Protein-Bound 3,4-Dihydroxyphenylalanine Is a Major Reductant Formed during Hydroxyl Radical Damage to Proteins†

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ABSTRACT: Proteins and aromatic amino acids previously exposed to hydroxyl radicals reduced cytochrome *c*, free iron, and copper ions. A major product of hydroxyl radical addition to tyrosine is 3,4-dihydroxyphenylalanine (DOPA), which has these reducing properties. The reduction of nitro blue tetrazolium by radical-damaged protein was consistent with the generation of quinones in the protein. By acid hydrolysis followed by high-performance C_{18} reversed-phase liquid chromatography we have shown that hydroxyl radical-damaged proteins contain significant amounts of protein-bound DOPA (PB-DOPA). The authenticity of the DOPA measured was confirmed by gas chromatography–mass spectrometry. PB-DOPA was also generated enzymatically using mushroom tyrosinase, which catalyzes the hydroxylation of tyrosine residues. By comparing the levels of DOPA in radical-damaged or enzyme-treated protein with that of cytochrome *c* reduction, we show that PB-DOPA is a major source of the observed reducing activity. PB-DOPA may have a role in the replenishment of reduced transition metal ions involved in free radical generating systems in vivo.

Protein damage by free radical exposure has usually been thought to produce relatively inert chemical species, with possible pathology resulting from the loss of enzymatic activity or structural integrity (Wolff et al., 1986; Pacifici et al., 1991). In contrast to this view, we have shown that proteins damaged by hydroxyl radicals contain significant amounts of protein peroxides (which can react with ascorbate and GSH) and a protein-bound reducing moiety (PBRedM)[1] which can reduce cytochrome *c*, free iron, and copper ions (Simpson et al., 1992a). This protein-bound reducing activity may have important biochemical effects, such as regeneration of reduced forms of redox-active metal ions which could induce subsequent metal-dependent radical reactions.

In previous studies we have shown that PBRedM, as measured by cytochrome *c* reduction, can be generated on a number of different proteins by exposure to free radicals generated by γ radiolysis, UV irradiation, and Fenton type systems (Simpson et al., 1992b). The reductive activity is associated with the protein and is not due to thiol formation. The acid stability of PBRedM and the ability of tyrosine to form an apparently similar reductive species after radical damage suggested that the catechol 3,4-dihydroxyphenylalanine (DOPA) is the protein-bound species reducing the metal ions. DOPA is a well-characterized reducing agent and a major product of hydroxyl radical addition to free tyrosine (Ishimitsu et al., 1990; Lynn & Purdie, 1976; Karam et al., 1984). Here we demonstrate the generation of PB-DOPA by hydroxyl radical attack and show that it is a major component of the reducing activity of PBRedM.

MATERIALS AND METHODS

Materials. Glassware was cleaned by soaking in concentrated nitric acid for 6–7 h, washing in purified water, and drying. Solutions were made up in water purified by passage through a four-stage Milli Q system (Millipore-Waters, Sydney, Australia) equipped with a 0.2-μm final filter. Catalase, fatty acid free bovine serum albumin (fraction 5), tyrosine, nitro blue tetrazolium, mushroom tyrosinase, and L-DOPA were obtained from Sigma (St. Louis, MO); bovine insulin was obtained from Boehringer Mannheim. Chelex-100 resin was supplied by Bio-Rad Laboratories (Richmond, CA). L-[$^2H_3$]DOPA (ring D^3, 98%) was supplied by Cambridge Isotope Laboratories (Cambridge, MA). Other materials were of AR or HPLC grade and were from Merck (Darmstadt, Germany), Mallinckrodt (St. Louis, MO), BDH (Poole, U.K.), Pharmacia (Uppsala, Sweden), or Aldrich (Milwaukee, WI).

