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Ascorbate Regeneration by the Reduced Form of 2-Amino-3-carboxy-1,4-naphthoquinone, a Strong Growth Stimulator for Bifidobacteria

Shin-ichi Yamazaki,[†] Kazumichi Iwasa,[†] Kenji Kano,[*,†] Tokuji Ikeda,[*,†] Naoki Taketomo,[‡] and Tsutomu Kaneko[‡]

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan, and Food Functionality Research Institute, Meiji Milk Products Company, Ltd., 1-21-3 Sakae-cho, Higashimurayama, Tokyo 189-8530, Japan

Nonenzymatic reduction of dehydroascorbate into ascorbate by the reduced form (quinol form) of 2-amino-3-carboxy-1,4-naphthoquinone, a strong growth stimulator for bifidobacteria, has been found. The bimolecular reaction rate constant was evaluated as 9 M^{-1} s^{-1} at pH 7.0. This reaction has been successfully coupled with enzymatic regeneration of the naphthoquinol by NAD(P)H in cell-free extracts of *Bifidobacterium longum* 6001. The overall reaction is a regeneration of $NAD(P)^+$ by dehydroascorbate [or a regeneration of ascorbate by NAD(P)H], in which the naphthoquinone/quinol redox couple functions as an electron transfer mediator. Kinetic study of the reduction of dehydroascorbate with related quinol compounds suggested the significance of the amino substituent of the naphthoquinol. A mechanism of the electron transfer from the quinol to dehydroascorbate is proposed, where the first step of the reaction is a nucleophilic addition of the C(2)-amino substituent of the naphthoquinol to the C(2)-position of dehydroascorbate to form a Schiff base intermediate.

INTRODUCTION

Bifidobacteria, predominant microorganisms in the large intestine, have many beneficial effects on human health (Gibson et al., 1995; Benno and Mitsuoka, 1992; Pool-Zobel et al., 1996; Yasui and Ohwaki, 1991; Reddy and Riverson, 1993; Saavedra et al., 1994), exemplified by detoxification of procarcinogens (Grill et al., 1995). To increase the population of bifidobacteria in the human gut, direct oral intake of bifidobacteria may be performed. However, most of the bifidobacteria in foods will be dead before they reach the large intestine due to their high sensitivity to the dioxygen and low pH in the stomach. As an alternative, selective growth stimulation of bifidobacteria has been proposed, and it remains a major concern in food chemistry and gastroenterology. For this purpose, growth stimulators for bifidobacteria, such as fructooligosaccharides and galactooligosaccharides, have been added to foods containing bifidobacteria (Gibson et al., 1995; Modler, 1994; Bouhnik et al., 1997; Saito et al., 1992; Roy et al., 1991). These growth stimulators work as specific substrates for bifidobacteria.

2-Amino-3-carboxy-1,4-naphthoquinone (ACNQ) has been discovered as a novel growth stimulator for bifidobacteria in methanol extractions and cell-free filtrates of *Propionibacterium freudenreichii*, which is used as a starter culture in making Swiss-type cheeses (Kaneko et al., 1994; Mori et al., 1997). ACNQ has exhibited a growth-stimulating effect toward all bifidobacteria tested: *Bifidobacterium longum*, *B. bifidum*, *B. adolescentis*, and *B. breve* (Kaneko et al., 1994). Compared to conventional growth stimulators such as fructooligosaccharides and galactooligosaccharides, ACNQ stimulates their growth at extremely low concentrations (0.5 nM), suggesting that ACNQ does not function as a substrate for bifidobacteria (Kaneko et al., 1994; Mori et al., 1997).

In our previous papers (Yamazaki et al., 1998, 1999), it has been demonstrated that ACNQ oxidizes NAD(P)H with the aid of diaphorase in cell-free extracts of bifidobacteria and that the reduced ACNQ ($ACNQ_{red}$) is reoxidized by dioxygen and/or hydrogen peroxide. The overall reaction is regarded as an $NAD(P)^+$ regeneration by exogenous electron acceptors, and ACNQ functions as an electron transfer mediator. Owing to the ACNQ-mediated $NAD(P)^+$ regeneration, pyruvate as a native electron acceptor for $NAD(P)^+$ regeneration is spared and can be utilized for various biosyntheses. Considering the fact that such a pyruvate-sparing effect stimulates the growth of certain anaerobes, including lactic acid bacteria (Condon, 1987), the ACNQ-mediated NAD-$(P)^+$ regeneration must be responsible for the growth stimulation of bifidobacteria at extremely low concentrations.

