Identification of Topaquinone and Its Consensus Sequence in Copper Amine Oxidases†

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ABSTRACT: The nature of the active site cofactor and the amino acid sequence flanking this structure have been determined in a range of copper amine oxidases. For enzymes from porcine plasma, porcine kidney, and pea seedlings, proteolytic digestion was performed on phenylhydrazone or *p*-nitrophenylhydrazone derivatives. Thermolysin treatment leads to relatively small active site peptides, which have been characterized by Edman degradation and by resonance Raman spectroscopy. Resonance Raman spectra of peptides show identical peak positions and intensities relative to each other and to a model *p*-nitrophenylhydrazone derivative of topaquinone hydantoin, establishing topaquinone as the cofactor in each instance. Edman degradation of peptides provides active site sequences for comparison to previous determinations with bovine serum and yeast amine oxidases. The available data establish a consensus sequence of Asn, Topa, Asp/Glu. Trypsin leads to significantly longer peptides, which reveal a high degree of sequence identity between plasma proteins from bovine and porcine sources (89%), with significantly decreased identity between the porcine serum and intracellular amine oxidases (56%). A lower degree of identity (45%) is observed between the pea seedling and mammalian enzymes. As an alternative to the isolation of active site peptides for topaquinone identification, visible spectra of intact proteins have been investigated. It is shown that *p*-nitrophenylhydrazone derivatives of native enzymes, active site-derived peptides, and a topaquinone model exhibit identical behavior, absorbing at 457–463 nm at neutral pH (pH 7.2) and at 575–587 nm in basic solution (1–2 M KOH). These spectral properties, which appear unique to topaquinone, provide a rapid and simple test for the presence of this cofactor in intact enzymes. Using this approach, the class of enzymes demonstrated to contain topaquinone has been expanded to include copper amine oxidases from sheep plasma and chick pea seedling.

The class of proteins designated copper amine oxidases (EC 1.4.3.6) catalyzes the oxidative conversion of amines to aldehydes and ammonia, concomitant with a two-electron reduction of dioxygen to hydrogen peroxide:

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

Although these enzymes are widely distributed in plants and animals, their physiologic roles have not been well-defined. Postulated functions in mammals include the removal of biogenic amines from blood plasma (Buffoni, 1966), the cross-linking of structural proteins such as collagen and elastin (Knowles & Yadav, 1984), and the regulation of intracellular polyamine concentrations (Mondovi et al., 1980). Due to the correlation of cell proliferation with high levels of polyamines, amine oxidases have been postulated to play a role in tumor suppression (Perrin et al., 1985).

A variety of copper amine oxidases have been isolated and characterized since the initial purification of bovine serum amine oxidase (Tabor et al., 1954). These include plant amine oxidases from chick pea, pea and lentil seedlings (Smith, 1985), serum amine oxidases from porcine and sheep (Buffoni, 1966), lysyl oxidase (Williamson et al., 1986), and porcine kidney diamine oxidase (Rinaldi et al., 1982). In addition to containing copper, each of these proteins has been demonstrated to contain a reactive carbonyl capable of derivatization with reagents such as phenylhydrazine (Knowles & Yadav, 1984). Functional studies of the copper amine oxidases have been hindered by the unknown nature of the carbonyl cofactor and of its participation in the catalytic cycle. Subsequent to reports that bovine serum amine oxidase contained bound pyrroloquinoline quinone as cofactor (Lobenstein-Verbeek et al., 1982; Ameyama et al., 1984), pyrroloquinoline quinone was implicated as the active site cofactor in the pea seedling (Glatz et al., 1987), porcine kidney (van der Meer et al., 1989), and lysyl oxidase (Williamson et al., 1986). However, the recent proof of the presence of the quinone of trihydroxyphenylalanine (topaquinone), Figure 1, in bovine serum amine oxidase (Janes et al., 1990), necessitated a closer examination of other members of the copper amine oxidase class.

As described in this report, we have isolated active site, cofactor-containing peptides for amine oxidases from pea seedling and porcine plasma and kidney. Characterization of these peptides by resonance Raman spectroscopy demonstrates the presence of topaquinone in each case, establishing this structure as a ubiquitous redox cofactor in nature. Edman degradation of available peptides provides active site sequences for comparison to those previously reported from bovine serum (Janes et al., 1990) and yeast (Mu et al., 1992) amine oxidases. The available data reveal a consensus sequence, Asn, Topa, Asp/Glu. In light of the recent demonstration that to-

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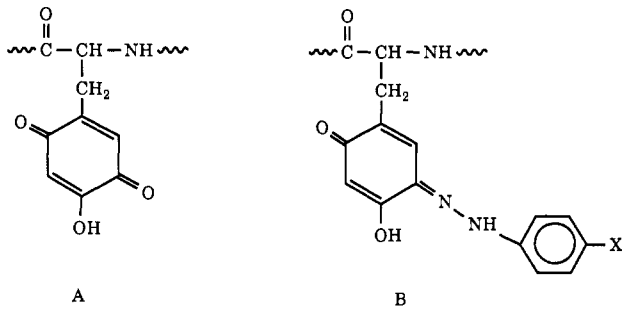


FIGURE 1: Structures for peptide-bound topaquinone (A) and the phenylhydrazone derivative of topaquinone, where X is either H or NO_2 (B). Recent studies indicate attack of phenylhydrazine at the C-5 carbonyl of topaquinone (Mure and Klinman, in preparation).

paquinone arises from a tyrosine precursor (Mu et al., 1992), the sequence Asn, Tyr, Asp/Glu is implicated as the requisite signal for topa biogenesis.

As previously shown by Brown et al. (1991), resonance Raman spectra of phenylhydrazone derivatives of intact copper amine oxidases may differ from spectra seen with active site-derived peptides and model compounds, necessitating the isolation of active site peptides for an unambiguous identification of protein-bound topaquinone. We now demonstrate that the visible absorbance properties of *p*-nitrophenylhydrazone derivatives of intact proteins can be used to identify topaquinone. Using this approach, a plasma amine oxidase from sheep and chick pea seedling amine oxidase are concluded to contain topaquinone. Overall, the data reported herein establish topaquinone as a ubiquitous cofactor in nature.

MATERIALS AND METHODS

Materials. [U-^{14}C]Phenylhydrazine hydrochloride and ultrapure urea were from ICN and phenylhydrazine hydrochloride, *p*-nitrophenylhydrazine hydrochloride, and pyrroloquinoline quinone (PQQ[1]) were from Fluka. Triethylamine, trypsin, thermolysin, potassium phosphate dibasic trihydrate, DEAE-Sephadex, and benzylamine hydrochloride were purchased from Sigma. High-pressure liquid chromatography-(HPLC-) grade trifluoroacetic acid (TFA) and acetonitrile were from Baker. Con-A Sepharose 4B and Q-Sepharose fast flow were purchased from Pharmacia/LKB. Ultrogel AcA-34 was from IBF Biotechniques. All other chemicals were of reagent grade unless otherwise noted.