Exposure of Protein to Radicals Generated by γ Radiation. Bovine insulin or BSA solution, at 1 mg/mL, in 10 mM sodium phosphate buffer, pH 7.4, was exposed for various times to γ radiation in a cobalt-60 facility. The dose rate was between 46 and 43.5 Gy min^{-1} (due to decay of the ^{60}Co source over the time of this study). In such a system HO$^\bullet$, e^-(aq), and H$^\bullet$ are the primary species formed, and manipulation of the radicals formed was achieved by bubbling either nitrogen, oxygen, or nitrous oxide through the solution during irradiation (von Sonntag, 1987). Saturation of the system with N_2O converts virtually all the primary species to hydroxyl radicals, whereas in the presence of O_2 an equimolar concentration of $O_2^{\bullet-}$ and HO$^\bullet$ is achieved. Irradiation of nitrogen-gassed solution produces equimolar amounts of e^-(aq) and HO$^\bullet$. The role of the hydroxyl radical in PB-DOPA formation was investigated by selective scavenging with 2-propanol.

G values are calculated as the number of DOPA molecules formed per 100 eV of γ irradiation (von Sonntag, 1987).

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[1] Abbreviations: BSA, bovine serum albumin; DOPA, 3,4-dihydroxyphenylalanine; e^-(aq), hydrated electron; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; HO$^\bullet$, hydroxyl radical; PB-DOPA, protein-bound DOPA; PBRedM, protein-bound reducing moieties; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

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Radiation-generated H_2O_2 was removed by the addition of a small volume of catalase (5 μg/mL final concentration). The catalase did not interfere with the cytochrome *c* reduction assay below.

Incubation of Proteins with Tyrosinase. Exactly as described by Ito et al. (1984), bovine insulin (100 nmol/mL) was incubated at 30 °C with mushroom tyrosinase (200 units/mL; 1 unit will cause an increase in A_{280nm} of 0.001/min at pH 6.5 and 25 °C in a 3-mL reaction mixture containing L-tyrosine) in 2 mL of sodium phosphate buffer (100 mM, pH 7.4). At various times the reaction was stopped by the addition of 0.5 mL of TCA (final concentration 5% (v/v), which was sufficient to denature the tyrosinase) and 200 μL (20 nmol protein) was removed for acid hydrolysis. The remaining protein solution was pelleted by centrifugation and dissolved in 250 mM sodium phosphate buffer (pH 7.4) to a final volume of 1.8 mL (pH 7.4) for measurement of cytochrome *c* reduction and PB-DOPA.

Copper-Catalyzed Radical Generation. BSA (1 mg/mL final concentration) in 10 mM sodium phosphate buffer (pH 7.4) was incubated at 37 °C with copper(II) chloride (0–100 μM) in the presence of 5 mM hydrogen peroxide for various times up to 3 h. The reaction was stopped by the addition of catalase (5 μg/mL final concentration, to remove H_2O_2) and Chelex resin (50 mg/mL final, to remove free metal) and incubation at 4 °C for 2 h with gentle agitation. The Chelex was removed by centrifugation, and the supernatant was assayed for reductive activity and PB-DOPA.

Nitro Blue Tetrazolium/Glycinate Assay for Quinones. Quinones were detected by nitro blue tetrazolium (NBT) reduction (Paz et al., 1991). To 1 mL of protein (in 10 mM sodium phosphate buffer, after the removal of hydrogen peroxide) was added 1 mL of NBT reagent (0.24 mM NBT and 2 M glycine, adjusted to pH 10 with potassium hydroxide). The mixture was incubated at room temperature in the dark for 1 h before the absorbance at 530 nm was measured.

Measurement of the Reductive Capacity of Radical-Exposed Samples. Reductive capacities were measured with cytochrome *c* by monitoring the increase in absorbance at 550 nm (Simpson et al., 1992a). The activities were confirmed by measuring reduction of free cupric ions using the neocuproine binding assay for cuprous ions (data not shown; Simpson et al., 1992a).

Gas-Phase Amino Acid Hydrolysis of Protein. DOPA is labile, and hence the addition of the reducing agent mercaptoacetic acid and the removal of all oxygen by extended flushing of the hydrolysis vessel with an inert gas was found to be essential for the detection of DOPA in the hydrolysates (Ito et al., 1984). The recovery of DOPA was also aided by using gas-phase hydrolysis, where only the acid vapor came into contact with the protein sample (Meltzer et al., 1987).

