Spectral Studies of Bovine Dopamine β-Hydroxylase

ABSENCE OF COVALENTLY BOUND PYRROLOQUINOLINE QUINONE

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Bovine dopamine β-hydroxylase was examined spectroscopically for the presence of covalently bound pyrroloquinoline quinone (PQQ). Pure dopamine β-hydroxylase had a featureless UV-visible spectrum above 300 nm. An equimolar solution of dopamine β-hydroxylase and exogenously added PQQ (1 PQQ/active site) had a strong absorption maximum at 333 nm. Dialysis removed the added PQQ, indicating that dopamine β-hydroxylase does not bind PQQ irreversibly. Reaction of dopamine β-hydroxylase with 6 mM phenylhydrazine in the presence of 15 mM ascorbate caused 96% inactivation within 20 min and did not produce any spectrally detectable amounts of the phenylhydrazine adduct of PQQ, as reported by van der Meer et al. (van der Meer, R. A., Jongejan, J. A., and Duine, J. A. (1988) FEBS Lett. 231, 303-307). The peptide profile of phenylhydrazine inactivated dopamine β-hydroxylase was monitored at 316 nm and did not reveal any peptides that might contain a PQQ-phenylhydrazine adduct. Thus, the absence of any spectrally detectable PQQ-phenylhydrazine adducts under these conditions demonstrates that the mechanism of phenylhydrazine inactivation does not involve covalent modification of PQQ at the active site of dopamine β-hydroxylase and provides strong evidence that the native enzyme does not contain PQQ.

Dopamine β-hydroxylase is a copper-containing monoxygenase that converts dopamine to norepinephrine within the chromaffin granules of the adrenal medulla (1, 2). The enzyme catalyzes the stereospecific benzylic hydroxylation of dopamine in a physiological reaction requiring ascorbate and dioxygen. Previous studies have demonstrated a strict stoichiometry of 2e−/O2/substrate and have shown that maximally active enzyme contains eight Cu/tetramer. However, the nature of the copper-oxygen species involved in catalysis has not been identified conclusively and may involve a binuclear copper peroxy species (1), an acylperoxide (1), or a hydroperoxide (2).

A recent study claims that dopamine β-hydroxylase also contains covalently bound pyrroloquinoline quinone (PQQ)1 and suggests that this organic cofactor may have a redox function in the enzyme in conjunction with copper and ascorbate (3). Specifically, it has been proposed that dopamine β-hydroxylase-bound PQQ may serve as either 1) an "anchoring" point for the amino group of the substrate, 2) an organic cofactor providing a redox link to a one copper center, or 3) an organic cofactor providing a redox bridge at a binuclear copper center.

A number of bacterial alcohol dehydrogenases have been reported to contain PQQ, and, more recently, PQQ has been suggested to be a cofactor in several eukaryotic enzymes. These studies have been reviewed (4). In eukaryotic enzymes, much of the evidence for enzyme-bound PQQ has consisted of UV-visible, fluorescence, and resonance raman spectroscopy. However, Duine and co-workers (5) have developed a procedure for PQQ derivatization and extraction that permits to provide easy identification and isolation of PQQ in small amounts. The procedure involves derivatization of PQQ with phenylhydrazine to form the C(5) hydrazone of PQQ and phenylhydrazine (Fig. 1) and subsequent digestion of the enzyme with Pronase to release the adduct. Hydrazone formation enhances the PQQ absorption maximum at 342 nm, and the hydrazone may be identified on HPLC systems by comigration with the authentic model compound prepared from pure PQQ and phenylhydrazine. Duine and co-workers have used this procedure to report the presence of PQQ in methylamine dehydrogenase (5), plasma amine oxidase (6), pig kidney diamine oxidase (7), lipoxygenase (8), lipoygenase (9), dopa decarboxylase (10), and dopamine β-hydroxylase (3).

The same laboratory has used hexanol derivatization and acid hydrolysis to extract PQQ from galactose oxidase (11).

