  Bull. Chem. Soc. Jpn., 63, 417—420 (1990)

Chemical Behavior of Coenzyme PQQ toward Aminoguanidine: Redox Reaction and Adduct Formation

Minae MURE, Kazumi NII, Shinobu ITOH, and Yoshiki OHSHIRO*
Department of Applied Chemistry, Faculty of Engineering, Osaka University, Yamadaoka 2-1, Suita, Osaka 565
(Received August 29, 1989)

The reaction of coenzyme PQQ with aminoguanidine, which is known as an inhibitor of quinoprotein amine oxidases, was investigated in vitro. The redox reaction predominantly proceeded at pH 10.0 to give $PQQH_2$ (quinol), whereas deactivation of PQQ occurred at pH 6.7 to give the triazine adduct. In the case of semicarbazide or acetohydrazide as the substrate, azo adduct formation was mainly observed even at pH 10.0. Importance of the C-5 carbinolamine-type intermediate **a** is discussed.

Copper-containing amine oxidases involve a second organic cofactor which is covalently bound to the enzymes and interacts strongly with carbonyl reagents such as hydrazine derivatives.[1] The structure of the organic cofactor has not been clearly identified for a long time, though pyridoxal phosphate (PLP) or a modified pyridoxal had been proposed initially as a possible cofactor without any conclusive evidence.[1] In 1984, two independent groups reported that the enzymes might involve PQQ (4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylic acid) as the cofactor.[2,3] Phenylhydrazine and 2,4-dinitrophenylhydrazine have been generally used to detect the coenzyme, because they give pronounced spectral changes during the inhibition process.[1,4] Aminoguanidine is also known as an irreversible inhibitor of the amine oxidases,[5] but details of its inhibitory action in the enzymatic system have not been clarified yet, because it does not produce such a strongly characteristic chromophore.

In this paper, we studied the reaction between PQQ and aminoguanidine in vitro to find that either redox reaction or adduct formation reaction occurred depending on the pH. The present results may give very important information not only for the enzymatic studies (inhibition mechanism) but also for the study on the oxidation mechanism of amine homologues by PQQ.

Results and Discussion

The reaction of PQQ and aminoguanidine was examined spectrophotometrically in an aqueous buffer solution at 30 °C under *anaerobic* conditions. A slow increase in absorption at 286 and 348 nm was observed at pH 6.7, and the final spectrum was quite different from that of reduced PQQ (Fig. 1). Product analysis was carried out by using PQQTME (trimethyl ester of PQQ), because PQQTME is easier to handle than PQQ itself. Treatment of PQQTME with 10-fold excess of aminoguanidine sulfate in refluxing MeOH for 6 h gave 3-amino-1,2,4-triazine derivative **1** (Eq. 1). IR spectrum shows typical absorption at 3456, 3312, 3200, and 1646 cm^{-1} of the 3-amino-1,2,4-triazine structure.[6] Although two possible regioisomers (**1** and **1'**) are considered, ^1H NMR and HPLC analyses indicate the presence of only one isomer. It is impossible to determine the actual structure with the spectral data available at the present. However, the structure of type **1** is considered to be plausible, since an absorption spectrum characteristic of C-5 adducts of PQQ[7] appears at the initial stage of the reaction (a in Fig. 1). It is well-known

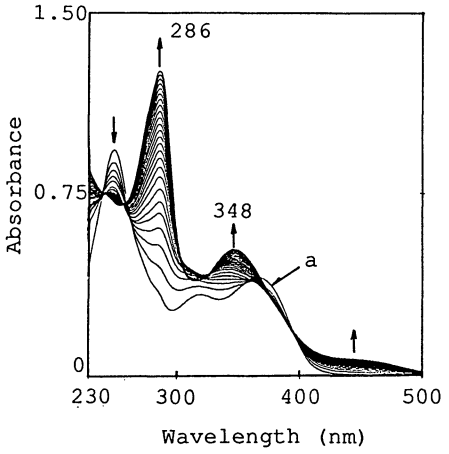
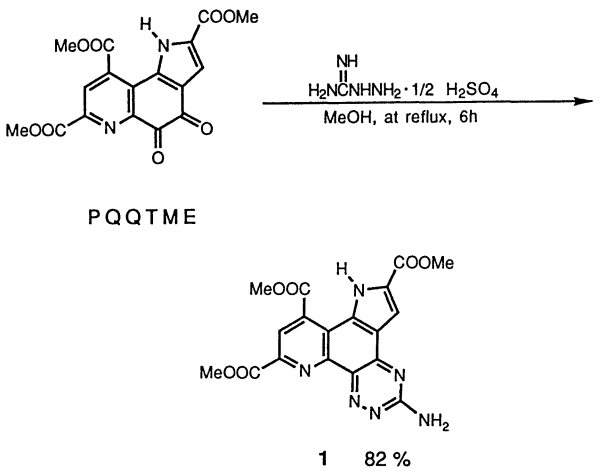
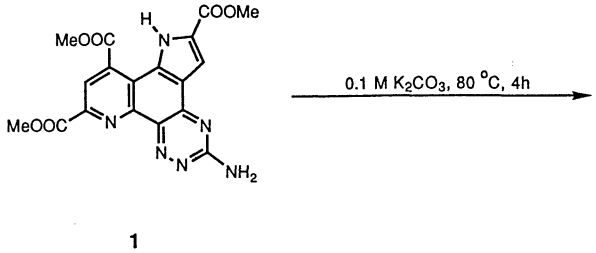


