Kinetics and Mechanism of the Oxidative Deamination of Amines by Coenzyme PQQ

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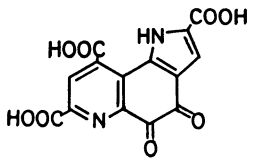
(Received January 27, 1986)

Kinetic studies on the oxidative deamination of amines by coenzyme PQQ are carried out under anaerobic conditions. The reaction is first-order in the appearance of the reduced PQQ and in the total amine concentrations. A large kinetic isotope effect (k_H/k_D=7.24) in the oxidation of α,α-dideuteriobenzylamine indicates that the C-H bond cleavage is a rate-determining step. From the results of formation of two types of reduced PQQ (quinol and aminophenol) and low reactivities of secondary and tertiary amines, we propose the mechanism via covalent addition of the amine to the quinone followed by rate-limiting α-proton removal.

Amine oxidases are known to catalyze the oxidative deamination of amines in the presence of molecular oxygen as an electron acceptor to produce the corresponding carbonyl compounds, hydrogen peroxide, and ammonia (Eq. 1).[1]

$$RCH_2NH_2 + O_2 + H_2O \longrightarrow RCHO + H_2O_2 + NH_3 \quad (1)$$

They are divided into two classes: Copper-containing amine oxidases and FAD-containing ones. However, the chromophore of the former enzymes, which has a carbonyl function,[2] has not been identified despite of the presence of many investigations extended over a long period of time.[1-3] Recently, Ameyama and his co-workers demonstrated that 4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylic acid (PQQ, methoxatin) is the coenzyme of a methylamine dehydrogenase and copper-containing amine oxidases from beef plasma, pig kidney, and *Aspergillus niger*.[4] Duine and his co-workers simultaneously indicated the possible occurrence of PQQ in bovine serum amine oxidase, a mammalian copper-containing amine oxidase.[5]



PQQ

PQQ is a novel coenzyme which was originally discovered and identified in methanol dehydrogenases of methylotropic bacteria.[6] In addition to methanol dehydrogenases from various methanol-grown microorganisms,[7] several types of alcohol, aldehyde, and glucose dehydrogenases have been revealed to be PQQ-containing enzymes (quinoproteins).[8] Methylamine dehydrogenase of *Pseudomonas* AM-1 has also been shown to be a quinoprotein in which PQQ is attached to the enzyme protein via a covalent bond.[9] Total syntheses[10] and some chemical properties[11] of PQQ have already been reported. A mechanism for alcohol and amine oxidation by quinoprotein, however, has not been clarified, though a few enzymological studies have been developed.[12,13]

As a model system of amine oxidases, we have already demonstrated the micellar enhanced oxidative deamination of amines catalyzed by coenzyme PQQ by two electron transferring from the amine to molecular oxygen.[14] We wish to demonstrate here the kinetics of the oxidative deamination of amines by PQQ and product analysis of the reduced PQQ to elucidate the mechanism of the reaction under anaerobic conditions.[15]

Experimental

PQQ was prepared according to the reported method.[10a] All amines investigated (except for α,α-dideuteriobenzylamine) and CTAB (hexadecyltrimethylammonium bromide) were obtained commercially and purified by the standard method.[16] α,α-Dideuteriobenzylamine was prepared according to the known method,[17] and the ^{1}H NMR indicated that the content of the deuterated compound was more than 99%.

Ultraviolet and visible absorption spectra were recorded on Shimadzu UV-240 spectrophotometer equipped with a temperature controlled cell holder, Shimadzu TCC-240. Measurements of pH were performed by using Horiba pH-meter F-8. HPLC analysis was performed by using Waters Model 510 (pump)-Lambda-Max Model 481 (UV-monitor)-Radial Compression Separation System (C_{18}).

