is consistent with our previous report in which K141 nucleophilically attacked the α,β-unsaturated intermediate of carbaglucose 6-phosphate giving a covalent complex with the enzyme.[18] The leaving phosphate was speculated to take a proton from ammonium of K141 forming HPO_4^{2-} and a nucleophilic free amine, followed by Michael addition to the α,β-unsaturated intermediate. Therefore, in the DOI synthase reaction, certain amino acid residues are likely to abstract a proton at C-5 of G-6-P in the β-elimination process, while in the DHQ synthase reaction a leaving phosphate of DAHP was reported to abstract the corresponding proton of substrate.[18,21] The lysine residue of DHQ synthase (K152 of DHQ synthase from *A. nidulans*) was proposed to be part of a phosphate-binding pocket and involved in the initiation of domain closure.[23] Therefore, both lysine residues in DOI synthase and DHQ synthase could be important for recognition of phosphate group of substrates, while direction of the side chain toward the substrate molecule and/or conformation of the reaction intermediates would be quite different from each other.

Table 1. Kinetic constants for G-6-P

	K_m (M)	k_{cat} (s^{-1})
Wild type	$2.3 \pm 0.4 \times 10^{-4}$	0.41 ± 0.05
E243Q	$1.1 \pm 0.2 \times 10^{-4}$	0.017 ± 0.002
K141Q	$>2 \times 10^{-2}$	< 0.007

2.2. Function of E243 of BtrC

Since the E243Q mutant significantly reduced the catalytic efficiency beside a little better substrate recognition, our attention was thus focused to which step of the DOI synthase reaction was slowed by this mutation. At first, the enzyme reactions with E243Q and the wild type were carried out under the single-turnover conditions (25 μM enzymes, 20 μM of G-6-P, and 2.5 mM of NAD^+) and the NADH formation in the oxidation at C-4 position

of G-6-P by NAD^+ was chased by using a Stopped-flow instrument (Fig. 4). To simplify the reaction model as a standard pre-steady state kinetic model with a substrate, an intermediate, and a product, a larger amount of NAD^+ was pre-incubated with enzyme to ignore the concentration of NAD^+ outside of the enzymes as shown in Scheme 1. By using the kinetic analysis software, Dyna-Fit,[26] the obtained data were fitted to the equations with the best-fitted kinetic constants (Table 2). As a result, k_1 and k_{-1} of E243Q were almost equal to the wild type, while k_2 of E243Q was eight times lower than that of the wild type. In other word, the rate of the NADH formation in the first oxidation was same, while the combined rate of β-elimination of phosphate and reduction by NADH was significantly slower than the wild type. Since the k_1 and k_{-1} values for the oxido-reduction were similar to each other, the rate of reduction by NADH involved in the k_2 value was presumed to be similar as well. Therefore, the reduction of the k_2 value is likely caused by basically slow down at the β-elimination step, in which abstraction of a proton at C-5 and elimination of a phosphate are involved.

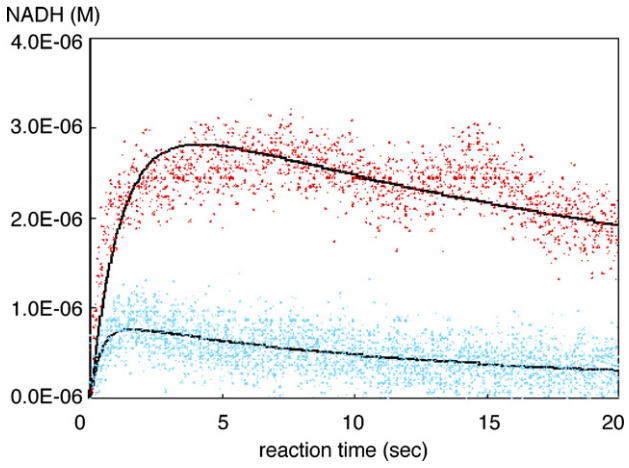

Figure 4. Pre-steady kinetic analysis of the wild type of BtrC (blue dots) and E243Q (red dots). The NADH production (M) was monitored by a stopped-flow instrument under the single turnover conditions. Data were fitted by regression analysis using DynaFit to the kinetic equations (Section 3) to give a fitted solid line, respectively.

$$[\mathrm{E\ NAD^+}] + [\mathrm{S}] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\mathrm{E\ NADH\ S'}] \xrightarrow{k_2} [\mathrm{E\ NAD^+\ S''}]$$

Scheme 1. Pre-steady kinetic model of the DOI synthase reaction in the present study. The k_1 is the oxidation rate by NAD^+; the k_2 is the rate containing the elimination step of phosphate and reduction step by NADH (see Fig. 1). Absorbance of 340 nm for NADH formed in the enzyme reaction was measured and fitted to the equations (see Section 3).

