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Factors required for the catalytic reaction of PqqC/D which produces pyrroloquinoline quinone

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Abstract

PqqC/D converts the biosynthetic intermediate purified from a *pqqC* mutant to pyrroloquinoline quinone (PQQ), and both NAD(P)H and cytosolic fraction, named as activating factor (ActF), are required to show its higher production. Dithiothreitol alone, as well as ActF plus NAD(P)H, enhanced the PQQ production by PqqC/D. Thioredoxin–thioredoxin reductase system with NADPH showed similar effect. PqqC/D made a tight complex with PQQ, however, in the presence of dithiothreitol, PQQ was dissociated from the protein. ActF showed NADPH oxidase activity which was enhanced by the addition of PQQ. These data suggest that PqqC/D produces the reduced PQQ from the intermediate *in vivo*, but *in vitro*, it is further oxidized by molecular oxygen and then the oxidized PQQ is trapped in PqqC/D to show product inhibition.

Keywords: Pyrroloquinoline quinone; PQQ; Methylotrophs; Biosynthesis

Pyrroloquinoline quinone (PQQ) is the prosthetic group of several bacterial redox enzymes, including quinoprotein methanol dehydrogenase of methylotrophs and quinoprotein glucose dehydrogenase of a number of bacteria [1]. Genes involved in PQQ synthesis, *pqq*, have been cloned from several bacteria [2]. However, the biochemical steps of PQQ synthesis in details are still unknown.

Methylobacterium extorquens AM1 has two gene clusters, *pqqABC/DE* and *pqqFG*, both of which are required to produce PQQ [2,3]. PqqC/D in *M. extorquens* AM1 appeared to be a fusion of two Pqq proteins [3] found in other bacteria, for instance, PqqC and PqqD in *Klebsiella pneumoniae* [4]. A *pqqC* mutant of *M. extorquens* AM1 accumulated a large amount of an intermediate of PQQ biosynthesis [3], which was also produced by all *K. pneumoniae pqq* genes but *pqqC* expressed in *Escherichia coli*, albeit in much less extent [4]. Recently, the chemical structure of the intermediate was determined [5]. The purified PqqC/D or PqqC could convert it to PQQ only to the stoichiometric amount of the enzyme, and its catalytic turnover seemed to occur in the presence of both NAD(P)H and an unknown protein factor named activating factor (ActF) [6]. Crystal structure of PqqC from *K. pneumoniae* was solved and reaction mechanism was proposed [7]. However, we have less information about ActF, and the reason is not clear why catalytic turnover of PqqC and PqqC/D does not occur *in vitro*.

In this report, we examined further about ActF from *E. coli* and *M. extorquens* AM1. We found that dithiothreitol (DTT) can enhance the PqqC/D reaction instead of ActF plus NADPH. We also found that thioredoxin–thioredoxin reductase (TrxAB) system can enhance PQQ production by PqqC/D like ActF. DTT has ability to keep PQQ in the reduced state even under aerobic conditions,

Abbreviations: PQQ, pyrroloquinoline quinone; ActF, a proteinous fraction which enhances the PQQ production from the intermediate by PqqC.

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therefore, we propose that the role of ActF in PqqC/D reaction is also to maintain the product PQQ in the reduced form and to lower the product inhibition of PqqC/D by the oxidized PQQ.

Materials and methods

Bacterial strains, plasmids, media and growth conditions. *Methylobacterium extorquens* AM1 rif wild-type strain and its *pqqC* and *pqqE* mutants (EMS12 and UV45) [8,9] were grown on the minimum medium described previously [3]. *E. coli* was grown on LB medium. Antibiotics were used at the following concentrations (μg/ml); rifamycin, 20; kanamycin, 25; tetracycline, 10; ampicillin, 50. The *E. coli* mutants defective in TrxA or TrxB (JW5856 or JW0871, respectively) were obtained from National Institute of Genetics, Japan.