Enzyme Sources and Assay. Pea seedling amine oxidase (McGowan & Muir, 1971) and porcine kidney diamine oxidase (Tamura et al., 1989; Coleman et al., 1991) were purified as referenced, with final specific activities of 0.58 and 1.1 units/mg, respectively. Protein concentrations were estimated with the Bradford method (Bradford, 1976) using bovine serum albumin as a protein standard. Calculations of active site stoichiometries employed molecular weight values of 170 000 for pig kidney diamine oxidase (Klutz & Schmidt, 1977) and 180 000 for pea seedling amine oxidase (Yanagisawa et al., 1981). Enzyme activity for pea seedling amine oxidase was determined spectrophotometrically as benzaldehyde production from 3.3 mM benzylamine ($\Delta\epsilon_{250}$ = 12.8 mM^{-1} cm^{-1}) in 50 mM potassium phosphate buffer at pH 7.0, 25 °C (Coleman et al., 1991). The assay for pig kidney diamine oxidase was carried out at 37 °C, in 100 mM KP_i buffer, pH 7.4, by monitoring H_2O_2 production coupled to the oxidation 2,2-azinobis(3-ethylbenzthiazoline sulfonate) ($\Delta\epsilon_{414}$ = 23 mM^{-1} cm^{-1}) using horseradish peroxidase (Szutowicz et al., 1984).

[1] Abbreviations: PQQ, pyrroloquinoline quinone; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid.

Whereas the pea seedling amine oxidase was found to be essentially homogeneous by SDS gel electrophoresis, pig kidney diamine oxidase indicated some trace bands at lower molecular weight.

Porcine serum amine oxidase with a specific activity of 0.05 units/mg was prepared by a modification of existing procedures. The dialyzed pellet (40 units, 82 g) obtained after ammonium sulfate fractionation of 18 L of pig blood was chromatographed on DEAE-Sephadex at pH 7.5 in potassium phosphate buffer and rechromatographed using Q-Sepharose fast flow. The amine oxidase activity thus obtained (29 units, 56 g) was applied to CON-A Sepharose in 100 mM potassium phosphate, pH 7.2, and eluted using 0.5 M methyl α-D-mannopyranoside in 100 mM potassium phosphate, pH 7.2. The active fractions were pooled and concentrated using an Amicon cell with a PM-30 membrane (15.5 units, 845 mg). The resulting protein was chromatographed on Ultrogel Ac34 to yield three protein pools: specific activity 0.048 unit/mg (4.8 units, 100 mg), specific activity 0.054 unit/mg (5.4 units, 99 mg), and specific activity 0.032 unit/mg (1.0 units, 30 mg) for a total yield of 28%. The purified pools of enzyme were concentrated using an Amicon cell and frozen. Only the enzyme with specific activity 0.054 unit/mg (≥95% pure by gel electrophoresis) was used for the isolation of peptides. Protein concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient of 12 for a 1% solution (1-cm path length), determined from Bio-Rad kit protein measurements using bovine serum albumin as a standard and from the corrected technique of Warburg and Christian (Yanagisawa et al., 1981; Falk et al., 1983). Calculation of active site concentration employed a molecular weight of 196 000 (Buffoni, 1966). Enzyme activity was determined using the assay described for pea seedling amine oxidase with slight modification (buffer contained 100 mM potassium phosphate, pH 7.2, and 10 mM benzylamine).

Chick pea amine oxidase was isolated from 2.7 kg of 5-day-old etiolated seedlings to a final specific activity of 1.6 units/mg using a modification of the method of Coleman et al. (1991). Isolation steps included homogenization with 50 mM potassium phosphate buffer, pH 7.2, polyvinylpolypyrrolidone treatment, $(NH_4)_2SO_4$ fractionation (30–65% pellet), and sequential chromatography on cellulose phosphate and DE-52 cellulose columns. The activity was based on benzylamine oxidation at 25 °C monitored at 250 nm for 3.3 mM benzylamine in 100 mM potassium phosphate buffer, pH 7.2.

Sheep plasma amine oxidase with a specific activity of 0.08 unit/mg was isolated as described previously (Coleman et al., 1991). The isoenzyme used for derivatization and spectroscopy eluted from the final hydroxyapatite column with 100 mM potassium phosphate buffer, pH 7.4, followed by elution from a DE-52 cellulose column with 30 mM potassium phosphate buffer, pH 7.4. Enzyme activity was determined spectrophotometrically by monitoring the increase in absorption at 250 nm with 3.3 mM benzylamine as a substrate in 100 mM potassium phosphate buffer, pH 7.6.

Preparation of [U-^{14}C]Phenylhydrazine-Labeled Enzyme and Isolation of Radiolabeled Active Site Peptides from Thermolytic Digests. Enzymes in pH 7–8 potassium phosphate buffer (10–25 mM) were labeled by the addition of a slight excess of [U-^{14}C]phenylhydrazine in at least four portions over a 30-min period. Excess radioactivity was removed by desalting on a Bio-Rad 10DG column in 100 mM NH_4CO_3, pH 7.8. The derivatized enzyme solutions were brought to 2 M urea and placed in a shaker bath at 37 °C. Proteolysis was initiated by the addition of 2–5% (w/w) thermolysin. The course of the proteolysis was monitored by

small-scale HPLC injections of digests. Large-scale purifications were initiated when the protein digests were judged to be complete. Digested enzymes were injected onto a Dynamax-C_8 column equilibrated with 0.3% (v/v) triethylamine acetate, pH 6.8; peptides were eluted using a gradient from 0 to 60% acetonitrile and fractions were collected at 1-min intervals. The elution of peptides was monitored at 214 nm (peptide bond absorbance), at 350 nm (cofactor–phenylhydrazone absorbance), or by the radioactivity in the collected fractions.

Isolation of Radiolabeled Active Site Peptides from Tryptic Digests. The [U-^{14}C]phenylhydrazine-derivatized enzyme solutions were brought to 2 M urea and placed in a shaker bath at 37 °C. Proteolysis was initiated by the addition of 2% (w/w) trypsin. The course of the proteolysis was monitored by small-scale HPLC injections of digests. When the reactions were judged to be complete, the digests were quenched with phenylmethanesulfonyl fluoride and large-scale purification was initiated. Digested enzymes were injected onto a Dynamax-C_4 column equilibrated with 0.11% TFA containing 5% (v/v) acetonitrile (solvent A). Peptides were eluted using a gradient to a solvent containing 80% (v/v) acetonitrile in 0.10% (v/v) TFA (solvent B). The elution of peptides was monitored as detailed above for the thermolytic digests.