Kinetics. The kinetic measurements of the oxidation of amines with PQQ were carried out in a carbonate buffer solution (0.05 M (1 M=1 mol dm^{-3}) pH 10.1, μ=0.2 with KCl) at 35 °C under anaerobic conditions. Typically, 1.5 ml of an aqueous bufffer solution containing CTAB (4.0×10^{-3} M) and an amine (8.0×10^{-3} M) was mixed with 1.5 ml of an aqueous buffer solution containing PQQ (8.0×10^{-5} M) in a Thunberg cuvette. Both solutions were degassed by bubbling N_2 through them before the reaction. The progress of the reaction was followed by monitoring the appearance of the reduced PQQ at 320 nm.

Product Analysis of Reduced PQQ. After the reaction of PQQ (20 mg, 5.7×10^{-2} mmol) and benzylamine (2.9×10^{-1} mmol) in a carbonate buffer solution (0.1 M, pH 10, 20 ml) under anaerobic conditions (N_2) for 24 h, the reduced product (19.2 mg) was precipitated by adding 2 M HCl (2 ml). HPLC analysis and UV spectrum revealed that there were at least two types of reduced compounds from PQQ;

HPLC, column: radial pack cartridge C_{18}(Waters), solvent: MeOH/H_2O/85% H_3PO_4, 45/54.5/0.5, v/v/v, flow rate: 2.0 ml min^{-1}, retention time: 4.9 min (for **1**) and 3.3 min (for **2**), (ratio of peak area, **1**:**2**=82:18), UV-spectrum (λ_{max}, CH_3CN), 320 nm (**1**) and 301 nm (**2**). Both products, **1** and **2**, were readily converted into PQQ by treating with an alkaline aqueous solution under aerobic conditions. The main product **1** was identical with $PQQH_2$ (quinol)[11b] in HPLC analysis, ^1H NMR spectrum, and UV spectrum in the mixture.

Results and Discussion

The reaction of benzylamine with PQQ was studied under pseudo-first-order conditions of total benzylamine concentration (4.0×10^{-3} M) greatly exceeding [PQQ]=4.0×10^{-5} M in the presence of CTAB (2.0×10^{-3} M) in a carbonate buffer solution (0.05 M, pH 10.1, μ=0.2 with KCl) at 35 °C. Monitoring of an appearance of the product from PQQ at 320 nm established a short lag phase followed by the first-order appearance of the product ($k_{obsd}=1.75\times10^{-3}$ s^{-1}, Fig. 1). Introduction of O_2 into the final reaction mixture led to quantitative regeneration of PQQ. The reaction was also first-order in total benzylamine concentrations (Fig. 2). In this oxidation reaction, the free amine is considered to be an active species.

$$k_{obsd} = k_2[\text{Amine}]_f = k_2\frac{K_a}{K_a+a_H}[\text{Amine}]_T \qquad (2)$$

$[\text{Amine}]_f$: free amine concentration

$[\text{Amine}]_T$: total amine concentration

Thus, the second-order rate constant k_2 is calculated to be 5.36×10^{-1} $M^{-1}s^{-1}$ (at pH 10.1) from Eq. 2 by using the pK_a value of 9.45 for benzylamine determined by volumetric titration under the kinetic conditions. The second-order rate constants at pH 10.1 for the other amines are listed in Table. Interestingly, β-phenethylamine and cyclohexylamine were oxidized faster than the benzylic amines. Incorporation of the substrates into the hydrophobic core of the micelle may be an important factor.[18]

It should be noted that *N*-methylbenzylamine was

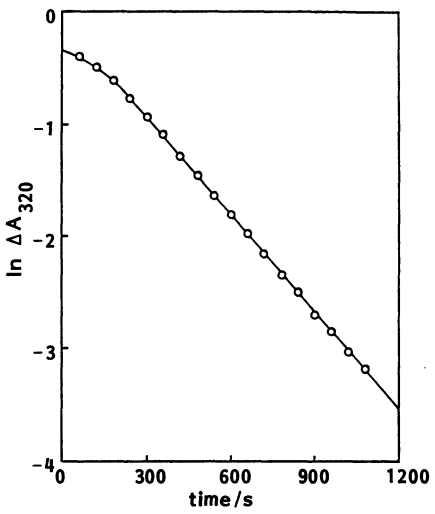


Fig. 1. Pseudo-first-order plot for the oxidation. PQQ (4.0×10^{-5} M), $PhCH_2NH_2$ (4.0×10^{-3} M), CTAB (2×10^{-3} M), pH 10.1 with 0.05 M carbonate buffer (μ=0.2 with KCl), 35 °C, anaerobic conditions (N_2).