Purification of PqqC/D, PqqC, and the intermediate. PqqC/D and PqqC were purified from *E. coli* transformant cells as described previously [6,7]. The intermediate was purified from the crude extract of the mutant EMS12 as described previously [3]. The amount of the intermediate was estimated by measuring PQQ produced after the reaction with an excess amount of the purified PqqC/D as described below. The protein content was measured by modified Lowry method [10] using bovine serum albumin as a standard.

Detection of the intermediate and assay of PqqC/D. The enzyme reaction was performed in 100 mM Tricine–NaOH (pH 8.3) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 2–3 μM intermediate at 25 °C. The reaction was stopped by adding a half volume of 1 M HCl, and before assaying PQQ, the reaction mixture was neutralized with the same volume of 1 M NaOH. The amount of PQQ produced was measured as described previously [11], by using the apo-form of soluble glucose dehydrogenase of *Acinetobacter calcoaceticus*, which was purified from the *E. coli* transformant cells [12].

Partial purification of the ActF of PqqC/D. The ActF from the *E. coli* was obtained by fractionation with 40–50%-saturated ammonium sulfate. In the case from the *pqqE* mutant of *M. extorquens* AM1, the precipitate was obtained by fractionation with 30–40%-saturated ammonium sulfate. The precipitate was dissolved with 20 mM Tris–HCl (pH 8.0) and dialyzed against the same buffer. The active fractions were determined as follows: a portion was added to the reaction mixture of PqqC/D (see above) with or without 0.4 mM NADPH, and PQQ production was compared.

Effect of TrxAB system on PqqC/D reaction. The reaction mixture contained 10 pmol of intermediate and 4.9 pmol PqqC/D, and incubated for 5 min at 25 °C. After stopping the reaction, the produced PQQ was determined by apo-sGDH. The complete TrxAB system contained TrxA (10 μM), TrxB (1 μM), and NADPH (500 μM).

Gel filtration. The purified PqqC/D of *M. extorquens* AM1 (ca. 2.4 nmol of subunit) were mixed with 2.4 nmol of PQQ and applied onto a Superdex S-200 (Amersham-Biotech) equipped on a HPLC system (Shimadzu). Elution was done with 20 mM Tricine–NaOH (pH 8.3) containing 1 mM EDTA and 150 mM NaCl with or without 1 mM DTT, and monitored by photodiode array detector SPD10A (Shimadzu).

Fluorescence spectra and determination of dissociation constant K_d. The fluorescence of PqqC/D (0.5 μM-subunit, 1 mL) in 20 mM Tricine–NaOH (pH 8.3) containing 1 mM EDTA and 150 mM NaCl was measured with excitation at 290 nm, and the quenching by the addition of PQQ (2 μL-each of 50 μM) was monitored at 335 nm. Relative fluorescence intensity (F) to the intensity without PQQ was plotted as a function of PQQ concentration. Relative fluorescence change (ΔF) should correspond to the ratio of the enzyme bound to PQQ, expressed as the following equation:

$$\Delta F = (1-F)/(1-F_\infty) = [\mathrm{E}\cdot\mathrm{PQQ}]/[\mathrm{E}]_0 = ([\mathrm{E}]_0 - [\mathrm{E}])/[\mathrm{E}]_0 \quad (1)$$

where F_∞ is the relative fluorescence intensity of the enzyme saturated with PQQ, $[\mathrm{E}\cdot\mathrm{PQQ}]$ is the concentration of the enzyme–PQQ complex, and $[\mathrm{E}]_0$ and $[\mathrm{E}]$ are the concentrations of the total enzyme capable to bind PQQ and the unbound enzyme free from PQQ, respectively. Dissociation constant (K_d) is expressed as follows:

$$\begin{aligned} K_d &= ([\mathrm{E}]\ [\mathrm{PQQ}])/[\mathrm{PQQ}\cdot\mathrm{E}] \\ &= ([\mathrm{E}]_0 - \Delta F\ [\mathrm{E}]_0)([\mathrm{PQQ}]_0 - \Delta F[\mathrm{E}]_0)/\Delta F[\mathrm{E}]_0 \end{aligned} \quad (2)$$

$$\Delta F = \frac{([\mathrm{E}]_0 + [\mathrm{PQQ}]_0 + K_d) - (([\mathrm{E}]_0 + [\mathrm{PQQ}]_0 + K_d)^2 - 4[\mathrm{E}]_0[\mathrm{PQQ}]_0)^{1/2}}{2[\mathrm{E}]_0} \quad (3)$$