Preparation of p-Nitrophenylhydrazine-Labeled Enzyme and Isolation of Active Site Peptides from Thermolytic Digests. Enzymes in 100 mM potassium phosphate, pH 7.2, were labeled by the addition of a slight excess of 1 mM *p*-nitrophenylhydrazine in at least four portions over a 30-min period. Unreacted *p*-nitrophenylhydrazine was removed by desalting on a Bio-Rad Econo-Pac 10DG column equilibrated with 50 mM NH_4HCO_3, pH 7.8. Desalted derivatized enzyme solutions were brought to 2 M urea and placed in a shaker bath at 37 °C. Proteolysis with thermolysin was carried out as described for [^{14}C]phenylhydrazine-labeled proteins. Large-scale purification of peptides was carried out with a Dynamax-C_8 column equilibrated with 0.3% (v/v) triethylamine acetate, pH 6.8. Peptides were eluted using a gradient from 0 to 60% acetonitrile, and fractions were collected at 1-min intervals. The elution of peptides was monitored at 214 (peptide bond absorbance) and 350 nm (cofactor–nitrophenylhydrazone absorbance).

Preparation of Peptide Samples for Analysis. The isolated peptides were often subjected to a second or third HPLC purification using more selective analytical columns (Vydac-C_4 or -C_{18}) to assure purity prior to sequencing or resonance Raman spectroscopy. In each case, the use of a shallow gradient of acetonitrile, calculated on the retention time of the peptide of interest from the first dimensional analysis, with either TFA- or triethylamine acetate-buffered solvent afforded good yields of pure peptides. Sequence analyses by Edman degradation (Janes et al., 1990) and cofactor structural analyses by resonance Raman spectroscopy (Brown et al., 1991) were carried out as previously described.

Preparation of p-Nitrophenylhydrazone Derivatives of Model Compounds. The synthesis of the topaquinone hydantoin model compound (Janes et al., 1990) was by a modification of existing procedures (Lee & Dickson, 1971). The *p*-nitrophenylhydrazone of topaquinone hydantoin was prepared by dissolving 200 mg (0.39 mmol) of crude quinone in 0.05 mL of methanol and adding a 1.1-fold excess of *p*-nitrophenylhydrazine hydrochloride (80 mg) in 5 mL of warm methanol. The resulting mixture was warmed for 1 min over a steam bath. The reaction was purified by HPLC using a semipreparative Ultrasil-C_{18} column with a 40-min gradient from 0.11% TFA/5% acetonitrile to 0.1% TFA/80% acetonitrile. The desired product (6.75 mg, 5%) eluted at 21 min and was obtained as a reddish-orange crystalline solid after evaporation of solvent: mp 260 °C dec; UV/visible 455 nm; MS, 372 (MH^+), major fragments at 272, 238, 156, 139, 123, 109, 94, 80; ^{1}H NMR (DMSO + 5 μL of D_2O) δ 2.66 (1 H, dd), 3.03 (1 H, dd), 4.25 (1 H, dd), 6.43 (1 H, s), 7.57 (1 H, s), 8.05 (2 H, d), 8.36 (2 H, d).

The preparation of the *p*-nitrophenylhydrazone derivative of pyridoxal employed 50 mg of pyridoxal hydrochloride, which had been recrystallized from acetone/water (gift of J. F. Kirsch), and was dissolved in 1 mL of methanol. This solution was added to 50 mg of *p*-nitrophenylhydrazine in 2 mL of methanol, warmed in hot water for 2 min, and let stand for 30 min. The insoluble orange *p*-nitrophenylhydrazone adduct which precipitated was collected by filtration and washed with methanol.

The *p*-nitrophenylhydrazone of PQQ was prepared using 8 mg of PQQ and 5 mg of *p*-nitrophenylhydrazine dissolved in 1 mL of methanol. The reaction mixture was warmed for several minutes, leading to the immediate formation of the *p*-nitrophenylhydrazone precipitate (Mure et al., 1990). The product was stored at 4 °C overnight, collected by filtration, and washed with methanol.

Absorbance Spectroscopy of Intact Proteins. Derivatization of enzymes (0.5–8.8 mg) was carried out in 1–2 mL of 100 mM potassium phosphate buffer, pH 7.2, by the addition of a slight excess of either 1 mM *p*-nitrophenylhydrazine or phenylhydrazine in at least four portions over a 30-min period. Unreacted phenylhydrazine was removed by desalting samples on a Bio-Rad 10DG column equilibrated with 50 mM NH_4HCO_3, pH 7.8; protein was recovered in a volume of 2–3 mL. For spectroscopic characterization, 0.1–0.5 mL of desalted enzyme was added to 0.9–0.5 mL of potassium phosphate buffer, pH 7.2 (final concentration 0.1 M), or 0.9–0.5 mL of KOH (final concentration 2 M). Spectra were obtained using either a Cary 118B or a Varian DMS 200 spectrophotometer.

RESULTS

Peptides from Thermolytic Digestion. (a) Pea Seedling Amine Oxidase. Pea seedling amine oxidase (3.4 mg, 37.8 nmol) was found to incorporate 39.5 nmol of phenylhydrazine. Thermolytic digestion of derivatized pea seedling amine oxidase yielded the profile shown in Figure 2. The bulk of the digest eluted in the first portion of the gradient (Figure 2A), followed by more hydrophobic peptides. As seen by the absorbance profile at 350 nm (Figure 2B), the derivatized organic cofactor eluted in the hydrophobic region of the elution profile. The 34-, 39-, and 46-min peaks contained 6%, 26%, and 13%, respectively, of the radioactivity initially contained in the digest. The sequence of the 39-min peak was Val-Gly-Asn-X-Asp-Asn-Val. The sequence of the 46-min peak was Val-Gly-Asn-X-Asp-Asn-Val-Ile-Asp-Thr/unknown-Glu. The 34-min peak gave a mixed sequence that contained Val-Gly-Asn-X-Asp-Asn-Val-Ile-Asp/Ile-X-Glu/Ile.