Previously, we demonstrated that phenylhydrazine is a mechanism-based inhibitor of dopamine β-hydroxylase, and therefore that it undergoes turnover in the catalytic center of the enzyme (12). These two separate lines of experimentation, that phenylhydrazine participates in catalysis and that it forms a hydrazene with dopamine β-hydroxylase-bound PQQ, potentially provide a unique combination for studying the role of PQQ as a cofactor in enzymatic catalysis. Consequently, we sought to provide additional physical and chemical evidence for the presence of PQQ in dopamine β-hydroxylase but have been unable to identify any adduct in dopamine β-hydroxylase inactivation by phenylhydrazine occurs through an enzyme-generated radical intermediate that covalently modifies amino acid residues at the active site of the protein (13).

EXPERIMENTAL PROCEDURES

Materials—Pyrroloquinoline quinone was from Fluka. Phenylhydrazine-HCl was from Aldrich. Methyl a-d-mannopyranoside was from Sigma, and ConA-Sepharose was from Pharmacia LKB Bio-
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Enzyme Purification—Bovine dopamine β-hydroxylase was purified as described using whole adrenal medulla as starting material (14). Enzyme activity was measured as described previously, with the exception that the furanart concentration was 20 mM rather than 16 mM (14). Enzyme concentrations were determined spectrophotometrically using an extinction coefficient of ε250 = 12.4 (15). Prior to use, the enzyme was dialyzed against three successive changes of 50 mM MES, pH 5.8, 100 mM NaCl containing 10 μM CuCl₂, 1 μM CuCl₂, and 0 μM CuCl₂, respectively. Enzyme used in these experiments had a specific activity of 45 μmol of O₂/min/mg, and a molecular weight of 290,000 was used for calculations involving enzyme concentration.

Spectrophotometric Measurements of PQQ and Dopamine β-Hydroxylase—Spectrophotometric measurements of PQQ and dopamine β-hydroxylase were made on a Gilford Response. A stock solution of PQQ was prepared by dissolving 2.7 mg of PQQ in 1 ml of 100 mM NaH₂PO₄, pH 7.0. The concentration of the stock solution was determined by its A257 = 19,122 M⁻¹ cm⁻¹ (16). Ten μl of stock solution were diluted 100-fold in 100 mM NaH₂PO₄, pH 7.0, and the A257 was determined against a buffer reference. The concentration of stock PQQ was 81 μM. The absorbance profile of 83 μM PQQ was obtained as solution of 2.6 μl of 81 μM PQQ in 250 μl of 50 mM MES, pH 5.8, and 100 mM NaCl. The absorbance profile was obtained in MES buffer to correspond to the buffer in our technology Inc. All other reagents were of the highest quality commercially available.

Enzyme Activity—To inactivate dopamine β-hydroxylase, a second identical inactivation reaction was performed with a 20-fold excess of dithiothreitol over dopamine β-hydroxylase sulfhydryl content. After 4 h of reduction under argon at 37 °C, the samples were dialyzed against a 1.5-fold excess of dithiothreitol over total sulfhydryl content, and dialysis was quenched after 1 h under argon at 37 °C by addition of 50 μl of β-mercaptoethanol. Each sample of alkylated enzyme was dialyzed exhaustively against 50 mM NaH₂PO₄, pH 7.8, and was made 4 mM in urea by addition of solid urea. The samples then were digested with 2% (w/w) S. aureus V8 proteinase at room temperature for 7 days.

Peptides from digested enzyme were separated on a Vydac C18 protein and peptide column connected to a Waters 501 solvent delivery system. Single injections contained peptides corresponding to 2.2 mg of dopamine β-hydroxylase (50 nmol of monomer). Peptide elution patterns were monitored with a Varian polychrom 9060 diode array detector. Peptides were separated in a two-solvent system with a linear gradient starting at 90% A-10% B to 10% A-90% B over 80 min. Solvent A contained 0.1% trifluoroacetic acid in water and solvent B contained 0.1% trifluoroacetic acid in acetonitrile.