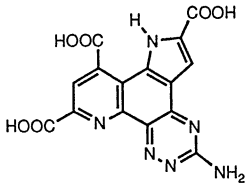
Fig. 1. Spectral change along the progress of the reaction of PQQ with aminoguanidine at pH 6.7. [PQQ]=4.0×10^{-5} M [aminoguanidine]=8.0×10^{-2} M, 0.1 M phosphate buffer (μ=0.2 with KCl), at 30 °C, under *anaerobic* conditions (N_2).



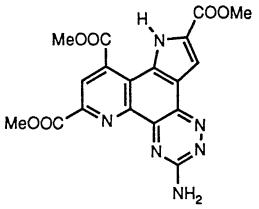
(1)



1



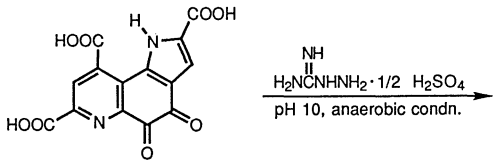
quant. (2)



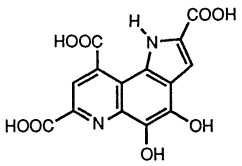
1'

that the C-5 adduct of H_2O or acetone shows such characteristic spectrum which lacks in either absorption of quinonoid n→π* transition at around 475 nm (very broad and weak) and a shoulder at around 270 nm but has two absorption maxima at around 330 and 360 nm. Alkaline hydrolysis of the three ester groups of **1** gave the same compound as in the reaction of PQQ and aminoguanidine, indicating that the product having λ_{max} 286 and 348 nm was the tricarboxylic acid derivative of **1** (triazine derivative of PQQ, Eq. 2).

On the other hand, the formation of $PQQH_2$ (reduced PQQ in quinol form) proceeded mainly at pH 10.0 (Fig. 2a), and PQQ was easily regenerated by aeration of the final reaction solution (Fig. 2b). The reduced PQQ ($PQQH_2$) was isolated in the preparative scale experiment under the same conditions (Eq. 3).



PQQ



quant. (3)

From these results, we propose the mechanism shown in Scheme 1. The amino group of aminoguanidine attacks the C-5 position of PQQ to form a carbinolamine type intermediate **a**. It is well-known that the C-5 quinone carbon is very reactive toward various nucleophiles.[7] This kind of intermediate is also proposed in amine-oxidations.[8] As mentioned above, a characteristic UV-vis spectrum of the C-5 adduct of PQQ[7] was observed at the initial stage of the reaction. Electron flow (shown as arrows in Scheme 1) at the stage of the intermediate **a** will lead to the generation of $PQQH_2$. If such electron flow is not fast enough, the dehydration from the intermediate **a**