(b) Porcine Kidney Diamine Oxidase. Pig kidney diamine oxidase (3.8 mg, 44.7 nmol) incorporated 17.1 nmol of phenylhydrazine. Thermolytic digestion of derivatized pig kidney diamine oxidase yielded the profile shown in Figure 3. This profile (Figure 3A) reveals more hydrophobic peptides than previously seen with either pea seedling amine oxidase (Figure 2) or bovine serum amine oxidase (Janes et al., 1990). As a result, the active site peptide, which can be readily identified from the profile at 350 nm (Figure 3B), was not as well separated from the bulk of the unlabeled peptides as for other amine oxidases examined; however, this peptide could

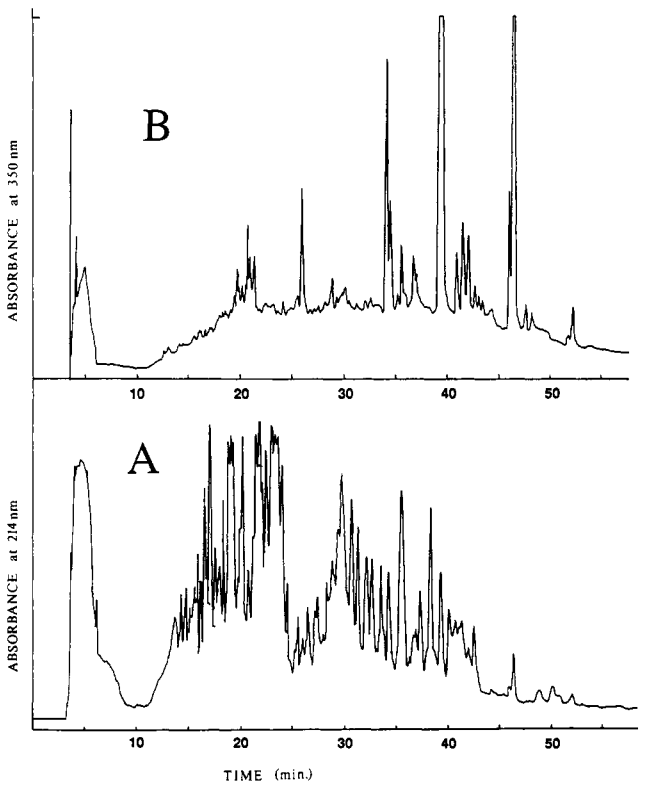


FIGURE 2: HPLC purification of a thermolytic digest of pea seedling amine oxidase. Digested, radiolabeled enzyme (12 nmol) was injected onto a Dynamax-C_8 column equilibrated with 0.3% (v/v) triethylamine acetate, pH 7 (solvent A), and eluted using a gradient to 20% solvent B [0.3% (v/v) triethylamine acetate, pH 7, with 60% (v/v) acetonitrile] at 10 min followed by an increase to 65% solvent B at 70 min. The bottom trace (A) was obtained by monitoring the elution profile at 214 nm, and the upper trace (B) was from a duplicate injection monitored at 350 nm.

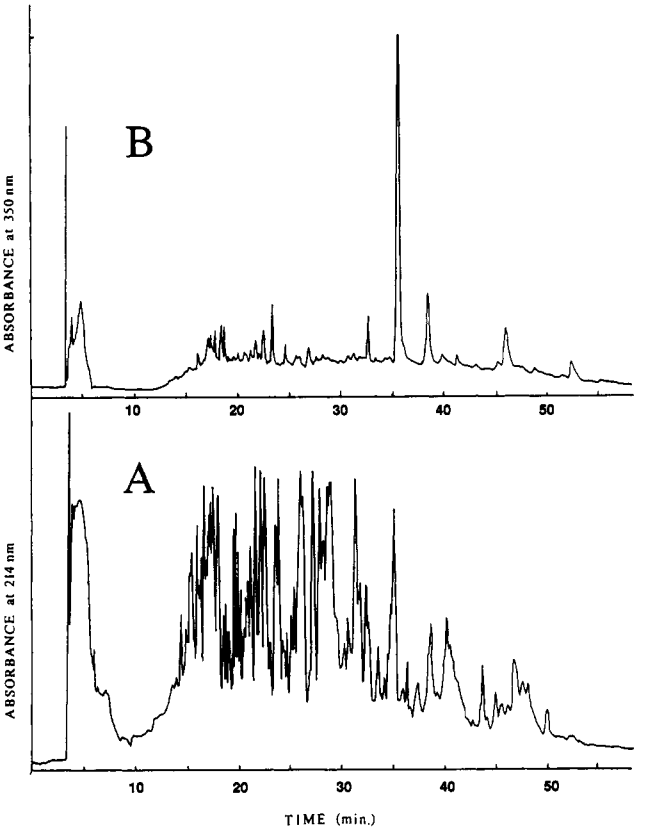


FIGURE 3: HPLC purification of a thermolytic digest of pig kidney diamine oxidase. Digested, radiolabeled enzyme (5 nmol) was injected onto a Dynamax-C_8 column equilibrated with 0.3% (v/v) triethylamine acetate, pH 7 (solvent A), and eluted using a gradient to 20% solvent B [0.3% (v/v) triethylamine acetate, pH 7, with 60% (v/v) acetonitrile] at 10 min followed by an increase to 65% solvent B at 70 min. The bottom trace (A) was obtained by monitoring the elution profile at 214 nm, and the upper trace (B) was from a duplicate injection monitored at 350 nm.

be readily purified by reinjection onto a Dynamax-C_8 column, followed by elution with a TFA/acetonitrile gradient. The 35-min peak contained 15% of the radioactivity initially contained in the digest and was the only labeled peptide obtained in greater than 3% yield. The sequence of the 35-min peak was Val-Tyr-Asn-X-Asp-Tyr. This sequence is completely different from one reported by Duine and coworkers (van der Meer et al., 1989); this discrepancy most likely reflects the low yield (only 0.6%) of cofactor-containing peptide in the previous work.

(*c*) *Porcine Serum Amine Oxidase.* Porcine serum amine oxidase (7.7 mg, 78.6 nmol) incorporated 46 nmol of radioactive phenylhydrazine. Thermolytic digestion of labeled porcine serum amine oxidase yielded the profile in Figure 4. The bulk of the peptides eluted in the first portion of the gradient (Figure 4A), followed by more hydrophobic peptides containing the derivatized cofactor (Figure 4B). The 33-min peak contained 32% of the radioactivity initially contained in the digest. The sequence of this peak was Leu-Asn-X-Asp-Tyr.

Isolation and Characterization of Tryptic Peptides. Active site-containing tryptic peptides were successfully isolated from tryptic digestions of pea seedling amine oxidase, pig kidney diamine oxidase, and porcine plasma amine oxidase. These were purified using linear gradients of acetonitrile as previously described (cf. Materials and Methods). Examination of the porcine kidney diamine oxidase indicated a radiolabeled peptide eluting at 54 min in 24% yield. The following sequence was obtained from this peptide: Asp/Thr-Thr-Ser-Thr-Val-Tyr-Asn-X-Asp-Tyr-Ile-unknown-Asp-Phe-Ile-Phe-Tyr-Tyr-(Asn). In the case of pea seedling amine oxidase, radiolabeled peptides resisted Edman sequencing and were assumed to be N-terminally blocked. Porcine serum amine oxidase was found to yield a very large number of radiolabeled peaks. Two major peaks were observed at 57 and 60 min, corresponding to 6% and 8% of the total radioactivity. The sequence of the 57-min peak was Ser-Val-Ser-Thr-Met-Leu-Asn-X-Asp-Tyr-Val-unknown-Asp-Met-Ile-Phe-His-Pro. A similar sequence was seen for the 60-min peak, except that the first round of sequencing indicated two amino acids (Ser/His).