K_d, initial PQQ concentration $[\mathrm{PQQ}]_0$, and $[\mathrm{E}]_0$ are used for expression of F as follows:

$$F = 1 - \frac{([\mathrm{E}]_0 + [\mathrm{PQQ}]_0 + K_d) - (([\mathrm{E}]_0 + [\mathrm{PQQ}]_0 + K_d)^2 - 4[\mathrm{E}]_0[\mathrm{PQQ}]_0)^{1/2}}{2[\mathrm{E}]_0} \times (1 - F_\infty) \quad (4)$$

Data of F plotted as a function of PQQ concentration were fitted to Eq. (4) with K_d and F_∞ as adjustable parameters using a non-linear regression analysis program (Excel™).

PQQ-dependent NADPH oxidase assay. The reaction was performed in 0.1 M Tricine–NaOH (pH 8.3) containing 1 mM EDTA and 100 μM NADPH, in the presence or absence of 100 nM PQQ. The reaction was started by the addition of ActF partially purified from *E. coli*. Decrease of absorbance at 340 nm was monitored.

Results and discussion

Partial purification of ActF from pqqE mutant of M. extorquens AM1

PqqC/D reacted with the intermediate only stoichiometorically and show no requirement of NAD(P)H, however, a protein fraction (ActF), which was obtained by fractionation with 40–50%-saturated ammonium sulfate from the crude extract of *E. coli*, enhanced the PQQ production by PqqC/D with NAD(P)H [6]. The activation by ActF was also observed when the purified PqqC of *K. pneumoniae* was used (data not shown). Further purification of ActF was failed because the activity decreased severely or disappeared after one or two different column chromatographies (data not shown).

We also found ActF activity in the crude extract from the wild-type of *M. extorquens* AM1. Due to the presence of large amount of intrinsic PQQ, it was difficult to analyze the crude extract from the wild-type strain, thus, a *pqqE* mutant was used for purification. The activity appeared together with PqqC/D in each step, suggesting that they may consist of a complex. Although the ActF activity decreased severely during purification as in the case in *E. coli*, we obtained a partially purified fraction with several protein bands on SDS–PAGE analysis. The N-terminal amino acid sequence of the main protein band showed an identity to hydroxypyruvate reductase (HPR), and in fact, the fraction showed HPR activity. However, the crude extract from the mutant defective in HPR gene still activated the production of PQQ by PqqC *in vitro*, in NADPH-dependent manner. Moreover, the purified HPR itself did not enhance the PqqC/D reaction with NAD(P)H (data not shown). N-terminal sequences of other minor protein bands were also analyzed, but all of them but PqqC/D itself seemed not to relate with PqqC/D reaction (data

not shown). Identification of ActF in *M. extorquens* AM1 was also not accomplished.

Effect of dithiothreitol

We found that dithiothreitol (DTT) alone increased the rate of PQQ production by PqqC/D of *M. extorquens* AM1 (Fig. 1A) and PqqC of *K. pneumoniae* (data not shown), when examined the effect of HPR, which was purified in the presence of DTT [13]. DTT also stimulated the final amount of PQQ produced (after incubated overnight, Fig. 1B). Therefore, DTT was shown to replace ActF plus NADPH. It seemed to be saturated at around 40 μM for shorter period (5 min) of the reaction, but at much higher