NMR Spectroscopy—Proton NMR spectra of PQQ, phenylhydrazine, and the reaction product of PQQ and phenylhydrazine were obtained on a Bruker 900 MHz NMR instrument at 25 °C. Fresh PQQ was dissolved in 0.5 μl of deuterated methanol to a final concentration of 10.6 mM, and the proton signals from PQQ were accumulated for 10 min at 25 °C with a pulse delay of 0 s. Similarly, recrystallized phenylhydrazine-HCl was freshly dissolved in 0.5 ml of deuterated methanol to a final concentration of 21.2 mM, and its proton NMR spectrum was obtained within the next hour under the same conditions as the spectrum for PQQ. Finally, the entire phenylhydrazine sample was mixed with the entire PQQ sample, and the spectrum of the reaction product was obtained after pulsing for 1 h. Tetramethylsilane was used as an internal standard.

An aliquot of the reaction corresponding to 30 nmol of PQQ-phenylhydrazine then was injected onto the HPLC under conditions used for the peptide separation, and the elution profile of the reaction product was monitored at 316 nm. Material eluting from the HPLC in previous injections of reaction product was collected and scanned to determine its UV-visible profile. The reaction product had a maximum at 316 nm in the solvent system used, and therefore 316 nm was chosen for peptide monitoring.

Oxidation of Phenylhydrazine—Oxidation of phenylhydrazine was measured spectrophotometrically on a Perkin-Elmer lambda array 3840 UV-visible spectrophotometer. Distilled phenylhydrazine or recrystallized phenylhydrazine-HCl was dissolved in freshly prepared 1:1 ethanol/phosphoric acid to a final concentration of 100 mM, and 2 μl of this solution was added to 2.5 ml of 100 mM NaH₂PO₄, pH 7.0. The water-jacketed cuvette holder was warmed to 40 °C and the instrumental base line was set by scanning 100 mM NaH₂PO₄, pH 7.0. One ml of phenylhydrazine solution then was
tivation was 0.167 min, and after 20 min dopamine hydroxylase forms a phenylhydrazine-inactivated dopamine β-hydroxylase. The absorption of the untreated dopamine β-hydroxylase was essentially identical to that of PQQ, and the absorption profile of dialyzed dopamine β-hydroxylase (83 active sites) was scanned over the range of 250-340 nm under reducing conditions. A high concentration of dopamine β-hydroxylase was used in order to detect any small absorption features in the range of 300-350 nm that might be attributable to enzyme-bound PQQ. Based on $A_{342} = 9630 \text{ M}^{-1} \text{ cm}^{-1}$ (16) and assuming one PQQ/active site, 83 μM dopamine β-hydroxylase would have an $A_{342} = 0.8$ if PQQ were bound to the enzyme. In comparison, profile B in Fig. 2 shows the absorption profile of 83 μM PQQ in 50 mM MES, pH 5.8, and 100 mM NaCl. In this buffer we obtained PQQ absorption maxima at 257, 275, and 333 nm. These agree with those previously reported, except for the maximum at 333 nm due to PQQ did not change, as shown in profile C. However, dopamine β-hydroxylase clearly exhibited no absorption maximum resembling the PQQ maximum at 333 nm, which occurred at a shorter wavelength than the maximum at 342 nm reported previously (16). However, dopamine β-hydroxylase clearly exhibited no absorption maximum resembling the PQQ maximum at 333 nm.

Authentic PQQ then was mixed in an equimolar ratio with concentrated dopamine β-hydroxylase, and the absorption profile of the solution was obtained. The absorption of the mixture was larger than the absorption of either dopamine β-hydroxylase or PQQ alone, but the absorption maximum at 333 nm due to PQQ did not change, as shown in profile C. However, extensive dialysis removed all absorption due to PQQ, and the absorption profile of dialyzed dopamine β-hydroxylase (profile D) was essentially identical to that of untreated dopamine β-hydroxylase.