Beneficial effects of this ACNQ-mediated pathway will not be limited to pyruvate sparing. This pathway is considered as a reaction to reduce the dioxygen stress by reductive detoxification of hydrogen peroxide with $ACNQ_{red}$. Probably, there may be other toxic compounds in the gut, which could be detoxified by the reduction with $ACNQ_{red}$.

Dehydroascorbate is one of these toxic compounds, at least for mammals, because it is reported to react with

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* Corresponding authors [fax +81-75-753-6456; e-mail kkano@kais.kyoto-u.ac.jp (K.K.) and tikeda@kais.kyoto-u.ac.jp (T.I.)].
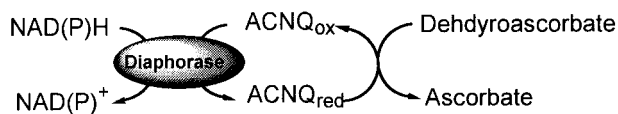† Kyoto University.
‡ Meiji Milk Products Co., Ltd.

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Figure 1. Proposed mechanism of ACNQ-mediated NAD(P)$^+$ regeneration by dehydroascorbate.

Figure 2. Cyclic voltammograms of (A) 25 μM ACNQ and (B) 19 mM dehydroascorbate plus 25 μM ACNQ in completely deaerated 0.1 M phosphate buffer of pH 7.0 at $v = 5$ mV s^{-1} with a bare glassy carbon electrode.

nucleophilic groups of proteins and cause certain types of diabetes (Tolbert and Ward, 1982) and cataracts (Slight et al., 1992). Of course, dehydroascorbate is easily generated by the oxidation of ascorbate with various electron acceptors including dioxygen. Ascorbate is reported to exhibit no remarkable effect on the growth of bifidobacteria (Dave and Shah, 1997), but it is an important vitamin for mammals and is consumed in large quantity through various foods and beverages. Therefore, the reductive detoxification of dehydroascorbate could be very important for mammals. However, nonenzymatic reductions of dehydroascorbate have usually a large kinetic barrier even in thermodynamically favorable cases, as exemplified by the difficulty of the direct reduction of dehydroascorbate with NAD(P)H. To our knowledge, only sulfides such as cysteine and glutathione can reduce dehydroascorbate directly (Borsook et al., 1937).

In this work, we examined the possibility of nonenzymatic reduction of dehydroascorbate into ascorbate by ACNQ$_{red}$ and other related naphthoquinol compounds. The result showed that ACNQ$_{red}$ works as a good electron donor to reduce dehydroascorbate. The nonenzymatic reduction of dehydroascorbate has been successfully coupled with diaphorase-catalyzed regeneration of ACNQ$_{red}$ by NAD(P)H (Figure 1). The overall reaction is an ACNQ-mediated electron transfer from NAD(P)H to dehydroascorbate. The mechanism of this reaction is proposed on the basis of kinetic study using electrochemical techniques. Furthermore, we will briefly discuss possible effects of this reaction in terms of the growth stimulation of bifidobacteria and detoxification of dehydroascorbate in the human gut.

MATERIALS AND METHODS

Materials. ACNQ was extracted from freeze-dried cells of *Propionibacterium freudenreichii* 7025 and purified according to the method of Kaneko et al. (1994). All other chemicals were purchased and used as received. ACNQ and other quinonoid compounds used were dissolved in DMSO and used as stock solutions, whereas dehydroascorbate was dissolved in 0.1 M acetate buffer (pH 4.0) just before use.

Media and Cultivation. The culture medium contained 11 g of bactopeptone, 5 g of yeast extract, 5 g of K$_2$HPO$_4$, 0.5 g of MgCl$_2$·6H$_2$O, 0.5 g of L-cysteine·HCl, 10 mg of FeSO$_4$·7H$_2$O, and 20 g of glucose per liter. The pH of the medium was adjusted to 6.5 with HCl. *B. longum* 6001 is a stock culture of Meiji Milk Products Co. Ltd., and was isolated from human feces. *B. longum* 6001 cells (20 mL) were inoculated into 1.0 L of culture and cultivated anaerobically at 37 °C. Cells were harvested at 16 h after the inoculation by centrifugation at 5000g for 10 min. The harvested cells were washed twice with 0.145 M NaCl solution and suspended in 0.1 M acetate buffer (pH 5.2).