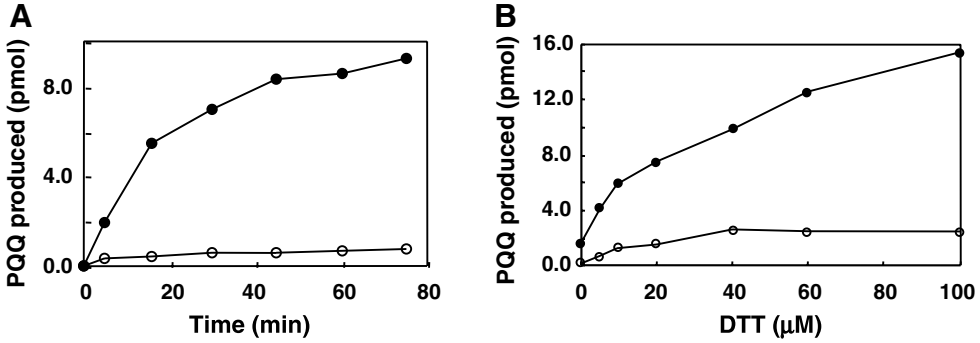

Fig. 1. Effect of DTT on PqqC/D reaction. (A) The purified PqqC/D of *M. extorquens* AM1 (2.78 pmol, left panel) was incubated with 11 pmol of the intermediate purified from the *pqqC* mutant of *M. extorquens* AM1 with or without 1 mM DTT (closed or open circle, respectively) in 0.1 M Tricine–NaOH containing 1 mM EDTA (pH 8.3). Aliquot was withdrawn time to time and the produced PQQ was determined enzymatically using apo-sGDH. (B) The purified PqqC/D (2.42 pmol) was incubated with 20 pmol of the intermediate and various concentration of DTT in 0.1 M Tricine–NaOH containing 1 mM EDTA (pH 8.3) for 5 min (open circle) or overnight (closed circle). Then the produced PQQ was determined using apo-sGDH.