Table 2. Kinetic parameters at the pre-steady state

	k_1 ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})	k_2 (s^{-1})
Wild type	$4.0 \pm 0.6 \times 10^3$	1.3 ± 0.4	0.94 ± 0.03
E243Q	$5.5 \pm 0.3 \times 10^3$	0.55 ± 0.04	0.12 ± 0.001

To examine the rate reduction in abstraction of a proton at C-5, the substrate analogue glucose-6-phosphonate was utilized, so that the enzyme reaction does not proceed after the abstraction step of a proton at C-5 of glucose 6-phosphonate.[27] The rate of H/D exchange of the proton at C-5 in a D_2O containing buffer was analyzed to see the effect of this mutation (Fig. 5). The enzyme reaction was quenched after 30, 60, and 300 min, and the treated glucose 6-phosphonate was recovered by DEAE A-25 chromatography. The exchange of H/D at C-5 was analyzed by ^1H NMR (Fig. 5) and was also confirmed by ^2H NMR (data not shown). In the enzyme reaction with the wild type of BtrC, a half of the proton was exchanged after 60 min and the exchange was completed after 300 min (Fig. 5A and Table 3). On the other hand, the exchange rate by E243Q was much slower than the wild type (Fig. 5B and Table 3). Although these rates cannot be simply compared as the rate for abstraction of a proton at C-5 of substrate analogue, this mutation reduced the rate about 10 times, which is similar order to the difference of the k_2 values mentioned above. Thus, it was clearly confirmed that E243 of BtrC is involved in the abstraction step of the H-5 in G-6-P, followed by β-elimination of phosphate (maybe via an enolate intermediate). The acidic proton of the formed carboxylic acid of E243 could be somehow taken by the formed free amino group of K141 to return to the catalytic cycle. In this process, the transiently formed uncharged K141 and E243 may be critical to fix an appropriate conformation of the reaction intermediate. While the wild type efficiently abstracts a proton at C-5 by the carboxylate group of E243, in the E243Q mutant, the proton placed at the α-position of the carbonyl group (C-4) might be spontaneously released due to the relatively high acidity. The glutamine or neighboring water may scavenge the proton for the catalytic cycle. Although the stereochemical course of β-elimination of the phosphate and the aldol condensation are still unclear, our mutational analysis clearly proved different roles of K141 and E243 of DOI synthase from the DHQ synthase. Also, G239 is proposed to be important to form a domain structure at the active site. To elucidate the precise reaction mechanism, it could be necessary to determine how DOI synthase recognizes the stereochemistry at the C-1 position and also direction of β-elimination of the phosphate.

In conclusion, our present mutational study gave significant information about three catalytically important amino acid residues, G239, E243, and K141, of BtrC. Especially, the conserved glutamate in DOI synthase was clearly found to be crucial in the DOI synthase reaction and a discriminatory residue against DHQ synthase as well.

3. Experimental

3.1. Site-directed mutagenesis

The plasmid vector pDS3,[2] which contains the *btrC* gene, was used as a template DNA for PCR based site-directed mutagenesis by QuikChange site-directed