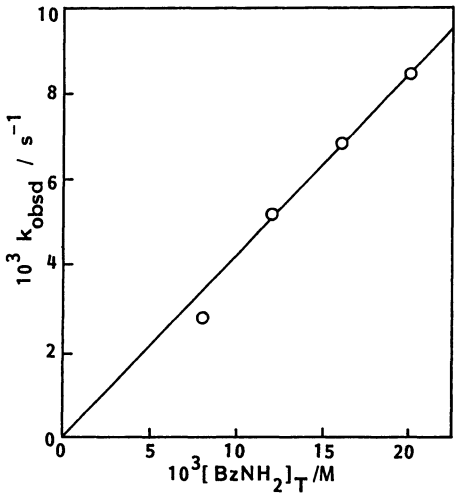
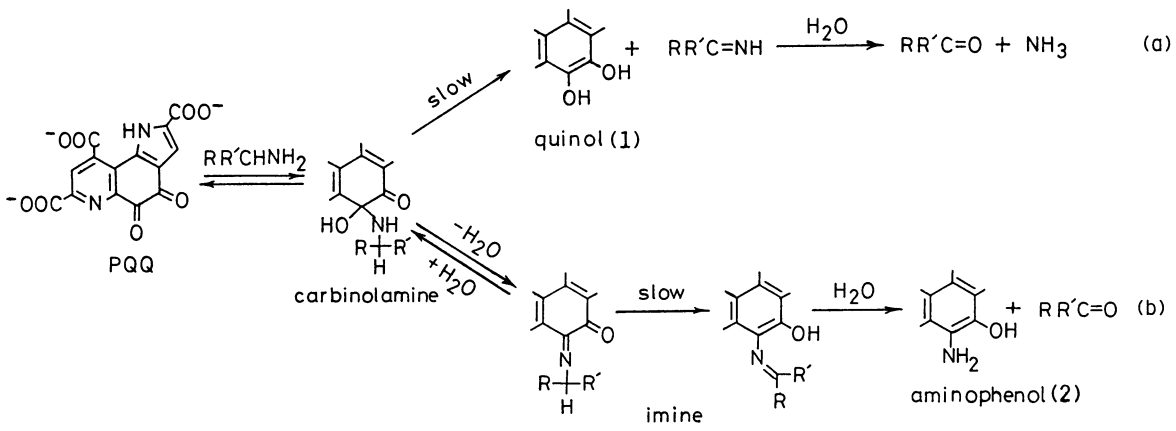


Fig. 2. Dependence on total benzylamine concentration of the pseudo-first-order rate constant (k_{obsd}). pH 10.1 with 0.05 M carbonate buffer (μ=0.2 with KCl), 35 °C.

Table. The Second-Order Rate Constant for the Oxidation of Amines by PQQ

Amine	$10^3 k_{obsd}$ / s^{-1}	pK_a	10^3[Amine]$_f$[a] / M	k_{obsd}/[Amine]$_f$ / $M^{-1}s^{-1}$
$PhCH_2NH_2$	17.5	9.45	3.26	0.536
$PhCH(CH_3)NH_2$	7.69	9.33	3.42	0.225
$Ph(CH_2)_2NH_2$	45.1	9.78	2.71	1.67
Cyclohexylamine	9.69	10.33	1.49	0.650
$PhCH_2NHCH_3$	0.81	9.57	3.10	0.026
$PhCH_2N(CH_3)_2$	0	9.02	3.70	0

a) Free amine concentrations at pH 10.1 calculated from the pK_a values determined by volumetric titration under the kinetic conditions. [PQQ]=4.0×10^{-5} M, [Amine]$_T$=4.0×10^{-3} M, 0.05 M carbonate buffer (μ=0.2 with KCl, pH 10.1) 35 °C, anaerobic conditions.