Thus, 200 μL of the protein solution (usually containing 200 μg of protein) was placed in a 0.7-mL glass autosampler vial (Alltech) and lyophilized in a vacuum centrifuge. The sample vials were placed in a Pico-Tag reaction vessel (Millipore-Waters, Australia) to which 1 mL of 6 M HCl containing 1% (w/v) phenol and 50 μL of mercaptoacetic acid were added. Oxygen-free nitrogen was then flushed through the reaction vessel for 10 min via a thin tube inserted through the inlet valve. The reaction vessel was evacuated and placed in an oven at 110 °C for 16 h. After hydrolysis, the residual acid was removed from the sample vials by vacuum centrifugation, and the hydrolysate was dissolved in 200 μL of 0.1% (v/v) trifluoroacetic acid and stored at 0 °C.

The incubation period of 16 h was found to release maximum levels of DOPA from radical-damaged protein. Spiking unirradiated and irradiated protein samples with authentic L-DOPA before hydrolysis resulted in DOPA recovery of approximately 98% on HPLC analysis.

HPLC Analysis of Protein Acid Hydrolysate for DOPA. Analytical HPLC was carried out on a Pharmacia HPLC system fitted with a prepump (low pressure) gradient mixer and using helium sparging of solvents. The column was a semipreparative LC-18 column (25 cm, i.d. 10 mm) supplied by Supelco with an LC-8 guard column. The mobile phase consisted of 5% (v/v) methanol containing 0.1% (v/v) TFA (adjusted to pH 2.5 with NaOH) and was run at 3 mL/min. Fifty microliters of hydrolysate was routinely injected onto the column. The eluted DOPA was detected by fluorometry (excitation 280 nm; emission 320 nm) using a Hitachi fluorescence spectrometer for LC (Model F-1050). Dityrosine could also be detected fluorometrically (excitation 280 nm; emission 405 nm) using a methanol gradient elution. The eluent absorbance at 210 nm was also monitored using a Pharamcia LKB VWM 2141 monitor. The detector signals were integrated by a Shimadzu C-R4A chromatopac.

The fluorescence peak with a retention time identical to authentic DOPA was quantified against standard (Sigma). The relationship between concentration and fluroescence peak area was linear in the range 5 pmol–10 nmol of DOPA. The detection limit was 5 pmol/50-μL injection of hydrolysate, which corresponds to 1 molecule of DOPA/300 molecules of BSA (BSA at 1 mg/mL). Duplicate hydrolyses and analysis gave peak areas for DOPA normally within 5% of each other. The DOPA concentration values from duplicate radiolyses were within 10% of each other.

GC–MS Analysis of Acid Hydrolysates. To confirm that the HPLC DOPA peaks observed were authentic DOPA, selected hydrolysates were chromatographed by HPLC as previously described, and the DOPA fractions were collected and lyophilized overnight. Each lyophilysate was then derivatized by adding 50 μL of trifluroethanol and 100 μL of pentafluoropropionic anhydride. The sample tubes were capped and placed into a heating block set at 80 °C for 60 min. The samples were cooled, dried under oxygen-free nitrogen, and dissolved in 20 μL of redistilled ethyl acetate. Samples (2 μL) were injected manually into the GC–MS system.

A Hewlett-Packard 5890 series II gas chromatograph directly interfaced with a Hewlett-Packard 5971A mass-selective detector was used for all GC–MS work. Chromatography was performed with a split/splitless capillary inlet on a cross-linked methyl silicon fused-silica capillary column (12 m × 0.22 mm i.d., PB1, 0.25-μm film thickness; SGE Scientific Instruments Pty Ltd). Helium was used as the carrier gas at an inlet pressure of 20 kPa (3 psi). The oven temperature was held at 40 °C for 1 min and then raised to 180 °C at 40 °C/min. Injections were made in the splitless mode with a split flow of approximately 50 mL/min and a septum purge of approximately 1 mL/min. The injection port was set at 250 °C, and the GC–MS interface was maintained at 280 °C. Data acquisition was delayed until 4 min after each injection. For the identification of DOPA one group of four masses (each at 50-ms dwell time; m/z 387, 415, 455, 554) were monitored. These are the major molecule-specific ions for this particular DOPA derivative (Goldstein et al., 1986). The presence of all four ions at the same retention time and at the same relative abundance as observed for