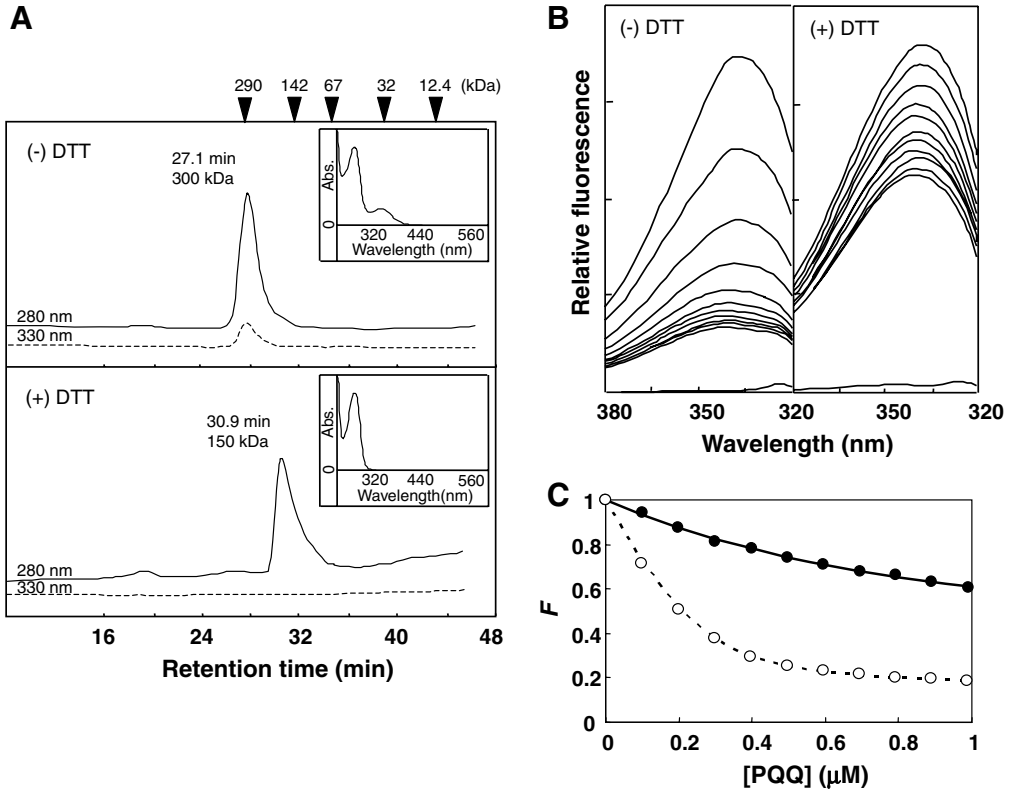

Fig. 2. Gel filtration analysis and fluorescence titration of PqqC/D with PQQ in the presence of DTT. (A) Gel Filtration. The purified PqqC/D (2.4 nmol) was mixed with 2.4 nmol of PQQ, and the mixture was applied onto a Superdex S-200 column and eluted with 20 mM Tricine–NaOH (pH 8.3) containing 1 mM EDTA and 150 mM NaCl with or without 1 mM DTT (lower or upper panel, respectively). Elution was monitored at 280 nm (solid line) and 330 nm (dashed line). The inserted panels are absorption spectra at the elution peaks. The closed triangles are the positions of elution peaks of marker proteins. (B) Fluorescence spectra of PqqC/D. The purified enzyme (0.5 μM in 1 ml) in 20 mM Tricine–NaOH (pH 8.3) containing 1 mM EDTA and 150 mM NaCl was titrated with 2 μl-each of 50 μM PQQ, in the absence (left panel) or presence (right panel) of 1 mM DTT. Fluorescence spectra were measured with excitation at 290 nm. (C) Data of relative fluorescence (*F*) at 335 nm were plotted as a function of PQQ concentration (open circle, −DTT; closed circle, +DTT).

concentrations after the reaction completed for longer time of incubation (Fig. 1B).

Effect of several other reagents on PqqC/D reaction was examined. Only DTT or its isomer, dithioerythritol (DTE), was effective, and 2-mercaptoethanol and other reducing reagents (H_2O_2, ascorbic acid, sodium hydrosulfate, and potassium metabisulfate) were ineffective or inhibitive. Effect of DTE is practically the same as that of DTT as described above (data not shown). Among amino acid sequences of PqqC known so far, no cystein residues were conserved [3], furthermore, only one cystein residue exists in PqqC/D of *M. extorquens* AM1 (C226). In the crystal structure of PqqC of *K. pneumoniae*, the complex formation with PQQ induces a large conformational change [7], but four cystein residues (C68, C136, C142 and C198) do not change in their positions or directions. Therefore, these residues seems not to be oxidized or reduced during the reaction, suggesting that these cystein residues do not associate with the PqqC activity, and thus DTT may not work for protection of –SH group in PqqC/D.

Complex formation of PqqC/D with PQQ

PqqC formed a tight complex with the oxidized form of PQQ in the crystal structure [7]. This was also demonstrated by gel filtration (Fig. 2A), where PqqC/D was eluted together with PQQ as a complex, showing absorption around 330 nm similar to the absorption spectrum of PQQ-containing quinoproteins. When DTT was added in the elution buffer, however, PqqC/D was eluted as a form free from PQQ. PqqC/D showed different molecular mass with or without DTT (150 and 300 kDa, respectively), both of which were larger than expected, since in the crystal structure PqqC was shown to be a dimer of 29 kDa [7]. The retention time of PqqC/D without PQQ was the same as that with PQQ and eluted in the presence of DTT (data not shown). In the case of PqqC, the separation was not so clear and some amount of PQQ was still retained; the amount of PQQ attached was much lower than that in the absence of DTT, judged from the decrease of the absorbance around 330 nm in the absorption spectrum (data not shown).