Resonance Raman Spectroscopy of Thermolytic Peptides. Resonance Raman spectroscopy was employed for identification of the blank in active site peptides derived from porcine serum amine oxidase, pig kidney diamine oxidase, and pea seedling amine oxidase. Previous studies of both bovine serum amine oxidase (Janes et al., 1990) and a yeast amine from *Hansenula polymorpha* (Mu et al., 1992) have shown the identity of spectra for active site-derived peptides to that of the spectrum for the phenylhydrazone of the model compound, topaquinone hydantoin (Janes et al., 1990). The results of resonance Raman spectroscopy on *p*-nitrophenylhydrazine-derivatized peptides obtained from porcine serum amine oxidase, pig kidney diamine oxidase, and pea seedling amine oxidase are summarized in Figure 5. As shown, the intensity of signals and their peak positions are essentially superimposable for the three peptide samples and the model hydantoin, establishing the presence of topaquinone in each case. The porcine serum amine oxidase peptide sample was also examined by the liquid secondary ion and tandem mass spectrometric techniques used with a bovine serum amine oxidase pentapeptide (Janes et al., 1990), indicating the expected ions for

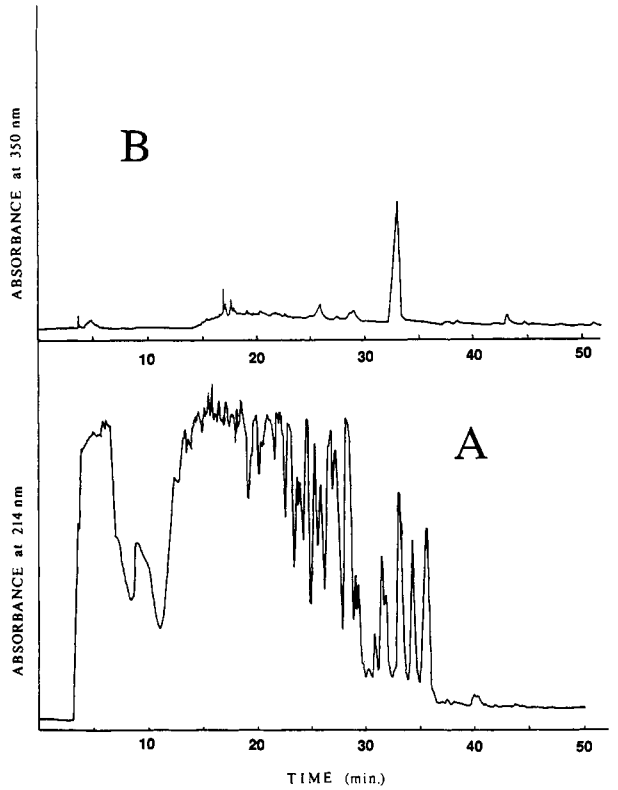


FIGURE 4: HPLC purification of a thermolytic digest of porcine serum amine oxidase. Digested, radiolabeled enzyme (15 nmol) was injected onto a Dynamax-C_8 column equilibrated with 0.3% (v/v) triethylamine acetate, pH 7 (solvent A), and eluted using a gradient to 20% solvent B [0.3% (v/v) triethylamine acetate, pH 7, with 60% (v/v) acetonitrile] at 10 min followed by an increase to 65% solvent B at 70 min. The bottom trace (A) was obtained by monitoring the elution profile at 214 nm, and the upper trace (B) was from a duplicate injection monitored at 350 nm.

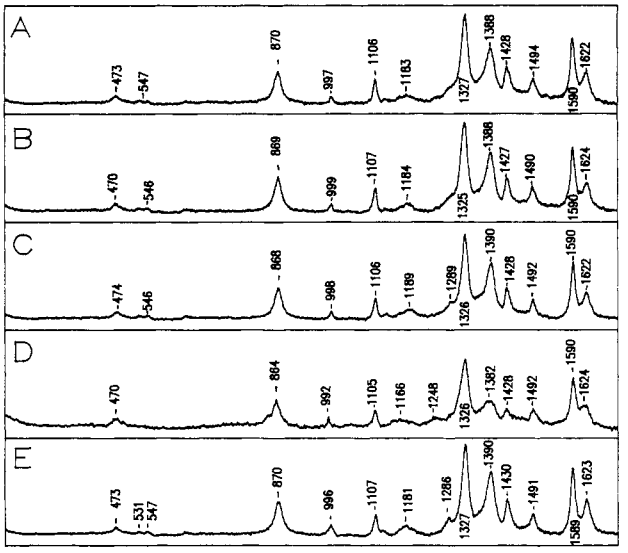


FIGURE 5: Resonance Raman spectra for active site thermolytic peptides derived from *p*-nitrophenylhydrazine-inhibited amine oxidases: (A) pea seedling amine oxidase, (B) pig kidney diamine oxidase, (C) porcine serum amine oxidase, (D) bovine serum amine oxidase (Brown et al., 1991), and (E) *p*-nitrophenylhydrazone of topaquinone hydantoin (Brown et al., 1991).

fragmentation of derivatized topaquinone and, hence, confirming the presence of this structure (data not shown).