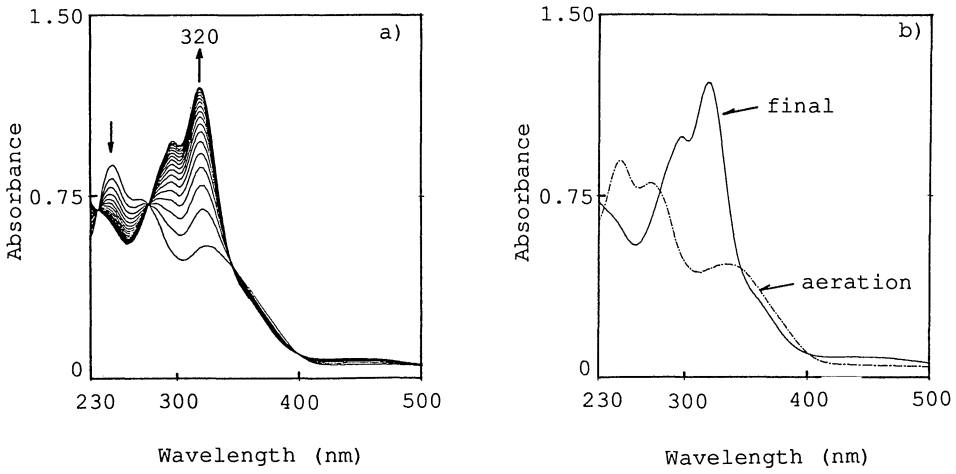
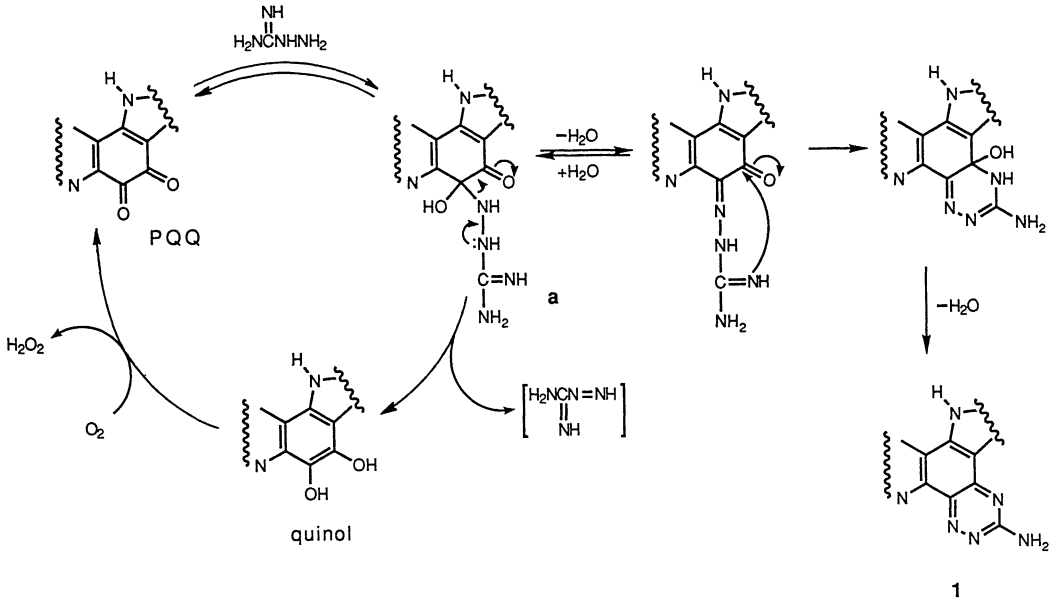


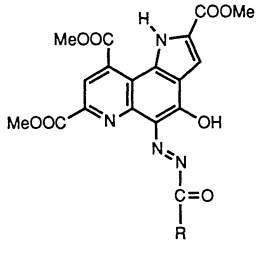
Fig. 2. Spectral change along the progress of the reaction of PQQ with aminoguanidine at pH 10.0. [PQQ]=4.0×10⁻⁵ M, [aminoguanidine]=4.0× 10⁻⁴ M, 0.1 M carbonate buffer (μ=0.2 with KCl), at 30 °C. a) Under *anaerobic* conditions (N_2). b) After aeration of the final reaction mixture.



Scheme 1.

predominantly proceeds to give the hydrazone adduct, which is converted into the 3-amino-1,2,4-triazine derivative by subsequent intramolecular cyclization and aromatization.

It is interesting that the pH of the solution controls the reaction path in the present reaction. At lower pH where almost all the guanidino groups are protonated, the electron flow must be suppressed because of the strong electron-withdrawing effect of the protonated guanidino group, and the acid-catalyzed dehydration could be accelerated. Consequently, the adduct formation mainly proceeded at pH 6.7. On the contrary, the protonation of the guanidino group and the acid-catalyzed dehydration would be depressed at higher pH (pH 10) to allow the electron flow to give the quinol as a major product. Importance of the electron-withdrawing effect of the substituent attached to hydrazino group was also found in the reaction between PQQ and semicarbazide or acetohydrazide. The azo adduct formation (**2** and **3**)[9] was mainly observed in the reaction of PQQ and semicarbazide or acetohydrazide under *anaerobic* conditions even at higher pH (pH 10). Thus, it could be said that the more electron-withdrawing nature the substituent attached to the hydrazino group has, the more preferable the adduct formation is.