**Results**

**UV-Visible Absorption Profile of PQQ and Dopamine β-Hydroxylase**—In order to compare the spectral properties of purified dopamine β-hydroxylase and authentic PQQ, a UV-visible spectrum of concentrated dopamine β-hydroxylase was obtained. A stock solution of 6.1 mg/ml dopamine β-hydroxylase (83 μM active sites) was scanned over the range of 250-600 nm, and profile A in Fig. 2 demonstrates that our enzyme had a featureless absorption profile above 300 nm. A high concentration of dopamine β-hydroxylase was used in order to detect any small absorption features in the range of 300-350 nm that might be attributable to enzyme-bound PQQ. Based on $A_{342} = 9630 \text{ M}^{-1} \text{ cm}^{-1}$ (16) and assuming one PQQ/active site, 83 μM dopamine β-hydroxylase would have an $A_{342} = 0.8$ if PQQ were bound to the enzyme. In comparison, profile B in Fig. 2 shows the absorption profile of 83 μM PQQ in 50 mM MES, pH 5.8, and 100 mM NaCl. In this buffer we obtained PQQ absorption maxima at 257, 275, and 333 nm. These agree with those previously reported, except for the maximum at 333 nm due to PQQ did not change, as shown in profile C. However, dopamine β-hydroxylase clearly exhibited no absorption maximum resembling the PQQ maximum at 333 nm.

**UV-visible Absorption Profile of Phenylhydrazine-inactivated Dopamine β-Hydroxylase**—In order to determine whether or not dopamine β-hydroxylase forms a phenylhydrazine adduct with a characteristic absorption maximum at 340 nm under reducing conditions, 8 mg of dopamine β-hydroxylase (15.7 μM active sites) was inactivated with 5 mM ascorbate and 0.125 mg/ml catalase. The first order rate constant for inactivation was 0.197 min$^{-1}$, and after 20 min dopamine β-hydroxylase was >95% inactive. Inactive enzyme then was dialyzed to remove reactants and chromatographed on ConA-Sepharose to remove catalase. Fig. 3 shows the UV-visible absorption profiles of dopamine β-hydroxylase before and after inactivation.

Absorption profile A in the presence of 15 mM ascorbate showed, again, that dopamine β-hydroxylase does not absorb significantly above 320 nm. Addition of catalase to dopamine β-hydroxylase increased the absorbance of the mixture and produced an absorption maximum at 404 nm due to catalase, as shown in profile B. After addition of 6 mM phenylhydrazine and inactivation for 45 min, the reaction mixture exhibited profile C. However, dialysis removed most of the absorption due to the phenylhydrazine, and chromatography on ConA-Sepharose removed all additional absorption due to phenylhydrazine and catalase, as shown in profiles D and E. Thus, the inactivated enzyme recovered from the ConA-Sepharose column showed no absorption above 300 nm that might be attributable to an enzyme-bound phenylhydrazine adduct of PQQ.

A parallel reaction done in the absence of ascorbate produced results similar to those shown in Fig. 3, except that dopamine β-hydroxylase exhibited increased absorbance over the entire range from 300 to 600 nm. However, there were no distinctive absorbance peaks in the spectrum that might represent absorbance due to the phenylhydrazine adduct of PQQ.

**1H NMR of PQQ and Phenylhydrazine**—The 1H NMR spectrum of PQQ in deuterated methanol contained two major resonances at 7.31 and 8.77 ppm, as shown in Fig. 4. After the reaction of PQQ and phenylhydrazine, the signal at 7.31 shifted downfield by about 0.2 ppm and the signal at 8.77 shifted upfield by about 0.05 ppm. The spectrum of the mixture also contained three new multiplets centered at 7.15, 7.35, and 7.55 ppm, presumably due to formation of the C(5) resonance of PQQ and phenylhydrazine. The resonances due to excess phenylhydrazine remained unchanged.

**Peptide Map of Dopamine β-Hydroxylase**—Inactivated and repurified enzyme (spectrum shown in Fig. 3E) was digested with S. aureus V8 protease to search for a peptide or peptides that might contain a PQQ-phenylhydrazine adduct. The peptides were separated by HPLC and monitored simultaneously.
of PQQ and phenylhydrazine.

obtained from used for the peptide separation immediately after obtaining as described under “Experimental Procedures.” Spectrum with the absorption profile at 215 and 316 nm. The peptide profile at 215 nm in Fig. 4 shows that there was no detectable absorption at 316 nm in the regions corresponding to the labeled peptides. This further supports the contention that phenylhydrazine modifies amino acid side chains in dopamine β-hydroxylase rather than covalently bound PQQ.