Absorbance Spectroscopy of Phenylhydrazone Derivatives of Native Enzymes, Thermolytic Peptides, and Model Compounds. Representative spectra of the *p*-nitrophenylhydrazone adduct of a native enzyme obtained at neutral pH and in 2 M KOH are shown for the pea seedling enzyme in

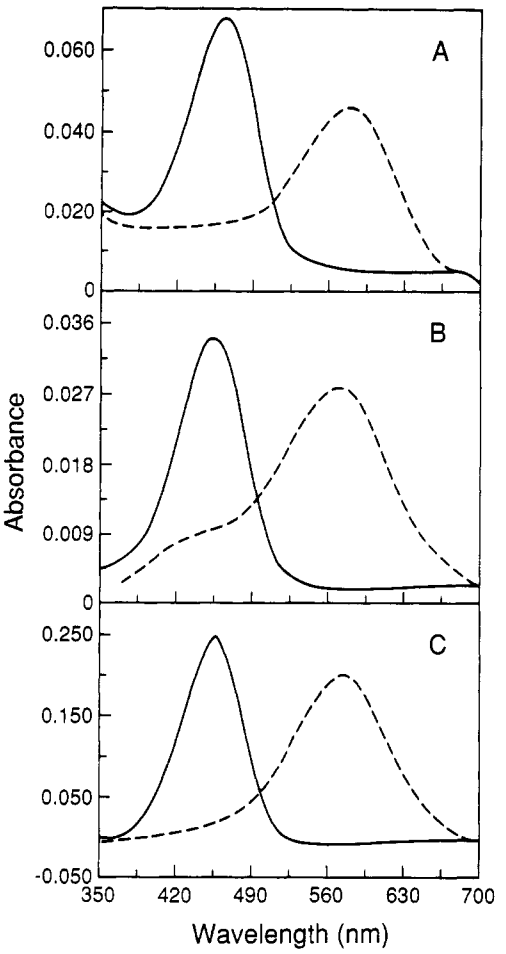


FIGURE 6: (A) Visible absorbance spectra of the *p*-nitrophenylhydrazone adduct of native pea seedling amine oxidase. Spectra were obtained with ca. 0.15 mg of protein in 1 mL of 0.1 M potassium phosphate buffer pH 7.2 (—) and in 2 M KOH (- - -). (B) Spectra of the *p*-nitrophenylhydrazone adduct of the active site peptide obtained from thermolytic digests of pea seedling amine oxidase. Spectra were obtained with peptide in 0.1 M potassium phosphate buffer pH 7.2 (—) and in 1 M KOH (- - -). (C) Absorbance spectra of the p-nitrophenylhydrazone derivative of topaquinone hydantoin. Spectra were obtained with the model compound in 0.1 M potassium phosphate buffer pH 7.2 (—) and in 1 M KOH (- - -).

Table I: Spectral Properties of *p*-Nitrophenylhydrazones of Native Enzymes and Thermolytic Peptides

	λ_{max} (nm)			
enzyme	protein (pH 7.2)	peptide (pH 7.2)	protein (2 M KOH)	peptide (1 M KOH)
bovine serum	457	457	585	584
porcine kidney	460	461	578	574
pea seedling	463	457	581	567
chick pea seedling	462	ND[a]	581	ND[a]
sheep serum	462	ND[a]	587	ND[a]
porcine serum	462	457	578	573

[a] Not determined.

Figure 6A. The λ_{max} is 463 nm for the neutral form, and this peak shifts to 581 nm in basic solution. The absorbance maxima of the *p*-nitrophenylhydrazones of six amine oxidases are listed in Table I. The adducts are all bright yellow at neutral pH and exhibit an absorbance peak at 457–463 nm. In 2 M KOH, the protein adducts turn a deep purple, reflecting a 120-nm shift of the absorbance peak maximum to 578–585 nm. Typical spectra of a peptide from the thermolytic digest of a *p*-nitrophenylhydrazone-derivatized protein are those of the pea seedling amine oxidase, Figure 6B. The λ_{max} of the *p*-nitrophenylhydrazone peptide adduct is 457 nm in neutral

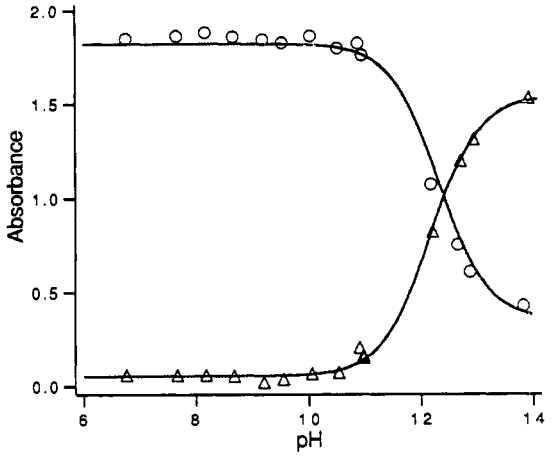


FIGURE 7: pH titration curve for the *p*-nitrophenylhydrazone of topaquinone hydantoin. Absorbance changes were monitored at 452 (○) and 576 nm (△).

buffer and 567 nm in basic solution. The spectral properties of all peptides examined are very similar, with a maximal absorbance at 457–461 nm at pH 7.2, shifting to 567–584 nm in 1 M KOH (Table I). *Comparison of the spectral properties of isolated active site peptides and native proteins, Table I, indicates that these are essentially identical.*

The spectra of the *p*-nitrophenylhydrazone derivative of topaquinone hydantoin, which are shown in Figure 6C to have absorbance maxima at 455 nm in neutral solution and 575 nm in base, display a close correspondence to the spectra for enzyme and peptide *p*-nitrophenylhydrazones, Figure 6A and B. This behavior is unique to topaquinone, with *p*-nitrophenylhydrazine derivatives of pyridoxal and pyrroloquinoline quinone revealing quite different behavior. In the case of the *p*-nitrophenylhydrazone of pyridoxal, we observe λ_{max} values at both neutral and alkaline pH which are blue shifted by 30 nm relative to topaquinone; additionally, the 541-nm band is unstable, decaying over a period of minutes. Although the absorbance properties of the *p*-nitrophenylhydrazone of pyrroloquinoline quinone resembles topaquinone at neutral pH (443 nm), this adduct undergoes only a modest red shift under basic conditions to 456 nm.

The pH-dependent changes in λ_{max} observed with native enzymes, active site peptides, and topaquinone hydantoin indicate a high pH ionization for the dyelike complex formed between the cofactor ring and *p*-nitrophenylhydrazine. The pH dependence of the shift in absorbance for the *p*-nitrophenylhydrazone of topaquinone hydantoin has been studied in detail between pH 7 and 14, indicating a pK_a of 12.2 (Figure 7). In contrast to topaquinone hydantoin and isolated peptides, which are fully ionized at 1 M KOH, native proteins require 2 M KOH for the appearance of the high-wavelength band. This could indicate an elevated pK_a for the protein-bound chromophore, but more likely is a consequence of the buffering capacity of protein.

In addition to *p*-nitrophenylhydrazine, phenylhydrazine has frequently been used to derivatize topaquinone enzymes. The absorbance maxima of the phenylhydrazones of most of the native enzymes at neutral pH are at 442–447 nm, although the porcine plasma enzyme peak absorbance is at a somewhat lower wavelength, 437 nm (Table II). A typical spectrum of the phenylhydrazone of a native enzyme is shown in Figure 8A for pea seedling amine oxidase. The isolated active site peptides of phenylhydrazone adducts all have absorbance maxima at 433–435 nm at pH 7.2 (Table II), typified by the pea seedling spectrum (Figure 8B). Peptide spectra in 1 M KOH indicate a spectral shift of about 50 nm to 482–484 nm

Table II: Spectral Properties of Phenylhydrazones of Native Enzymes and Thermolytic Peptides

enzyme	λ_{max} (nm) protein (pH 7.2)	peptide (pH 7.2)	peptide (1 M KOH)
bovine serum	447	435	482
porcine kidney	443	434	483
pea seedling	443	433	484
chick pea seedling	442	435	482
sheep serum	444	ND[a]	ND[a]
porcine serum	437	435	482

[a] Not determined.