Binding of PQQ to PqqC/D was monitored by fluorescence change (Fig. 2B). Both PqqC and PqqC/D showed fluorescence at around 335 nm when excited at 290 nm, and by the addition of PQQ, this fluorescence was quenched. The amplitude of quenching was repressed by the addition of DTT (Fig. 2C). The dissociation constant (K_d) was determined by plotting the fluorescence at 335 nm as a function of PQQ concentration. When $[E]_0$ was fixed as 0.5 μM which was estimated from the protein concentration, the theoretical curve did not fit well to the data (not shown), especially in the absence of DTT. When $[E]_0$ was also included as an adjustable parameter, the curve was fit well and $[E]_0$ obtained after curve fitting was shown to be about 0.25 μM (data not shown). Thus, K_d values of PqqC/D for PQQ were determined. PqqC/D showed larger K_d value in the presence of DTT (752 nM) than that in its

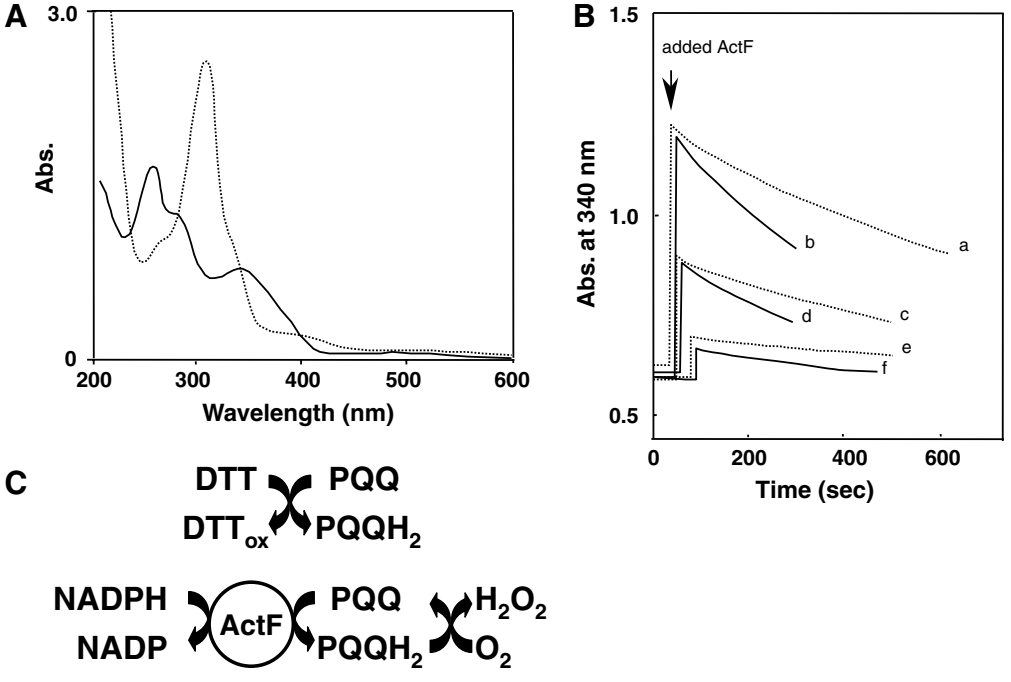

Fig. 3. PQQ reduction by DTT and $PQQH_2$ oxidation by molecular oxygen. (A) DTT can reduce PQQ under aerobic conditions. Solid line: 90 μM PQQ. Dotted line: 90 μM PQQ was mixed with 1 mM DTT under aerobic conditions. (B) PQQ-dependent NADPH oxidase activity. Decrease of absorbance at 340 nm was monitored. The reaction was performed in 0.1 M Tricine–NaOH (pH 8.3) containing 1 mM EDTA and 100 μM NADPH, in the presence (b, d, and f) or absence (a, c, and e) of 100 nM PQQ. The reaction was started by the addition of partially purified ActF (a and b, 1.27 mg-protein/ml; c and d, 0.636 mg/ml; e and f, 0.212 mg/ml). (C) Reaction scheme. DTT makes PQQ in the reduced form until it is exhausted. Reduced PQQ is easily oxidized by molecular oxygen to produce H_2O_2. ActF reduces PQQ depending on NADPH.

absence (42 nM), indicating that DTT lowers the affinity of the enzymes to PQQ. It is clearly demonstrated that binding of PQQ to PqqC/D was inhibited by DTT.