Oxidation of Phenylhydrazine—It is well known that phenylhydrazine oxidizes in air, and we observed that solutions of freshly distilled phenylhydrazine turned yellow at the surface even during storage at −20 °C. Therefore, we incubated phenylhydrazine under previously published conditions that were used for the reported PQQ detection in dopamine β-hydroxylase (3) to determine the extent of phenylhydrazine oxidation. We found almost total conversion of phenylhydrazine to two other products within 2 h at 40 °C in 100 mM NaH2PO4, pH 7.0, as shown in Fig. 6. Over the course of the 2-h incubation, the initial maximum at 233 nm decreased to 10% of its starting value, and the initial maximum at 280 nm shifted sequentially to two new maxima at ≈270 and ≈275 nm.

DISCUSSION

Recently, Duine’s laboratory (3, 5–10) has reported that PQQ is a covalently bound cofactor in at least seven eukaryotic enzymes, including dopamine β-hydroxylase. The discovery of PQQ in amine oxidases was based on repeated observations over many years that these enzymes contain a carboxyl cofactor that does not necessarily correspond to pyridoxal phosphate (17). Similarly, the discovery of PQQ in dopa decarboxylase was based on the observation that transaminases and amino acid decarboxylases contain pyridoxal phosphate, and in some cases have additional unexplained chromophores that absorb in the 330–340 nm range (10). Based on evidence of this type, it also has been speculated that three other enzymes, including rat liver ornithine aminotransferase, pseudomonas ω-amino acid pyruvate amin-
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Nevertheless, while published reports suggest that dopamine \(\beta\)-hydroxylase-bound PQQ would have a detectable free carbonyl, we did not observe it, nor did we observe that exogenously added PQQ irreversibly binds to dopamine \(\beta\)-hydroxylase. We interpret this to mean that there is no spectroscopically detectable PQQ in dopamine \(\beta\)-hydroxylase and that we have not prepared an apoenzyme that might be reconstituted by simple addition of PQQ. Covalently bound PQQ would be expected to result from a cell-mediated process, and therefore this experiment does not rule out the possibility of PQQ-binding sites on dopamine \(\beta\)-hydroxylase, but it does show that there is no adventitious PQQ binding to dopamine \(\beta\)-hydroxylase, and it does eliminate the possibility that an apodopamine \(\beta\)-hydroxylase might be reconstituted by simple PQQ addition.

Furthermore, the peptide maps of enzyme inactivated with or without ascorbate showed no absorbance at 316 nm that would indicate a PQQ-phenylhydrazine adduct. More than 2 mg of proteolyzed enzyme digest (30 nmol of dopamine \(\beta\)-hydroxylase monomer) was injected in a single run to ensure that even a small amount of PQQ-phenylhydrazine adduct would be detected if present. Data in Fig. 5 show that the equivalent amount of PQQ-phenylhydrazine adduct prepared from pure PQQ and phenylhydrazine was easily detected at 0.02 absorbance units full scale.

While our data are self-consistent (we detect no chromophores and find no PQQ-phenylhydrazine adducts) the literature is contradictory. For instance, unexplained chromophores in the region of 330-340 nm have been provided as the rationale for experiments leading to the extraction of PQQ from several enzymes (10), yet native lysyl oxidase (8), plasma amine oxidase (6), and dopamine \(\beta\)-hydroxylase have no absorption peaks at 330-340 nm. Nevertheless, Duine and co-workers claim to have extracted PQQ from these three enzymes. We cannot explain the last result. We note, also, that absorption spectra of native dopa decarboxylase (10), diamine oxidase (7), and galactose oxidase (11) have not been published in the studies reporting extraction of PQQ from these three enzymes.