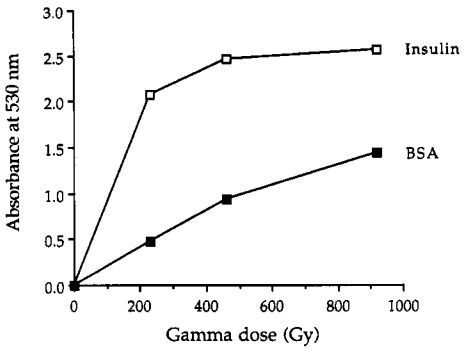


FIGURE 1: Detection of protein-bound catechol/quinones in radical-damaged BSA and insulin. Insulin (□) or BSA (■) (1 mg/mL solutions in 10 mM sodium phosphate buffer, pH 7.4) was exposed to free radicals generated by 0, 230, 460, and 920 Gy of γ radiation corresponding to irradiations of up to 20 min while oxygen gas was bubbled through the solution. One-half milligram of each of the resulting oxidized protein samples was assayed for quinones using the NBT reduction assay as in Materials and Methods. The values shown are means of duplicate samples from a single experiment representative of several. Absorbance values cannot be converted precisely into moles of reduced NBT because of protein binding of the relatively insoluble product. The variation in values was less than 5% of the mean shown.

authentic DOPA was considered to confirm the presence of DOPA in the sample.

On the basis of the HPLC, we estimated that our samples contained between 0.2 and 2 ng/μL DOPA; therefore, for the quantitative GC–MS studies [$^2H_3$]DOPA (1 ng) was added to each sample of the hydrolyzed protein. Derivatization was performed as described previously. The same four ions were monitored together with four additional ions derived from the stable isotopomer standard (i.e., [$^2H_3$]DOPA; m/z 390, 418, 458, 557). Abundance ratios for the four pairs of ions (i.e., 387/390, 415/418, 455/458, and 554/557) were calculated. A sample of DOPA [$^2H_3$]DOPA (1:1) was used to determine the relative response factor for each ion pair, and results were corrected by this factor to give four separate estimates of the DOPA concentration.

RESULTS

Protein-Bound Catechol/Quinone Formation during Radiolysis. To test our hypothesis that a catechol/quinone might be produced on proteins damaged by free radicals, we subjected BSA and insulin to γ radiolysis and measured alkaline-NBT reducing activity. We found that after irradiation, insulin and BSA were able to reduce significantly more NBT than unirradiated controls (Figure 1). NBT reduction was related to the extent of radical exposure and was observed with both oxygen-saturated and nitrous oxide-saturated protein solutions (data not shown). γ-Irradiated insulin reduced more NBT than γ-irradiated BSA. This can be explained by the higher PB-DOPA content of the insulin as measured by HPLC. This data is consistent with the presence of catechols/quinones on radical-damaged protein.

Reducing Activity of Tyrosinase-Treated Insulin. To assess more directly whether the catechol DOPA might be key among the reductants produced, we incubated insulin with mushroom tyrosinase and observed a concurrent time-related increase in PB-DOPA and PBRedM activity (Figure 2). Though tyrosinase can react with protein-bound tryptophan (as well as tyrosine), there is no tryptophan in insulin; so PB-DOPA is expected to be the major reaction product in this instance.

DOPA Formation by Radicals Generated by γ Irradiation. Our central objective was to establish whether DOPA is a