Scheme.

oxidized very slowly (k_2=2.6×10^{-2} M^{-1}s^{-1}) and *N*,*N*-dimethylbenzylamine was not oxidized under the same conditions. Thus, the order of the reactivities of the amines is primary>secondary≫tertiary. Furthermore, a large kinetic isotope effect (k_H/k_D=7.24) was observed for the reaction with benzylamine-d_2, indicating the C–H bond cleavage is a rate determining step.

Bruice and his co-workers recently investigated the chemical behavior of model compounds of PQQ and their reactions with various amines.[19,20] They suggested that the oxidation of primary amines by the model compounds proceeds via two competing covalent addition base-catalyzed elimination mechanisms through intermediate carbinolamine and imine, respectively, and they confirmed the formation of two types of reduced compounds (quinol and aminophenol).[20] Thus, we tried to isolate the reduced compound of PQQ in the reaction of PQQ with an amine, and found the formation of two types of reduced PQQ. Since the main product (**1**) was identical with $PQQH_2$ (quinol), the minor product (**2**) is assumed to be an aminophenol type one, though these two compounds could not be separated because of their instability in the mixture.

From these results, the mechanism of the reaction of the amines and PQQ is considered to be similar to that proposed by Bruice and his co-workers for the reaction of a PQQ-model, 7,9-didecarboxymethoxatin with amines (Scheme). That is to say, the oxidation of the amine by PQQ proceeds mainly via covalent addition of the amine to the quinone to form the carbinolamine intermediate followed by rate determining α-proton removal providing the quinol **1** (path a).[21] The reaction through the formation of the imine intermediate between PQQ and the amine followed by successive rearrangement and α-proton removal to provide the aminophenol **2** (path b) is considered to compete with the path a, but this may be the minor process. As in fact, appearance of absorptions at 385 and 403 nm which may be due to the imine intermediate were observed in the course of the reaction at pH 7 under anaerobic conditions. These absorptions are not for a semiquinone radical of PQQ because these absorptions do not disappear by aeration of the reaction mixture. On the other hand, the reaction was much decelarated above pH 12, which may be attributed mainly to hydration of the quinone group of PQQ.

At the present time, the mechanism via rate-determining one-electron transfer from the amine to PQQ may be dismissed because of lower reactivity of the secondary amine and of no reactivity of the tertiary amine. Considering the fact that the peak oxidation potentials of amines increase in general as in following order: primary>secondary>tertiary, the secondary and the tertiary amines should be oxidized more smoothly if the reaction should proceed via electron-transfer mechanism. It has been shown that the rate for chlorine dioxide oxidation of amines, which proceeds via electron transfer mechanism, increases with decrease of the peak oxidation potentials of amines.[22] A large kinetic isotope effect also contradicts the rate-limiting one-electron-transfer mechanism.

A possibility of the hydride-transfer mechanism from the α-position of the amine to the quinone is also considered to be discarded, since the oxidation potential of PQQ is relatively low ($E_{1/2}$=90 mV)[11b] compared with those of ordinary quinones in a neutral aqueous solution. As in fact, dehydrogenation of allylic and benzylic alcohols and carbonyl compounds such as succinate does not occur in the reaction with PQQ under suitable conditions. To obtain further insight into the mechanism of the oxidation, we are now synthesizing varieties of fused heteroaromatic *o*-quinones and examining their reactivities.

The present work was partially supported by a Grant-in-Aid for Special Project Research (No. 60104005) from the Ministry of Education, Science, and Culture to which our thanks are due.

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