R = NH_2 : **2**
R = CH_3 : **3**

Slow formation of the 3-aminotriazine adduct was also observed even at pH 10.0 when the reaction was carried out under *aerobic* conditions. This means the reduction of PQQ (major reaction) and the formation of the 3-amino-1,2,4-triazine (minor reaction) competitively occurred, and PQQ is gradually converted into the redox inactive derivative during an aerobic autorecycling process (Scheme 1). The similar inhibition process is presumed to take place in the enzymatic system.

Experimental

PQQ and its trimethyl ester (PQQTME) were prepared according to the reported method.[10] Aminoguanidine sulfate, semicarbazide hydrochloride, and acetohydrazide hydrochloride were obtained commercially and used without further purification. Ultraviolet and visible absorption spectra were recorded on a Shimadzu UV-265 spectrophotometer equipped with a temperature controlled cell holder, Shimadzu TCC-260. HPLC analysis was performed by using a Waters Model 510 (pump), a Lambda-Max Model 481 (UV-monitor), and a Radial Compression Separation System (C_{18}). Infrared spectra were recorded on a Hitachi 270-30 infrared spectrophotometer, and ^1H NMR spectra were obtained on a JEOL FT-NMR JNX-FX90Q (90 MHz) and a JEOL FT-NMR GSX 270S (270 MHz) spectrophotometers. Mass spectra (MS) were obtained on a JEOL JNX DX 303 HF mass spectrophotometer.

Reaction of PQQ and Aminoguanidine (UV-vis Scale). Spectrophotometrical examination of the reaction of PQQ and aminoguanidine was performed under the conditions of [PQQ]=4.0×10^{-5} M, (1 M=1 mol dm^{-3}) and [aminoguanidine]=4.0×10^{-4} M at 30 °C. Typically, 1.5 ml of an aqueous buffer solution containing PQQ (8.0×10^{-5} M) was mixed with 1.5 ml of an aqueous buffer solution containing

aminoguanidine (8.0×10^{-4} M) in a Thunberg cuvette. Both solutions were degassed by bubbling N_2 before the reaction if necessary.

Reaction of PQQTME and Aminoguanidine. PQQTME (5 mg, 1.34×10^{-5} mol) was treated with 10-fold excess of aminoguanidine sulfate in refluxing methanol for 6 h. The resulting precipitates were collected by centrifugation, washed with water, and dried in vacuo to yield an orange solid **1** (4.5 mg, 82%): mp (decomp) 278—280 °C; ^{1}H NMR (DMSO-d_6) δ=4.03, 4.17, 4.21 (each s, 3H, OCH_3), 6.61 (br s, 2H, NH_2), 8.08 (d, J=2.3 Hz, 1H, aromatic 3-H), 9.10 (s, 1H, aromatic 8-H), 12.60 (br s, 1H, pyrrole NH); IR (KBr, cm^{-1}) 1724 (-$COOCH_3$) 3456, 3312, 3200, 1646 (aminotriazine NH_2); Mass spectrum m/z 410 (M^+).

Product Analysis of the Reaction of PQQ and Aminoguanidine. Hydrolysis of the methyl ester groups of **1** (2.0 mg) was carried out in 0.1 M K_2CO_3 at 80 °C and was followed by HPLC. When no further change was observed (4 h), the aliquot was diluted with 0.1 M carbonate buffer (pH 10.0) and the UV-vis spectrum was taken. The hydrolysate showed the same absorption (λ_{max} at 286 and 348 nm) and the same retention time in HPLC analysis with those of the product in the reaction of PQQ and aminoguanidine at pH 10.0.; HPLC, solvent: MeOH/H_2O/85% H_3PO_4, 45/54.5/0.5 (v/v/v). flow rate: 1.0 ml min^{-1}, retention time: 12.8 min, UV-vis spectrum (λ_{max}, 0.1 M carbonate buffer, pH 10.0), 284 and 348 nm.