Table III: Sequences for Active Site Thermolytic Peptides from Copper Amine Oxidases

bovine serum[a]	porcine serum[b]	porcine kidney[b]	pea seedling[b]	yeast[c]
		Val	Val	Val
Leu	Leu	Tyr	Gly	Ala
Asn	Asn	Asn	Asn	Asn
X	X	X	X	X
Asp	Asp	Asp	Asp	Glu
Tyr	Tyr	Tyr	Asn	Tyr
			Val	Val

[a] Janes et al., 1990. [b] This study. [c] Mu et al., 1992.

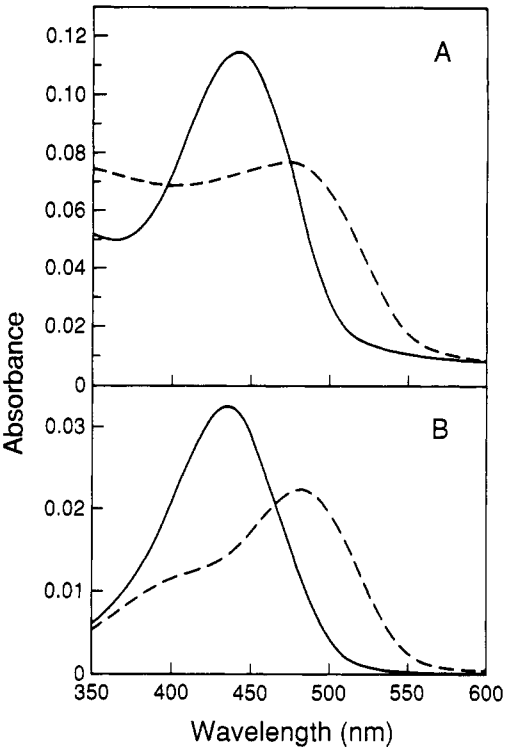


FIGURE 8: (A) Visible spectra of the phenylhydrazone of native pea seedling amine oxidase. Spectra were obtained on ca. 0.3 mg of protein in 0.1 M potassium phosphate buffer pH 7.2 (—) and in 2 M KOH (- - -). (B) Visible spectra of the phenylhydrazone of the active site peptide from pea seedling amine oxidase. Spectra were obtained in 0.1 M potassium phosphate buffer pH 7.2 (—) and in 1 M KOH (- - -).

(Table II and Figure 8B), reduced from the 120-nm shift observed for *p*-nitrophenylhydrazone adducts. When the spectra of native enzyme phenylhydrazones are measured in 2 M KOH solution, the spectral shift to 483 nm is incomplete, with a significant absorbance remaining at 443 nm (Figure 8A). This is attributed to a greater pK_a for protein phenylhydrazone than for *p*-nitrophenylhydrazone adducts, such that phenylhydrazone derivatives are incompletely converted to the high-pH form with 2 M KOH.

DISCUSSION

In this study we have applied the proteolytic conditions previously used with bovine serum amine oxidase (Janes et al., 1990) and yeast amine oxidase (Mu et al., 1992) to obtain active site peptides from other members of the copper amine oxidase class: pea seedling amine oxidase, pig kidney diamine oxidase, and porcine serum amine oxidase. In each case thermolytic digestion was performed on phenylhydrazine-derivatized proteins under mildly denaturing conditions which left the disulfide-bonded structure of protein intact.[2] The yield of the major radiolabeled peptide from each protein varied from 32% with porcine serum amine oxidase to 26% for pea seedling amine oxidase and 15% for pig kidney diamine oxidase. The low peptide yield with pig kidney diamine oxidase is attributed to the large number of hydrophobic peptides, Figure 3, and the difficulty of separating the active site peptide from background. The titration of enzyme active sites with pig kidney diamine oxidase was also low, 38%, which we ascribe to some trace protein contaminants (<10% of total protein), together with the copurification of active and inactivated enzyme[3] [cf. Janes and Klinman (1991)]. The yield of 26% with pea seedling amine oxidase is due to the partitioning of radiolabeled peptides among three peaks, Figure 2, which together represent a 45% yield. In all digestions, the appearance of active site peptides occurred without evidence for larger, precursor peptides. Although we cannot rule out the possibility of kinetically unstable peptide precursors, this observation raises the possibility that thermolysin has direct access to residues in or near the active site cleft to the copper amine oxidases. Given the success to date using thermolysin to obtain active site peptides from phenylhydrazine-derivatized proteins, this proteolysis protocol may serve as a general one for the isolation of topaquinone-containing peptides from copper amine oxidases.

Previous studies have established resonance Raman spectroscopy as a suitable tool for the identification of topaquinone in active site-derived peptides. In the case of bovine serum amine oxidase (Janes et al., 1990), mass spectrometric characterization of an active site-derived pentapeptide, together with comparative NMR studies of this peptide with a synthetic model topaquinone compound, led to the unambiguous identification of topaquinone. Subsequent resonance Raman studies provided spectra for the active site, bovine serum amine oxidase pentapeptide which were superimposable on spectra obtained for the model topaquinone compound (Brown et al., 1991). Resonance Raman has also been used to demonstrate topaquinone in the yeast amine oxidase from *H. polymorpha* (Mu et al., 1992). The resonance Raman data in Figure 5 now extend this finding to a plant enzyme (from pea seedling) and two additional mammalian proteins (from porcine serum and kidney).

A comparison of the sequences obtained from automated Edman degradation of peptides from bovine serum amine oxidase (Janes et al., 1990), porcine seedling amine oxidase, pig kidney diamine oxidase, pea seedling amine oxidase (this study), and yeast amine oxidase (Mu et al., 1992) indicates significant homology, Table III. In all instances, X was associated with radioactivity from the phenylhydrazone derivative of the active site cofactor. Alignment with regard to X reveals a high degree of homology, *leading to the identification of a consensus sequence of Asn-Topa-Asp/Glu, which is flanked on either side by hydrophobic amino acids.* The replacement of Asp by Glu in the consensus sequence has only been seen in a single enzyme (from yeast). It will be extremely interesting to see if other yeast enzymes and, in particular, bacterial enzymes utilize Glu in place of Asp. This would imply an evolutionary trend from Glu to Asp in proceeding from single-celled to more complex organisms.