Preparation of Cell-Free Extracts of *B. longum*. The harvested cells of *B. longum* 6001 were disrupted using a Microson XL-2005 ultrasonic cell disrupter (Misonix, Inc., New York). Disruption was carried out in 0.1 M acetate buffer (1 mL) containing 0.5 mg of *B. longum* cells with an optical density of 70−100 at 610 nm. The suspension was cooled with ice during ultrasonication. The suspension was centrifuged at 5000g and 4 °C to remove undisrupted cells, deaerated by
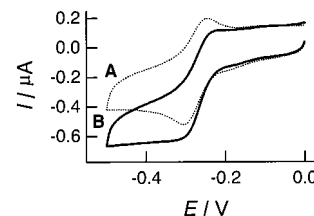
passing moistened argon gas through a needle for 10 min, and stored in a vial equipped with a rubber seal at 4 °C. This suspension was used as cell-free extract of *B. longum*. Protein contents of the cell-free extracts were determined according to a modified Lowry method (Markwell et al., 1978).

Electrochemical Methods. All electrochemical experiments were performed using a Bioanalytical Systems (BAS) 50 W electrochemical analyzer. A glassy carbon disk electrode (BAS, no. 11-2012) or a gold electrode (BAS, no. 11-2014) was polished with 0.05 μm alumina powder (Buehler, no. 3) and used as a working electrode for electrochemical measurements. Platinum disk and Ag|AgCl|KCl (sat.) electrodes were used as the counter and reference electrodes, respectively. The electrolysis basal solution was 0.1 M acetate or phosphate buffer, in which the ionic strength was adjusted to 0.5 M with KCl. Before each measurement, the electrolysis solution was completely deaerated with moistened argon gas.

Continuous monitoring of the concentration of ascorbate and quinones was carried out by the following amperometric method in a three-electrode system. A polished working electrode was covered with a dialysis membrane (20 μm thick in the dry state, pore size = 2.4 nm, no. 20/32, Union Carbide Corp.) as described previously (Ikeda et al., 1996). The membrane-covered electrode was used as a working electrode. Amperometry of quinones and ascorbate was performed at −0.4 and 0.3 V, respectively, under stirring at ∼500 rpm to yield the steady state limiting current. Under these conditions, the reduction current of quinones (at −0.4 V) and the oxidation current of ascorbate (at 0.3 V) were controlled by the dialysis membrane permeability of the analytes and proportional to the bulk concentration (Ikeda et al., 1996). The measurements were carried out under argon atmosphere, and each stock solution was added to the electrolysis solution anaerobically with an airtight syringe.
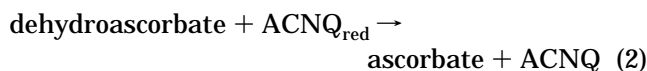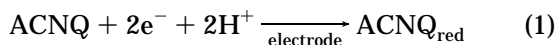
The rate of ACNQ-mediated reduction of dehydroascorbate with NADPH was measured by monitoring ascorbate generation with the membrane-covered electrode at 0.3 V in the test solution containing cell-free extracts of *B. longum* 6001 as well as ACNQ, NADPH, and dehydroascorbate. It is noteworthy that NADPH did not interfere in the oxidative amperometric detection of ascorbate because of the large overpotential of NADPH. This is an advantage of the electrochemical method for this use.

ACNQ$_{red}$ was prepared by bulk electrolysis using a carbon felt electrode as a working electrode in light of the electrolysis efficiency (Kato et al., 2000). A basal electrolysis solution (0.1 M phosphate buffer, pH 7.0) containing ACNQ (1.2 mM) was carefully deaerated, and the electrolysis was performed at −0.65 V vs Ag|AgCl|KCl (sat.) under stirring. With the progress of electrolysis, the color of the solution changed from red to yellowish green, which is characteristic of ACNQ$_{red}$.