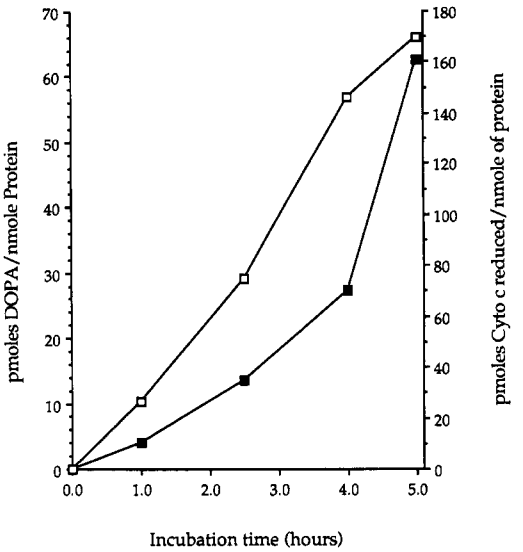


FIGURE 2: PB-DOPA and PBRedM formation by the action of mushroom tyrosinase on insulin. Bovine insulin (1 mg/mL) was incubated at 30 °C with mushroom tyrosinase (200 units/mL). At the indicated time points the reaction was stopped by the addition of TCA, and a sample was taken for acid hydrolysis and HPLC DOPA analysis as described in Materials and Methods; values are expressed as pmol DOPA/nmol treated protein (■). The TCA-precipitated protein fraction was pelleted by centrifugation and dissolved in sodium phosphate buffer before assaying for cytochrome *c*-reducing activity (PBRedM), as described in Materials and Methods; values are expressed as pmol cytochrome *c* reduced/nmol of treated protein (□). All results are means of duplicate values, and the maximum variation observed was less than 5% of the mean.

reductant generated by radical attack on proteins. Therefore, we next demonstrated the formation of DOPA. γ irradiation of bovine insulin (or albumin) under oxygen saturation caused a dose-related appearance of protein-bound DOPA, as detected by reversed-phase (C_{18}) HPLC fluorometric analysis of protein acid hydrolysates (Figure 3). Protein-bound DOPA was formed in all radiolysis systems we examined (Figure 4), including anoxic (data not shown).

The highest G value (see Materials and Methods) obtained for DOPA formation was with insulin at small radical doses, which is consistent with the loss of DOPA at higher doses (see below). With insulin at 1 mg/mL, a G value of 0.22 was obtained during oxygen gassing and of 0.07 with N_2O gassing, in each case after 460 Gy irradiation. Under the same conditions G values of 0.09 and 0.15 for 1 mg/mL BSA during oxygen and N_2O gassing, respectively, were obtained. At the higher BSA concentration of 10 mg/mL the maximum yield of reductant activity per incident radicals is achieved (Simpson et al., 1992a). The G values obtained for this BSA concentration for DOPA formation were 0.11 during oxygen gassing and 0.06 during N_2O gassing (at a comparable radical dose). At higher radical doses, the G values in all these cases declined due to the loss of DOPA (see below). This was most notable with N_2O gassing (Figure 4b,d), where the amount of DOPA present on the proteins declined, indicating a net destruction of DOPA. This is also seen when free DOPA in solution is exposed to γ-generated HO$^\bullet$ radicals and has been described during the irradiation of tyrosine-containing dipeptides (Lynn & Winchester, 1970).

DOPA is probably formed under oxic conditions through the formation of a peroxide after H abstraction by the hydroxyl radical (Cudina & Josimovic, 1986). The process involved in the hydroxylation of tyrosine to DOPA during anoxic γ irradiation of proteins is less clear (Karam et al., 1984), but