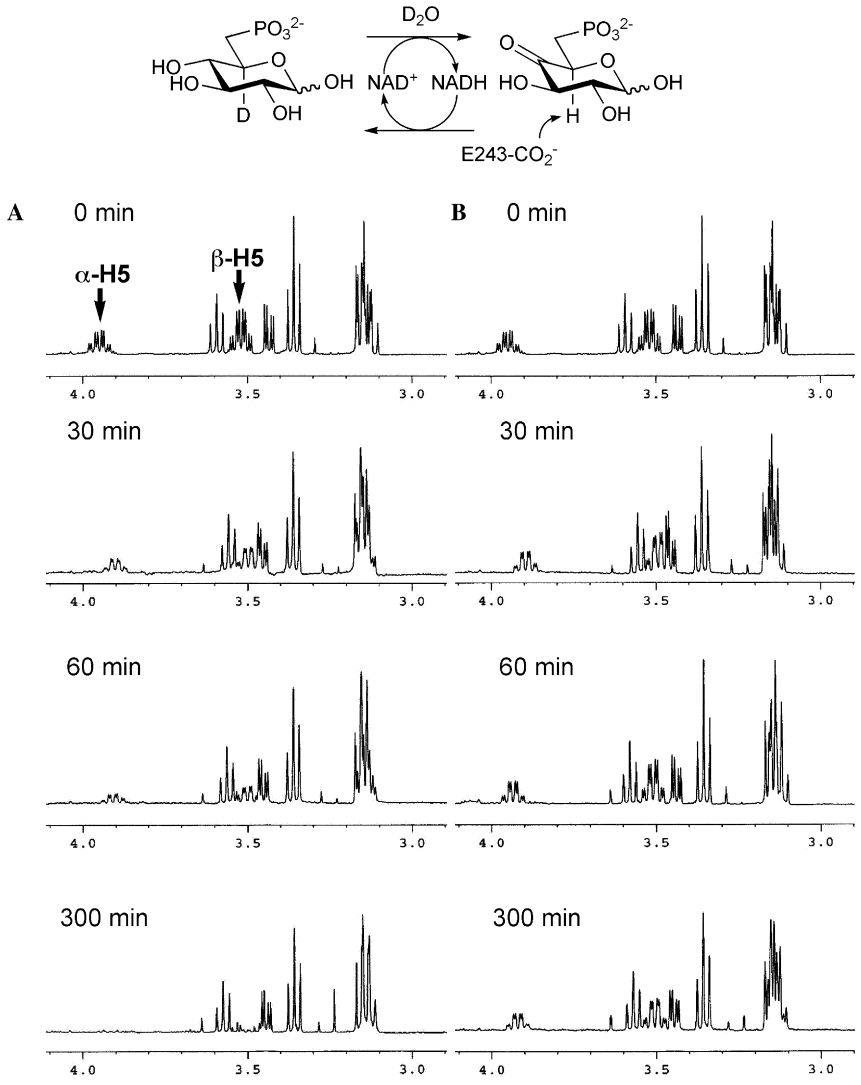

Figure 5. H/D exchange at C-5 of glucose 6-phosphonate with wild type of BtrC (A) and E243Q (B) in a D_2O containing buffer. The enzyme reactions were quenched after 30, 60, and 300 min. The recovered glucose 6-phosphonate substrate was analyzed by ^1H NMR in D_2O.

Table 3. The H/D exchange rate of a proton at C-5 in glucose 6-phosphonate in a D_2O buffer

Enzyme	Reaction time (min)			
	0	30	60	300
Wild type	100%	77%	47%	0%
E243Q	100%	~100%	91%	77%

These values were calculated from the percentage of α-H5/a proton of C-6 at 1.7 ppm.

mutagenesis kit (Stratagene). The following oligonucleotides were used as primers.

K141Q: 5′-CCGATTCGGTTCTTTCGATTCAG CAGGCTG-3′ and its complement.

G239R: 5′-CGAGAAAAAGAAGCGCCTGATCTTT GAGTACGG-3′ and its complement.

E243Q: 5′-GCCTGATCTTTCAGTACGGCCATAC GATCGG-3′ and its complement.

After confirmation of the point mutation by DNA sequencer, the resultant plasmids were digested with *Nde*I/*Hin*dIII and introduced into pET30b(+) for expression. The recombinant mutant proteins of BtrC, except for an insolubly expressed mutant (G239R), were purified to homogeneity according to previously reported method for the wild type of BtrC.[2] Protein concentration was determined by the Lowry method with bovine serum albumin as standard.

3.2. Steady state kinetic analysis

Incubation (final volume 100 μl each) was carried out with 1.0 μM of enzyme (wild type or mutants), various concentrations of G-6-P (50–800 μM for wild type, 30–120 μM for E243Q, and 5–10 mM for K141Q), and 5 mM NAD^+ in a 50 mM Tris buffer containing 0.2 mM $CoCl_2$ (pH 7.7) at 37 °C for 10 or 30 min. The reaction mixture was subjected to the same manipulations as described previously.[28] The kinetic constants

(k_{cat} and K_m for G-6-P) were determined by fitting to the standard Michaelis–Menten equation.

3.3. Pre-steady state kinetic analysis

Absorption of NADH (340 nm) produced in the catalytic cycle was analyzed by a stopped-flow instrument, Photal (OTSUKA ELECTRONICS). After pre-incubation of the wild type or E243Q (25 μM each) and NAD^+ (2.5 mM) at 37 °C for 10 min, G-6-P (20 μM) was mixed to start the single-turnover enzyme reaction (triplicate). To simplify the reaction model, a larger amount of NAD^+ was pre-incubated with enzyme to ignore the concentration of NAD^+ outside of enzyme. A kinetic model was then ascribed to a simple reaction with a substrate and a product shown by Eqs. 1 and 2, where k_1 is the rate of oxidation by NAD^+; k_{-1} is the rate of the reverse; k_2 is the combined rate of the phosphate elimination step and the reduction step by NADH. Data were fitted by regression analysis using DynaFit[26] to the equations giving each kinetic constant.

$$[E] + [S] \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [ES'] \xrightarrow{k_2} [ES'']$$

$$\frac{d[ES']}{dt} = k_1[E][S] - (k_{-1} + k_2)[ES'] \quad (1)$$

$$\frac{d[ES'']}{dt} = k_2[ES'] \quad (2)$$

3.4. Enzyme reaction with glucose 6-phosphonate

Glucose-6-phosphonate was prepared according to the known method.[27] Incubation was carried out at 37 °C with 20 μM of enzyme (wild type or E243Q), 5 mg of glucose-6-phosphonate, and 10.4 mg of NAD^+ in 8 ml of the deuterium oxide buffer. The enzyme reaction (2 ml) was terminated by addition with 2 ml EtOH after 30, 60, and 300 min. The inactivated proteins were eliminated by ultracentrifugation using VIVASPIN 20 (30 K) concentrator. A mixture of compounds with low molecular weight was loaded onto DEAE A-25 (OH^- form, Sigma–Aldrich). The adsorbed glucose 6-phosphonate was eluted with a linear gradient of 5 mM (80 ml) to 500 mM (80 ml) NH_4HCO_3 and obtained from the fractions containing 195–205 mM NH_4HCO_3. The solution was lyophilized and the residue was dissolved in D_2O and analyzed by ^1H NMR. The solvent signal HDO was suppressed by decoupling and was set to δ 4.65 ppm.

Acknowledgments

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