Product Analysis with HPLC. Products of the reaction between ACNQ$_{red}$ and dehydroascorbate were analyzed with an HPLC system consisting of a Shimadzu LC-10ADVP chromatograph pump, a Shimadzu DGU-12A degasser, a Rheodyne 7125 sample injector, and a Shimadzu SPD-M10AVPR on-line photodiode array spectrophotometer. A Shimadzu STR-ODS column (250 × 4.6 mm) was used for the HPLC separation at room temperature with a mobile phase (0.1% trifluoroacetic acid) at a constant flow rate (0.5 mL min^{-1}).

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RESULTS AND DISCUSSION

Kinetic Study on Dehydroascorbate Reduction by ACNQ$_{red}$. Curve A in Figure 2 shows a cyclic voltammogram of ACNQ at a bare glassy carbon electrode in pH 7.0 phosphate buffer solution. ACNQ gave a reversible wave ascribed to a two-electron redox reaction. In the presence of dehydroascorbate, the wave shape drastically changed to exhibit catalytic characteristics (curve B). The reductive electrode reaction can be written as follows:

$$ACNQ + 2e^- + 2H^+ \xrightarrow{\text{electrode}} ACNQ_{red} \qquad (1)$$

$$\text{dehydroascorbate} + ACNQ_{red} \rightarrow$$
$$\text{ascorbate} + ACNQ \quad (2)$$

The bimolecular rate constant (k) of the electron transfer from ACNQ$_{red}$ to dehydroascorbate was evaluated from the catalytic steady-state limiting reduction current (I_s), according to

$$I_S = 2FAC_Q\sqrt{kDC_D} \qquad (3)$$

where C_Q and C_D represent the concentrations of ACNQ and dehydroascorbate, respectively, D is the diffusion constant of ACNQ, and F and A are the Faraday constant and the electrode surface area, respectively. The $A\sqrt{D}$ value was evaluated by potential-step chronoamperometry. The evaluated k value was 9.2 ± 0.7 M^{-1} s^{-1} at pH 7.0.

The k value was also evaluated by the following amperometric method. After the complete reduction of ACNQ with a carbon felt electrode at -0.65 V, a membrane-covered glassy carbon electrode was immersed in the test solution as a working electrode in place of the carbon felt electrode, and an excess amount of dehydroascorbate was added to the ACNQ$_{red}$ solution. During the reaction, the concentration change of ACNQ was monitored with the membrane-covered electrode at -0.4 V under stirring, where dehydroascorbate did not interfere in the reductive amperometric detection of ACNQ. On the addition of dehydroascorbate, the reduction current of ACNQ increased with time (eq 2). The reaction kinetics evaluated from the initial slope of the current were pseudo-first-order (data not shown). The bimolecular rate constant was 9.0 ± 0.3 M^{-1} s^{-1}, which is in good agreement with the value evaluated by the voltammetric method.

To identify the reduced product of dehydroascorbate, this reaction mixture was subjected to HPLC analysis with 0.1% trifluoroacetate aqueous solution. The reaction solution gave a single chromatographic peak at 244 nm as shown in Figure 3. The retention time and UV–vis spectrum (Figure 3, inset) of peak 1 were identical to those of the ascorbate standard. This result clearly indicates that ascorbate was generated in the reaction. Dehydroascorbate was not detected due to its low absorption coefficient. ACNQ was difficult to elute under the present conditions, although an acetonitrile gradient elution was applicable for the detection of ACNQ (data not shown). ACNQ$_{red}$ was very rapidly oxidized to ACNQ by dioxygen. These are the reasons the chromatogram gave a single peak under the present conditions.

We also examined the reactivity of some reduced naphthoquinones toward dehydroascorbate in comparison to ACNQ$_{red}$ using the cyclic voltammetric method. The results are summarized in Table 1. The reduced
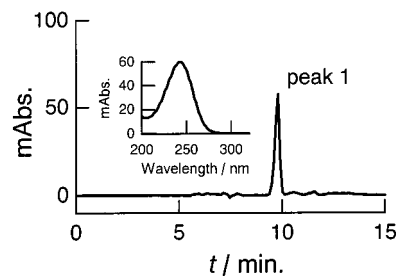

Figure 3. HPLC chromatogram of a test solution after reaction between ACNQ$_{red}$ (1.2 mM) and dehydroascorbate (390 μM). The reaction was performed in completely deaerated 0.1 M phosphate buffer (pH 7.0). HPLC separation was carried out using an ODS column with 0.1% trifluoroacetate aqueous solution as a mobile phase at a flow rate of 0.5 mL min^{-1}. The chromatogram was monitored at 244 nm, which is the maximum wavelength of the absorption of ascorbic acid. (Inset) UV–vis spectrum of peak 1 at $t = 9.8$ s after injection of the sample.