Previous studies from our laboratory strongly indicate that dopamine \(\beta\)-hydroxylase inhibition by phenylhydrazine occurs by mechanism-based inactivation (12). These studies, enzyme was inactivated by phenylhydrazine alone or by phenylhydrazine in the presence of 12 mM ascorbate. The inactivation data contradict the claim that reduced enzyme does not incorporate phenylhydrazine (8). Previous studies also show that turnover in the presence of tyramine and ascorbate blocks inactivation by phenylhydrazine, and that the absence of oxygen greatly reduces \(^{14}C\)-labeling (12). Thus, all the data implicate mechanism-based inactivation by phenylhydrazine. In this paper we repeated the enzyme inactivation in the presence and absence of ascorbate and analyzed the inactivated enzyme for any evidence of a PQQ-phenylhydrazine adduct. Figs. 3 and 5 provided no spectral evidence for a PQQ-phenylhydrazine adduct in the inactive enzyme or in peptides derived from this enzyme.

The additional argument has been made that, while phenylhydrazine inactivation occurs rapidly, derivatization of the cofactor to the hydrazine requires prolonged incubation. Initially, we performed the phenylhydrazine incubation for 16 h exactly as described previously, and we were unable to reproduce the characteristic spectral changes in dopamine \(\beta\)-hydroxylase attributed to incubation with phenylhydrazine (3). Instead, we observed a broad increase in absorption from 300 to 600 nm that varied in repeated experiments. We concluded

transferase, and mammalian 4-aminobutrate transaminase, potentially contain PQQ (10). In short, many well-studied eukaryotic enzymes with interesting or unusual spectroscopic properties have now all become candidates for a new class of proteins termed "quinoproteins."

However, the bulk of the evidence for eukaryotic quinoproteins has accumulated in a single laboratory (3, 5-10). Independent studies are consistent with PQQ involvement in plasma amine oxidase (18) and bovine lysyl oxidase (19), but as yet there has been no independent confirmation of covalent attachment of PQQ to soybean lipoxygenase, dopa decarboxylase, galactose oxidase, or dopamine \(\beta\)-hydroxylase, nor has there been any definitive evidence on the nature of the covalent attachment or the catalytic role of PQQ in any of these enzymes. Despite the contention that kinetic studies have provided a one-sided view of dopamine \(\beta\)-hydroxylase (3), our experience with the kinetics of highly purified dopamine \(\beta\)-hydroxylase isolated from fresh adrenal glands has permitted us to study well-characterized enzyme, in contrast to commercial preparations others have used (3), and has permitted us to evaluate independently the evidence for covalent attachment of PQQ to dopamine \(\beta\)-hydroxylase.

A high concentration of our copper-saturated enzyme had a featureless UV-visible absorption profile above 300 nm. This differs from the profile reported previously (3), which contained a broad peak between 325 and 400 nm attributed to copper. Occasionally, in our hands, an aged enzyme sample or one that contained residual ammonium sulfate had anomalous broad absorption bands similar to those attributed to copper. However, in freshly dialyzed, highly active enzyme, we did not observe any spectral features above 300 nm. It is possible that protein-bound PQQ would have a lower extinction coefficient than free PQQ. Nevertheless, the extinction coefficient of protein-bound PQQ would have to be reduced at least 50-fold before PQQ would be below the limits of detection in our experiment.

Presumably, enzyme-bound PQQ would have a detectable absorption in the region of 330-340 nm, as evidenced by the high absorption of pure PQQ at 333 nm. This absorption may be attributed to the carbonyl chromophore and represents R band, or \(n \rightarrow \pi^*\), absorption. Covalent attachment to dopamine \(\beta\)-hydroxylase at any of the three carboxylates of PQQ via amide or ester bonds, or at a site other than the carboxylates via ether or thioether linkages (2), would not be expected to alter substantially the electronic spectrum of the chromophore beyond a shift of 5-10 nm, and would not be expected to eliminate R band absorption.