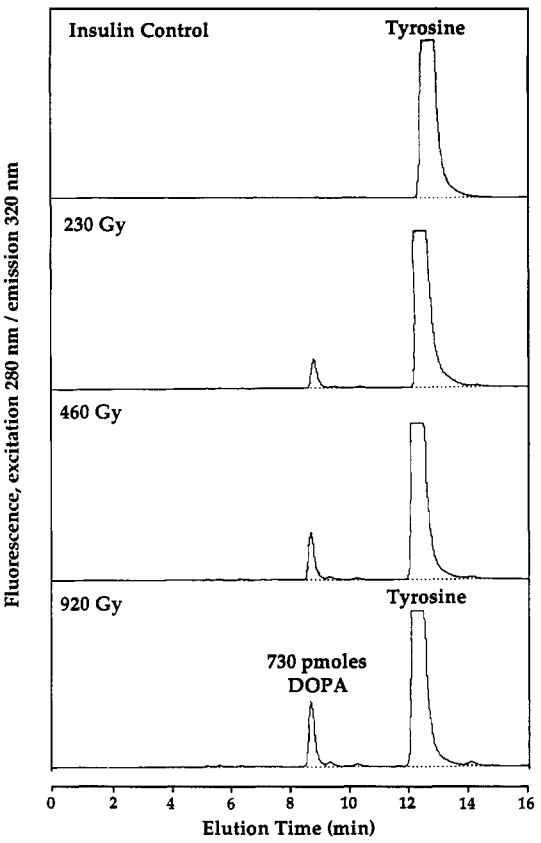


FIGURE 3: HPLC detection of DOPA after acid hydrolysis of insulin exposed to radicals generated by radiolysis. A 1 mg/mL solution of bovine insulin was exposed to increasing doses of radicals generated by γ radiation while being gassed with oxygen. The irradiated solution was acid hydrolyzed to release the individual amino acids, which were chromatographed by reversed-phase HPLC (see Materials and Methods). DOPA was detected by fluorometric monitoring of the eluent (excitation 280 nm; emission 320 nm). The values shown each represent the DOPA content of 50 μg of insulin sample.

we have shown (Simpson et al., 1992b) that the addition of a HO• scavenger such as 2-propanol to the protein solution during both oxic and anoxic irradiation prevents the formation of PBRedM, and we have now confirmed by HPLC that it also reduces the formation of PB-DOPA (data not shown), again demonstrating the requirement for HO•.

We have found by HPLC analysis of hydrolyzed γ-irradiated protein that dityrosine formation only occurs when oxygen is excluded during irradiation (results not shown). Therefore, during N_2O-gassed anoxic γ irradiations, there is a competition between the formation of DOPA and dityrosine from tyrosine.

PB-DOPA Formation by Copper-Catalyzed Haber–Weiss Reactions. PB-DOPA and PBRedM were formed simultaneously during copper-catalyzed Haber–Weiss reactions (data not shown) to an extent dependent on copper concentration (Figure 5). No PB-DOPA or PBRedM formation was observed below 15 μM copper [consistent with Simpson and Dean (1990)].

GC–MS Confirmation of the Generation of PB-DOPA. GC–MS analysis of derivatized DOPA peaks collected during HPLC analysis of protein hydrolysates of γ-irradiated (O_2, N_2, or N_2O gassing) BSA and insulin and copper/H_2O_2-oxidized insulin all showed a component eluting at 5.2 min. The mass spectrum of the component was identical to that obtained for authentic L-DOPA derivatized and run under identical conditions (major ions at m/z 554, 455, 415, and 387; results not shown). This confirmed the presence of DOPA in the radical-damaged protein.

Quantification of the DOPA content employed a stable isotope standard and was based on four separate ion abundance ratios. The excellent agreement between these values provides further confirmation that the analyte is DOPA. Four separate acid hydrolysates were examined by this GC–MS procedure (Figure 6). The mean of all four ion abundance values of each hydrolysate was within 15% of that obtained by the HPLC DOPA analyses. This further validates the HPLC fluorometric measurement of DOPA that was employed for this work.

PB-DOPA Is a Major Reductant of Cytochrome c Reduction on Radical-Damaged Proteins. In all conditions studied above (Figure 1–5), the formation of PB-DOPA is concurrent with the formation of PBRedM. Tyrosinase is known to act primarily on tyrosine in proteins, and it may also act on tryptophan. Since there is no tryptophan (or cysteine) in insulin, we assume that the reductant generated by the action of tyrosinase on insulin is almost entirely PB-DOPA. The molar ratio of cytochrome *c* reduced to PB-DOPA for the experiment of Figure 2 was about 5.5 for the first three time points and fell slightly after longer incubations. For tyrosinase treated BSA, values were comparable. Thus on the basis of these observations and theortical considerations (Scheme I) we conclude that, in the absence of other neoreductants, one molecule of PB-DOPA can reduce about 6 molecules of cytochrome *c*.

Using this value, we can calculate the expected contribution of PB-DOPA to the observed reducing activity based on the measured PB-DOPA content. In the case of insulin exposed to hydroxyl radicals under aerobic conditions, all the reductant generated can be explained by the PB-DOPA (perhaps again because of the lack of tryptophan). In all other cases above but one, PB-DOPA accounts for at least 50% of the determined PBRedM. The exception is the highest radical dose during anoxic irradiation of BSA (Figure 4d). In this instance, there has been a net loss of DOPA, and so we presume that some of its products are highly reductive also. We noted nevertheless, that the cytochrome *c* reduction:DOPA ratio does vary in most systems with the extent of radical attack, confirming some heterogeneity of reductants. We conclude that PB-DOPA is a major reductant on radical-damaged proteins. This is consistent with the ready formation of PB-DOPA by a single hydroxylation of tyrosine.