Reaction of PQQ with Aminoguanidine at pH 10 under *Anaerobic* Conditions. After quantitative hydrolysis of PQQTME (7.2 mg, 19.3 μmol) to PQQ in 0.1 M Na_2CO_3 (5 ml) at 60 °C for 6 h, the solution was placed in the bottom of a Thunberg vessel (30 ml), and 50-fold excess of aminoguanidine sulfate in 0.1 M $NaHCO_3$ (0.56 ml) was deposited in the side arm of the vessel. Both solutions were degassed by bubbling Ar for 30 min and then they were mixed to start the reaction. After 24 h, the reaction mixture was acidified with 2 M HCl to adjust the pH of the solution to 2 under Ar. The resulting precipitates were collected by centrifugation, washed with water, and dried in vacuo. ^{1}H NMR, HPLC analysis, and UV-vis spectrum revealed that the product was identical with reduced PQQ in quinol form ($PQQH_2$).[11]

Reaction of PQQ and Semicarbazide or Acetohydrazide. PQQTME (10 mg, 2.69×10^{-5} mol) was treated with 10-fold excess of semicarbazide hydrochloride in methanol at 60 °C for 5 h to yield 1 : 1 azo adduct **2**[9] as a yellow solid. **2** (64%): mp (decomp) 225—230 °C; ^{1}H NMR (DMSO-d_6) δ=4.00, 4.13, 4.18 (each s, 3H, -OCH_3), 7.11 (br s, 2H, -NH_2), 7.17 (d, J=2.3 Hz 1H, aromatic 3-H), 8.40 (s, 1H, aromatic 8-H), 12.40 (br s, exchangeable, pyrrole NH), 14.39 (br s, exchangeable, -OH); IR (KBr, cm^{-1}) 1730 (-$COOCH_3$), 1650 (-NHCO-); Mass spectrum m/z 430 (M^++1).

Reaction of PQQTME and acetohydrazide hydrochloride was carried out in the same manner (reaction time 20 h). **3** (85%): mp (decomp) 210—220 °C; ^{1}H NMR (DMSO-d_6) δ=3.91, 3.99, 4.07 (each s, 3H, -OCH_3), 7.29 (br s, 1H, aromatic 3-H), 8.48 (s, 1H, aromatic 8-H), 12.42 (br s, exchangeable, pyrrole NH), 14.41 (br s, exchangeable, -OH); IR (KBr, cm^{-1}) 1724 (-$COOCH_3$), 1668 (-NHCO-), 1630 (C=O); Mass spectrum m/z 429 (M^++1).

Methyl ester groups of **2** and **3** were hydrolyzed in 0.1 M K_2CO_3 for 6—10 h at 60 °C to give the corresponding tricarboxylic acid derivatives, respectively (**4** and **5**). Each of the products showed the same UV-vis spectrum and the same retention time in HPLC analysis with those of the product in the reaction with PQQ and semicarbazide and acetohydrazide at pH 10.0, respectively. **4**: UV-vis spectrum (λ_{max} 289 and 326 nm); HPLC retention time (12.2 min). **5**: UV-vis spectrum (λ_{max} 259, 287, and 365 nm); HPLC retention time (11.5 min).

References

1) G. Petterson, "Structure and Functions of Amine Oxidases," ed by B. Mondovi, CRC Press, Boca Raton, FL, 5 (1982).

2) M. Ameyama, M. Hayashi, K. Matsushita, E. Shinagawa, and O. Adachi, *Agric. Biol. Chem.*, **48**, 561 (1984).

3) C. L. Lobenstein-Verbeek, J. A. Jongejan, J. Frank, and J. A. Duine, *FEBS Lett.*, **170**, 305 (1984).

4) R. A. van der Meer, J. A. Jongejan, and J. A. Duine, *FEBS Lett.*, **221**, 299 (1987).

5) T. Bieganski, Z. Osinska, and C. Maslinski, *Int. J. Biochem.*, **14**, 949 (1982).

6) H. Neunhoeffer, "Comprehensive Heterocyclic Chemistry," ed by A. R. Katritzky and C. W. Rees, Pergamon Press, Oxford (1984), Chap. 2.19, p. 395.

7) R. H. Dekker, J. A. Duine, J. Frank. Jzn, and J. Westerling, *Eur. J. Biochem.*, **125**, 69 (1982).

8) S. Itoh, Y. Kitamura, Y. Ohshiro, and T. Agawa, *Bull. Chem. Soc. Jpn.*, **59**, 1907 (1986).

9) The product has the possibility to exist as hydrazone tautomer, but the azo form is likely to predominate because of the absence of C=O stretching band in IR spectrum and the existence of δ 14.39 resonance in ^{1}H NMR spectrum which is characteristic OH proton in azo form: J. E. Kuder, *Tetrahedron*, **28**, 1973 (1972). F. D. Saeva, *J. Org. Chem.*, **36**, 3842 (1971).

10) E. J. Corey and A. Tramontano, *J. Am. Chem. Soc.*, **103**, 5599 (1981).

11) S. Itoh, Y. Ohshiro, and T. Agawa, *Bull. Chem. Soc. Jpn.*, **59**, 1911 (1986).