Table 1. Bimolecular Reaction Rate Constants (k) between Dehydroascorbate and Reduced Naphthoquinones^a

quinone	$E^{o\prime}{}_{pH7.0}$ (mV vs NHE)	k (M^{-1} s^{-1})
4-amino-1,2-naphthoquinone	-96	2.8
2-farnesyl-3-methyl-1,4-naphthoquinone	-74	ND^b
ACNQ	-71	9.2
2-methyl-1,4-naphthoquinone	-10	ND

^{a} The bimolecular reaction rate constants were determined from the catalytic current observed by cyclic voltammetry. The measurements were performed in completely deaerated phosphate buffer solutions (pH 7.0). The concentrations of dehydroascorbate and quinones were fixed at 19 mM and 25 μM, respectively. The redox potentials of quinones were determined by cyclic voltammetry. ^{b} Not determined due to too small catalytic current.

forms of 2-farnesyl-3-methyl-1,4-naphthoquinone (vitamin K$_2$) and 2-methyl-1,4-naphthoquinone (vitamin K$_3$) exhibited too low reactivity compared with ACNQ$_{red}$ to evaluate the rate constant. In contrast, the reduced form of 4-amino-1,2-naphthoquinone was active, while the k value was one-third of that of ACNQ$_{red}$. The reactivity of these reduced naphthoquinones toward dehydroascorbate was not correlated with their redox potentials as judged from Table 1. Rather, the amino substituent seems to be important for this reaction.

Proposed Mechanism. On the basis of the above consideration, we considered a mechanism of the oxidation of ACNQ$_{red}$ by dehydroascorbate. The first step of the reaction is a nucleophilic addition of the amino group of ACNQ$_{red}$ to the C(2) position of dehydroascorbate to yield a substrate Schiff base (**1**) in Figure 4. Similar nucleophilic addition of amines to dehydroascorbate is well-known in glycation of the lysine residue of lens proteins, although the dehydroascorbate–protein complex as a Schiff base is easily hydrolyzed unless the Schiff base is reduced by certain reductants (Slight et al., 1992). The Schiff base **1** undergoes a base-catalyzed tautomerization and a C–O bond cleavage to give an *o*-quinone-type Schiff base (**2**) in Figure 4. These reactions closely resemble a transamination reaction proposed for the amine oxidation by quinonoid cofactors (Kano et al., 1997). Considering that ACNQ prefers a *p*-quinone-type electronic structure to an *o*-quinone type (Yamazaki et al., 1998), the Schiff base **2** undergoes a tautomerization to give a *p*-quinone-type Schiff base (**3**), which is hydrolyzed to yield ACNQ and ascorbate. This is in contrast with the transamination reaction for the amine oxidation by quinones, by which a Schiff base like
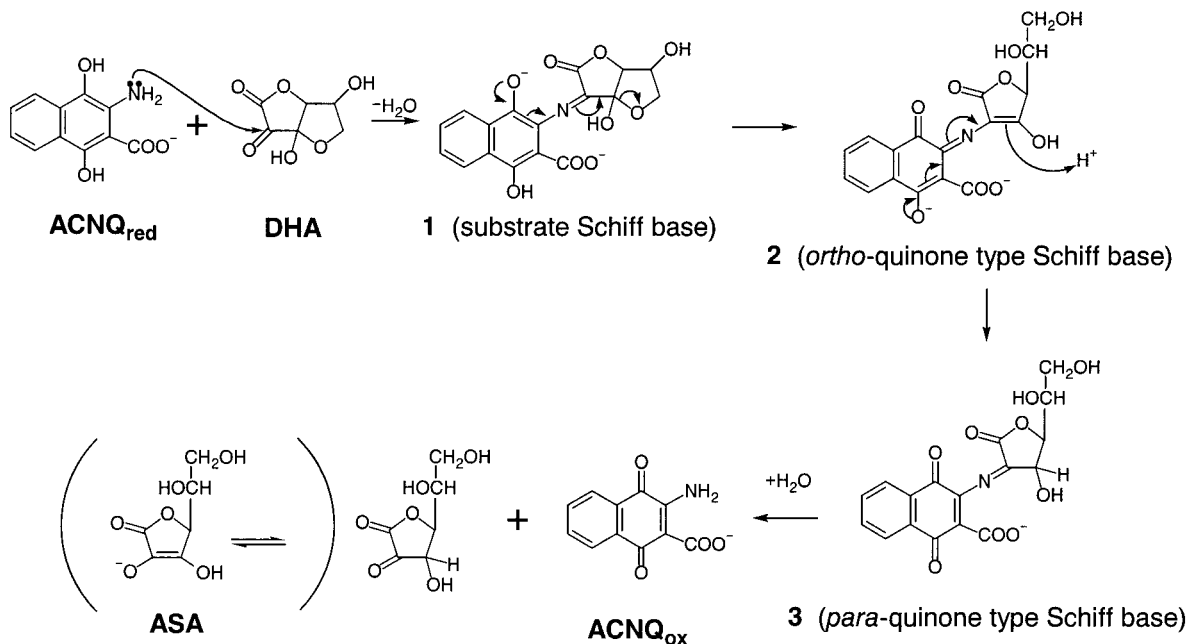
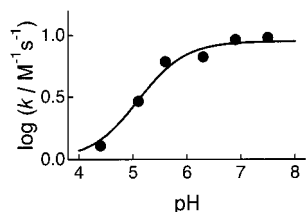**Figure 4.** Proposed mechanism of the reduction of dehydroascorbate by $ACNQ_{red}$.