PQQ-dependent NADPH oxidase activity of ActF

DTT alone stimulated the PqqC reaction, and its mechanism would be similar to that by ActF plus NADPH. DTT was able to reduce PQQ under aerobic conditions (Fig. 3A), although free $PQQH_2$ is reported to be quickly oxidized under aerobic conditions at neutral pH [14].

The partially purified fraction of ActF exhibited NADPH oxidase activity, which was enhanced by the addition of PQQ (Fig. 3B). This activity with PQQ should represent the PQQ-reduction activity (Fig. 3C), because $PQQH_2$ is successively oxidized by molecular oxygen. Increase of the NADPH oxidation rate by the addition of PQQ seemed proportional to the protein amount of ActF added in the reaction mixture. Thus, ActF seems to reduce PQQ in the presence of NADPH. It is difficult to show the reduction of PQQ by ActF plus NADPH photometrically, because both ActF and NADPH show absorption around 300 nm (data not shown). The oxidase activity without PQQ might be from thioredoxin reductase (see below), since the enzyme from *Sulfolobus solfataricus* showed NADPH oxidase activity [15].

Table 1
Effect of TrxAB system on PqqC/D reaction

Int	PqqC/D	TrxA	TrxB	NADPH	PQQ produced (pmol)
+	–	+	+	+	0
+	+	–	–	–	0.073
+	+	+	+	–	0.084
+	+	+	–	+	0.142
+	+	–	+	+	0.168
+	+	+	+	+	0.773

The complete reaction mixture contains 10 pmol of intermediate (Int), 4.9 pmol PqqC/D, 100 pmol TrxA, 10 pmol TrxB and 5 nmol NADPH in 10 μL. After incubated for 5 min at 25 °C, the produced PQQ was determined by apo-sGDH.

Effect of TrxAB system

The TrxAB system is widely distributed in nature and known to provide reducing equivalent from NAD(P)H to several biological cellular functions such as maintenance of the reduced state of cytoplasmic proteins [16], and defense against oxidative stress [17]. Both TrxA and TrxB from *E. coli* were obtained and effect of the TrxAB system on PQQ production by PqqC/D reaction was examined (Table 1). The complete TrxAB system enhanced the PQQ production, whereas omission of either component resulted in no effect. These results indicate that the TrxAB system assists PqqC reaction, probably in a similar manner by DTT or ActF plus NADPH. However, the crude extract of the *E. coli* mutants defected in either TrxA or TrxB enhanced the PQQ production by PqqC/D as well as that of the wild-type (data not shown), indicating that TrxAB system is not the sole system to activate PqqC/D reaction *in vitro*.

Reaction mechanism of PqqC or PqqC/D

Combined data obtained here all together, a reaction mechanism of PqqC was proposed as illustrated in Fig. 4.