Table IV: Sequences for Active Site Tryptic Peptides from Copper Amine Oxidases

bovine serum[a]	porcine serum[b]	pig kidney[b]	pea seedling[c]
Ser	Ser	Asp/Thr	
Val	Val	Thr	
Ser	Ser	Ser	
Thr[d]	Thr	Thr	
Met	Met	Val	Val
Leu	Leu	Tyr	Gly
Asn	Asn	Asn	Asn
X	X	X	X
Asp	Asp	Asp	Asp
Tyr	Tyr	Tyr	Asn
Val	Val	Ile	Val
unk[e]	unk[e]	unk[e]	Ile
Asp	Asp	Asp	Asp
Met	Met	Phe	unk[e]
Val	Ile	Ile	Glu
Phe	Phe	Phe	
Tyr	His	Tyr	
Pro	Pro	Tyr	
Asn		(Asn)	
Gly			
Ala			
Ile			
Glu			

[a] Mu et al., 1992. [b] This study. [c] Tryptic peptides from pea seedling amine oxidase were found to be N-terminally blocked. We therefore include the longer peptide obtained from thermolytic digestion (see Results) for comparison to the other amine oxidases. [d] This residue was originally identified as Asp. New mass spectrometric analysis (Medzihradszky and Burlingame, unpublished data), together with reevaluation of Edman data, indicates THR. [e] Unidentified amino acid.

Among the peptides summarized in Table III, only the functionally similar bovine and porcine amine oxidases indicate the same behavior, with both enzymes yielding pentapeptides of identical sequences. In order to explore possible species differences further, we extended our analysis of porcine serum amine oxidase to include tryptic digestion. A comparison of tryptic peptides from bovine serum amine oxidase (Mu et al., 1992) and porcine serum amine oxidase is given in Table IV, along with sequences for a tryptic peptide from pig kidney diamine oxidase and an extended thermolytic peptide from pea seedling amine oxidase. Among 18 amino acids in the porcine serum amine oxidase tryptic peptide, there is identity with bovine serum amine oxidase at 16 positions. This high degree of structural homology contrasts with the intracellular pig kidney diamine oxidase, which shows differences at 8 out of 18 positions when compared to the serum amine oxidase from the same species. This point may bear on the tissue origin of porcine serum amine oxidase, which is currently unknown but has been postulated to arise from the vascular smooth muscle [cf. Hysmith and Boor (1987)]. The absence of sequence homology between porcine serum amine oxidase and pig kidney diamine oxidase rules out a direct relationship between intracellular diamine oxidases and serum proteins. Not unexpectedly, the plant enzyme has also diverged significantly from bovine and porcine serum amine oxidases, showing identical residues at only 5 out of 11 positions.

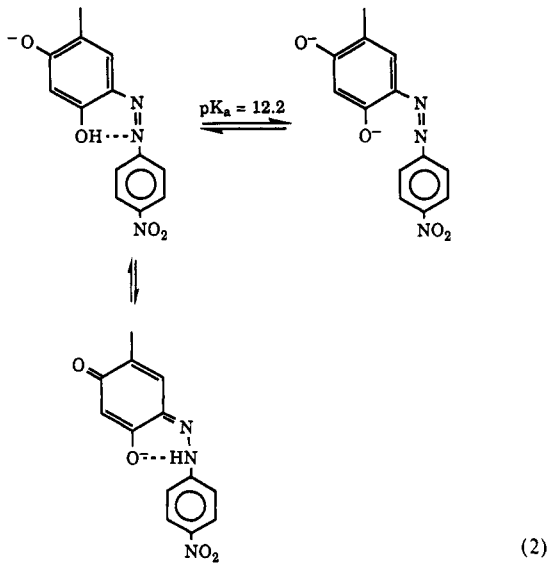
An unexpected feature of the tryptic peptide data in Table IV is the recurring presence of an unidentified amino acid, appearing at position +4 from topaquinone in bovine and

[2] We have observed that bovine serum amine oxidase remains fully active in 2 M urea.

[3] This enzyme was found to be less stable upon storage than other amine oxidases.

porcine serum amine oxidases and pig kidney diamine oxidase, and at position +6 in pea seedling amine oxidase. It should be noted that this residue is free from radioactivity and, hence, lacks a functional group capable of forming a stable derivative with [^{14}C]phenylhydrazine. The finding of a second unknown residue raises a number of questions, ranging from its conceivable role in the active site chemistry of copper amine oxidases to the possibility of oxidative damage within the active site in the course of isolation and analysis of protein/peptide samples. Attempts to characterize this amino acid by subdigestion of tryptic peptides and analysis of peptide fragments using Edman degradation and mass spectrometric analysis are currently in progress.

Although resonance Raman spectroscopy of active site-derived peptides has proven to be a valuable tool for cofactor identification in copper amine oxidases, data presented in this paper indicate that absorbance spectroscopy can be applied to intact proteins. As illustrated in Figure 6 and summarized in Table I, *p*-nitrophenylhydrazone derivatives of native proteins exhibit a pH-dependent, 120-nm red shift in absorbance which is identical to that observed with active site-derived peptides and model compounds. The properties of this absorbance shift in 1–2 M KOH appear unique to topaquinone and are attributed to the ionization of the azo group of the derivatized cofactor with a pK_a of 12.2:



(2)

Using this new method for cofactor identification, we now show that an amine oxidase from sheep plasma and a chick pea seedling amine oxidase are topaquinone-containing.

While phenylhydrazone derivatives also show pH-dependent shifts in absorbance, the effects are less dramatic than those observed with *p*-nitrophenylhydrazone derivatives. In the case of native enzyme phenylhydrazones, addition of 2 M KOH is found to give incomplete conversion to the high-pH form (Figure 8). It is, therefore, recommended that only the spectral properties of *p*-nitrophenylhydrazone adducts be used as the basis for the identification of topaquinone in a new enzyme. Caution should be exercised in using phenylhydrazone adduct spectra as indicators of topaquinone, and these should only be considered for cofactor identification when there is no reaction of enzyme with *p*-nitrophenylhydrazine.

From the data presented herein, it can be seen that two plant enzymes (from pea seedling and chick pea seedling), two plasma proteins (from porcine and sheep), and an intracellular diamine oxidase (from pig kidney) contain topaquinone as the active site cofactor. Taken together with the earlier demonstration of topaquinone in bovine serum amine oxidase (Janes et al., 1990) and yeast amine oxidase (Mu et al., 1992), we conclude that topaquinone is a ubiquitous cofactor in nature. It is anticipated that all copper amine oxidases will thus be shown to contain topaquinone as the active site prosthetic group.

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