Figure 5. Bimolecular reaction rate constant (k) between $ACNQ_{red}$ and dehydroascorbate as a function of pH. The values were measured by using cyclic voltammetry or amperometry under completely deaerated conditions. The electrolysis basal solutions used were 0.1 M phosphate (pH >6.0) and 0.1 M acetate buffers (pH <6.0).

product **2** is hydrolyzed and then the nitrogen atom is transferred to the electron acceptor moiety (Kano et al., 1997).

The proposed mechanism involves pH-dependent processes such as the nucleophilic addition of the amino substituent ($pK_a = 4.5$ for $ACNQ_{red}$; Yamazaki et al., 1998) and the dissociation of the phenolic hydroxyl group in the substrate Schiff base **1**. It is reasonably expected that basic conditions are favorable for these processes. Therefore, we examined the effect of pH on the k value. The result is depicted in Figure 5. The k value increased with pH up to 5.5, and the values became practically constant above pH 5.5. The pH dependence of the k value seems to be reasonable on the basis of Figure 4. Under acidic conditions, the nucleophilic addition of $ACNQ_{red}$ would not occur, or, if it occurs, the Schiff base **1** would be hydrolyzed before the tautomerization into the Schiff base **2**, as in the case of the dehydroascorbate–protein complexes.

ACNQ-Mediated NAD(P)$^+$ Regeneration by Dehydroascorbate in Cell-Free Extracts of *B. longum*. In bifidobacteria, $ACNQ_{red}$ is generated by NAD(P)H with the aid of diaphorase-like activity (Yamazaki et al., 1999). Therefore, in principle, dehydroascorbate can be reduced indirectly by NAD(P)H in the presence of ACNQ in cell-free extracts of bifidobacteria as shown in Figure 1. We tried to verify this point and investigated the reaction from the kinetic viewpoint. The reaction was monitored by amperometry using the
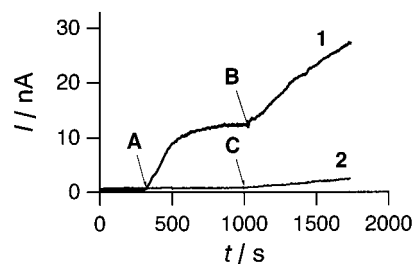

Figure 6. Amperometric monitoring of the reduction of dehydroascorbate and ACNQ by NADPH ($250 \mu M$) in cell-free extracts of *B. longum* 6001 (0.038 mg of protein/mL) in the presence (curve 1) and absence (curve 2) of ACNQ. The test solutions (0.1 M phosphate buffer, pH 7.0, 1.0 mL) containing NADPH and cell-free extracts were carefully deaerated before experiments. Curve 1: 12 μM ACNQ and 1.2 mM dehydroascorbate were added into the test solution at points A and B, respectively. Curve 2: 1.2 mM dehydroascorbate was added into the test solution at point C in the absence of ACNQ. The amperometric detection of ascorbate and $ACNQ_{red}$ was performed with a membrane-covered gold electrode at 0.3 V vs Ag|AgCl|KCl (sat.) under stirring.

membrane-covered gold electrode at 0.3 V vs Ag|AgCl|KCl (sat.), at which NAD(P)H gave no detectable current, and then the oxidation current is ascribed to $ACNQ_{red}$ and/or ascorbate alone.