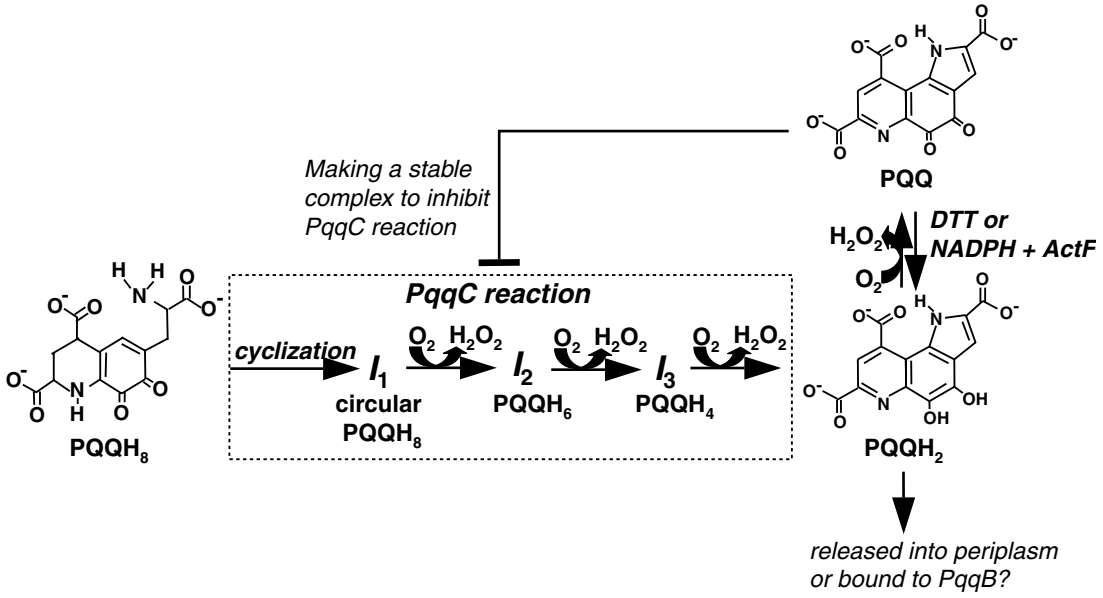

Fig. 4. Reaction scheme of PqqC and inhibition by PQQ. PqqC reaction is illustrated. The box with dotted line corresponds to the enzyme reaction of PqqC. The intermediate ($PQQH_8$) is converted to $PQQH_2$ by three successive oxidation with molecular oxygen, via I_1 (circular $PQQH_8$), I_2 ($PQQH_6$) and I_3 ($PQQH_4$). $PQQH_2$ is released from PqqC, and probably accepted by PqqB and/or transported into periplasm, *in vivo*. *In vitro*, $PQQH_2$ is further oxidized instantly by molecular oxygen, and the oxidized PQQ makes a tight complex with PqqC to inhibit binding of $PQQH_8$. Either DTT alone or ActF plus NADPH can reduce PQQ to $PQQH_2$, which leads to release the product and enhances the production of $PQQH_2$ from $PQQH_8$.

As proposed previously in Ref. [7], PqqC or PqqC/D reacts with the intermediate (equivalent to the 8H-reduced PQQ, $PQQH_8$) to produce the reduced form of PQQ ($PQQH_2$) via three intermediates, I_1 (circular intermediate), I_2 and I_3 (corresponding $PQQH_6$ and $PQQH_4$, respectively), the structures of which are not identified yet. Molecular oxygen is a primary acceptor of hydride ions *in vitro* to produce hydrogen peroxides under aerobic conditions [7], although it is possible that the physiological electron acceptor is not molecular oxygen because some bacteria produced PQQ under anaerobic conditions [2]. After completion of the reaction, the enzyme probably releases the product as $PQQH_2$. Under aerobic conditions *in vitro*, $PQQH_2$ is quickly oxidized further and the oxidized PQQ makes a stable complex with PqqC or PqqC/D, because PqqC or PqqC/D has high affinity to the oxidized PQQ but low to $PQQH_2$ as shown in Fig. 2. DTT alone and ActF plus NADPH enhance the PqqC reaction by reduction of PQQ *in vitro*. If PqqC or PqqC/D was mixed with equimolar PQQ prior to add the intermediate ($PQQH_8$), no further production of PQQ was detected (data not shown), indicating that the affinity of PqqC or PqqC/D to $PQQH_8$ is lower than PQQ. In general, since cytoplasm is in reductive circumstances, where TrxAB system works, $PQQH_2$ should keep in the reduced state. Therefore, ActF seems not to be directly involved in PqqC or PqqC/D reaction and might be dispensable *in vivo*. $PQQH_2$ might be caught by PqqB, which is shown to be dispensable for biosynthesis of PQQ *per se* and proposed to be involved in transport of PQQ into periplasm, since the *pqqB* mutant cells produced small amount of PQQ only inside the cells [4,18]. The proposed role of PqqB was required to be examined by further experiments.

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