It is known that R band absorption undergoes a blue shift as a result of hydrogen bonding, and enzyme-bound PQQ might well be hydrogen bonded at the active site of dopamine \(\beta\)-hydroxylase. However, hydrogen bonding would have to result in a large blue shift of 30-40 nm to obscure the carbonyl chromophore by shifting it to shorter wavelengths below 300 nm. Alternatively, PQQ might reside on the enzyme in a reduced or hydrated form such as the quinol, PQQH2, or PQQ-2H2O adduct, or in the form of a hemiketal with alcohol containing amino acid side chains, in which case the carbonyl chromophore would not be visible. However, it is unlikely that all the enzyme-bound PQQ would be reduced. All enzyme solutions were open to air and no special procedures were used to reduce the enzyme before spectroscopic determinations. If PQQ were reduced, this would preclude derivatization with phenylhydrazine, and would be inconsistent with the results in Ref. 3. Alternatively, a reversible adduct of PQQ could account for the absence of any UV-visible absorption and still permit derivatization with phenylhydrazine.
that these absorbance changes represent nonspecific protein modification.

In control experiments, we found that phenylhydrazine rapidly decomposes in less than 2 h in buffer at 40 °C. Autoxidation of phenylhydrazine produces N₂, H₂O₂, and benzene radicals (20, 21), and in the presence of trace metals will produce biphenyl and azobenzene (22). These data question the validity of the hydrazine extraction procedure, which requires exposure of the enzyme to phenylhydrazine for 16 h at 40 °C. Incubation for this time period merely exposes dopamine β-hydroxylase side chains and glycosylation sites to attack by free radicals and H₂O₂, and such attacks might account for the absorption changes observed under prolonged incubation conditions (3). In contrast, rapid inactivation in the presence of ascorbate and catalase (12) protects the enzyme against nonspecific oxidation and attack by free radicals, and therefore provides a rationale for the lack of spectral changes observed here (Fig. 3).

Our data and the data of others appear to rule out the proposal that PQQ might have a catalytic role in “anchoring” the amino group of the substrate in the catalytic site. Hydrogen bonding between the protons of the amino group in the substrate and the di-carbonyl of PQQ might certainly be possible, but a covalent hydrazone anchor should have been detected and was not. Other inhibitors of dopamine β-hydroxylase have been synthesized that do not have the amino group corresponding to the one in dopamine (23, 24), and yet these inhibitors turnover in the active site. If catalysis requires the PQQ “anchor,” these compounds should not convert to product.

Lastly, in other studies, we have sequenced almost the entire polypeptide chain of dopamine β-hydroxylase and the peptides labeled by [U-¹⁴C]phenylhydrazine.² We have not identified any unusual amino acid derivatives or blanks in the peptide sequences from native or labeled enzyme that might represent covalently bound PQQ. These data argue against the possibility of a spectrally silent PQQ that does not interact with the substrate. Moreover, a clone of the bovine enzyme has been obtained (25), and successful expression of the clone will provide unequivocal evidence of any cofactor requirement for PQQ in dopamine β-hydroxylase. In this regard, the human 5'-lipoxygenase gene has been cloned and expressed in a baculovirus/insect cell system, and it has been shown that its sequence demonstrates significant homology with the sequence of soybean lipoxygenase (26). The cloning data are additional evidence that human 5'-lipoxygenase and soybean lipoxygenase do not require PQQ unless it can be shown subsequently that the baculovirus/insect cell expression system contains both PQQ and the mechanistic systems required for protein modification by PQQ, or that this expression system has been contaminated fortuitously by PQQ. Together, our data and the cloning data for human 5'-lipoxygenase provide the first independent assessments of the claims for covalently bound PQQ in these enzymes, and both sets of data fail to support these claims.

In the absence of any spectral evidence for formation of a PQQ-phenylhydrazine adduct under our inactivation conditions with or without ascorbate, it does not appear necessary to reconsider the original interpretation of our data, namely that phenylhydrazine inhibition occurs by mechanism-based inactivation and derivatization of amino acid residues. Therefore, PQQ does not appear to have a role in dopamine β-hydroxylase catalysis, and from the weight of the evidence in this paper it seems unlikely that dopamine β-hydroxylase contains covalently bound PQQ.

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