Figure 6 shows the time dependence of the generation of $ACNQ_{red}$ and/or ascorbate in cell-free extracts of *B. longum* 6001. On the addition of ACNQ into the buffer solution (pH 7.0) containing NADPH and cell-free extracts of *B. longum* (Figure 6, point A on curve 1), the oxidation current started increasing and then reached a constant value. The increase in the current indicates an increase in the concentration of $ACNQ_{red}$ generated by NADPH diaphorase-like activity of *B. longum*. After ACNQ was completely reduced to $ACNQ_{red}$, the current reached a plateau. The subsequent addition of an excess amount of dehydroascorbate caused a subsequent increase in the oxidation current (Figure 6, point B on curve 1). The increase in the current is reasonably ascribed to the generation of ascorbate by the reduction of dehydroascorbate with $ACNQ_{red}$. As shown in Figure 1, the product ACNQ is

re-reduced by NADPH with the aid of diaphorase in the cell-free extracts; that is, ACNQ functions as a mediator in the electron transfer from NADPH to dehydroascorbate. This is the reason the current increased continuously without plateauing-off.

A similar experiment was performed in the absence of ACNQ (Figure 6, curve 2). After the addition of dehydroascorbate into a suspension of cell-free extracts of *B. longum* containing NADPH, the oxidation current increased only slightly (Figure 6, point C on curve 2). The rate of the ascorbate generation is ~10 times lower than that observed in the presence of ACNQ (Figure 6, curve 1). The very slow generation of ascorbate in the absence of ACNQ (8.3 nM s^{-1}) might be ascribed to putative NADPH−dehydroascorbate oxidoreductase activity. These data support the assumption that ACNQ assists NAD(P)$^+$ regeneration by dehydroascorbate according to Figure 1. From the difference in the initial slopes of curves 1 and 2 in Figure 6, the rate of the ACNQ-mediated electron transfer was evaluated to be 68 nM s^{-1}. The bimolecular rate constant between dehydroascorbate and ACNQ$_{red}$ was roughly evaluated as 4.7 M^{-1} s^{-1} under an assumption that all of the ACNQ is in a fully reduced state during the mediated reaction. This value is ~50% of those determined by voltammetry and amperometry. The discrepancy would be due to an overestimation of the (steady-state) concentration of ACNQ$_{red}$ during the mediated reaction.

Summary. We have clearly demonstrated that dehydroascorbate is nonenzymatically reduced by ACNQ$_{red}$. The reaction has been successfully coupled with the diaphorase-catalyzed oxidation of NAD(P)H to regenerate ACNQ$_{red}$ using cell-free extracts of *B. longum* 6001. The overall reaction is the oxidation of NAD(P)H by dehydroascorbate. The first step of the reaction seems to be the nucleophilic addition of the amino substituent of ACNQ$_{red}$ to dehydroascorbate to generate a Schiff base. The subsequent reaction mechanism has been proposed by referring to the mechanism of the amine oxidation by *o*-quinones. The fact that ACNQ has a *p*-quinone type of electronic structure seems to be important to yield ACNQ and ascorbate from the final Schiff base intermediate.

The ACNQ$_{red}$ oxidation by dehydroascorbate could occur in bifidobacterial cells and can be considered as a novel NAD(P)$^+$ regeneration pathway. The pathway could spare pyruvate and then have an important role in the possible growth stimulation of bifidobacteria in the gut. Another important point is that dehydroascorbate, a cause of cataracts and certain diabetes, could be detoxified rapidly in the gut in the presence of ACNQ and bifidobacteria. The product ascorbate is an important vitamin for mammals. Therefore, some beneficial effects may be expected for mammals.

ABBREVIATIONS USED

ACNQ, 2-amino-3-carboxy-1,4-naphthoquinone; ACNQ$_{red}$, the fully reduced form of ACNQ; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

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