DISCUSSION

The data presented show that PB-DOPA is a product of radical attack on proteins and a major component of generated PBRedM. This does not exclude minor contributions to PBRedM by other reducing residues. We previously reported that exposure of free tryptophan to hydroxyl radicals produces species capable of reducing cytochrome *c*. However, this process only gives rise to about 30% of the reductant activity generated in corresponding treatments of tyrosine (Simpson et al., 1992a). The reductant generated from tryptophan is most probably 3-hydroxykynurenine (Lynn & Winchester 1970; Christen et al., 1990). In the proteins we have used in this paper, and in most characterized proteins, tyrosine content greatly exceeds that of tryptophan; so in general the contribution to PBRedM by tryptophan oxidation products is expected to be far less than that of PB-DOPA.

It is essential for the preservation of DOPA during acid hydrolysis of protein to include a reductant. Some previous studies of protein oxidation have failed to detect DOPA (Guptasarma et al., 1992; Levine, 1983), and this is probably because no such provisions were made to prevent DOPA loss

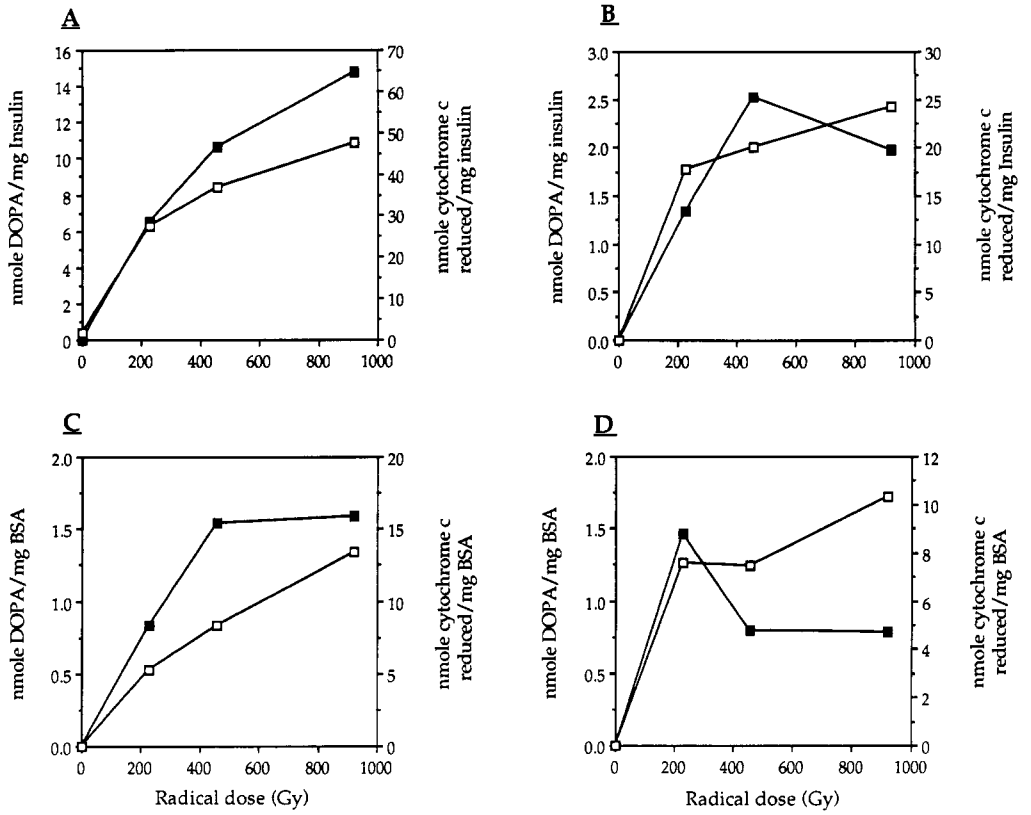


FIGURE 4: Comparison of PB-DOPA content and PBRedM activity in solutions (1 mg/mL, 10 mM sodium phosphate buffer, pH 7) of BSA or bovine insulin after increasing doses of radicals generated by γ radiation. PB-DOPA content (■) and PBRedM activity (□) were determined. The conditions examined were (A) insulin with oxygen gassing, (B) insulin with nitrous oxide gassing, (C) BSA with oxygen gassing, and (D) BSA with nitrous oxide gassing. A single experiment is shown, representative of several. Variation between experiments was less than 10%.

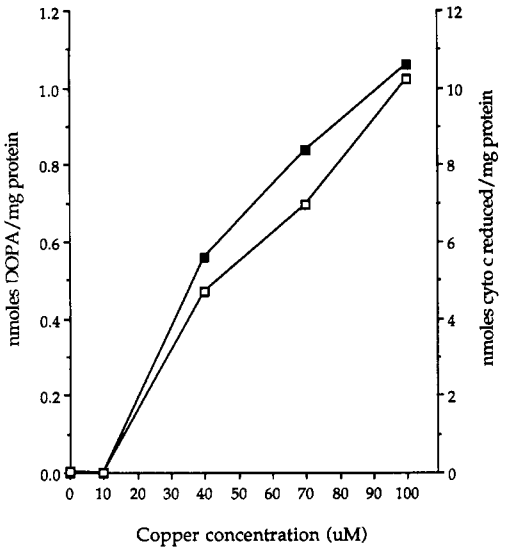


FIGURE 5: Effect of copper concentration on PB-DOPA and PBRedM formation on BSA by copper-catalyzed Haber–Weiss radical generation. BSA (1 mg/mL) was incubated at pH 7.4 with increasing concentrations of copper chloride in the presence of 5 mM hydrogen peroxide for 30 min at 37 °C. The reaction was stopped by the addition of catalase and Chelex resin before assaying for cytochrome *c*-reducing activity (PBRedM) (□) and PB-DOPA (■) were assayed as described in Materials and Methods. Values shown are means of duplicates. The maximum variation observed was less than 5% of the mean.

during acid hydrolysis. In addition, only a small portion of the tyrosine residues consumed are detected as DOPA. For example, when insulin was exposed to various doses of hydroxyl radical in oxic conditions, tyrosine consumption was progressive and reached 20% at 860 Gy; DOPA generation at each point corresponded to approximately 15% of the tyrosine consumption. In agreement with our findings on protein oxidation, several early studies have demonstrated the formation of peptide-bound DOPA from tyrosine during oxidation (Lynn & Purdie, 1976; Karam et al., 1984). On the other hand, Stadtman and Berlett (1991) have argued that, during amino acid oxidation by Fenton reagents, tyrosine is not particularly active, nor is it converted to DOPA. There was no oxygen supplied in these experiments (other than from the decomposition of peroxide), and they may thus not be comparable to the oxic Fenton conditions in which we generate reductants from both protein and free tyrosine. Futhermore, Stadtman and Berlett appear not to have attempted to protect DOPA during the analysis.

Although PB-DOPA is a major reductant, there are probably other active reducing species generated during protein oxidation. For example, Figure 4d, and to a lesser degree Figure 4b, indicates that PBRedM increases while PB-DOPA declines. We have already shown that free phenylalanine and tryptophan can be converted into reducing moieties by oxidation (Simpson et al., 1992a), and this may occur to some degree on proteins. In addition, it is quite likely that further oxidation products of protein-bound DOPA (discussed below) are themselves reductants, by analogy with the components known in the melanin pathway (Tsukamoto et al., 1992; Rodriguez-Lopez et al., 1992; Korner & Pawelek, 1982).

A key question is the nature of the reducing reaction of PB-DOPA. Since tyrosine-treated insulin gives a reductant which can be expected to be almost entirely PB-DOPA, it is notable that 1 mol of this product can reduce about 5 mol of cytochrome *c*. Similarly, free DOPA can reduce approximately 6 cytochrome *c* molecules. Presumably these reactions do not involve redox cycling, since no separate reductant

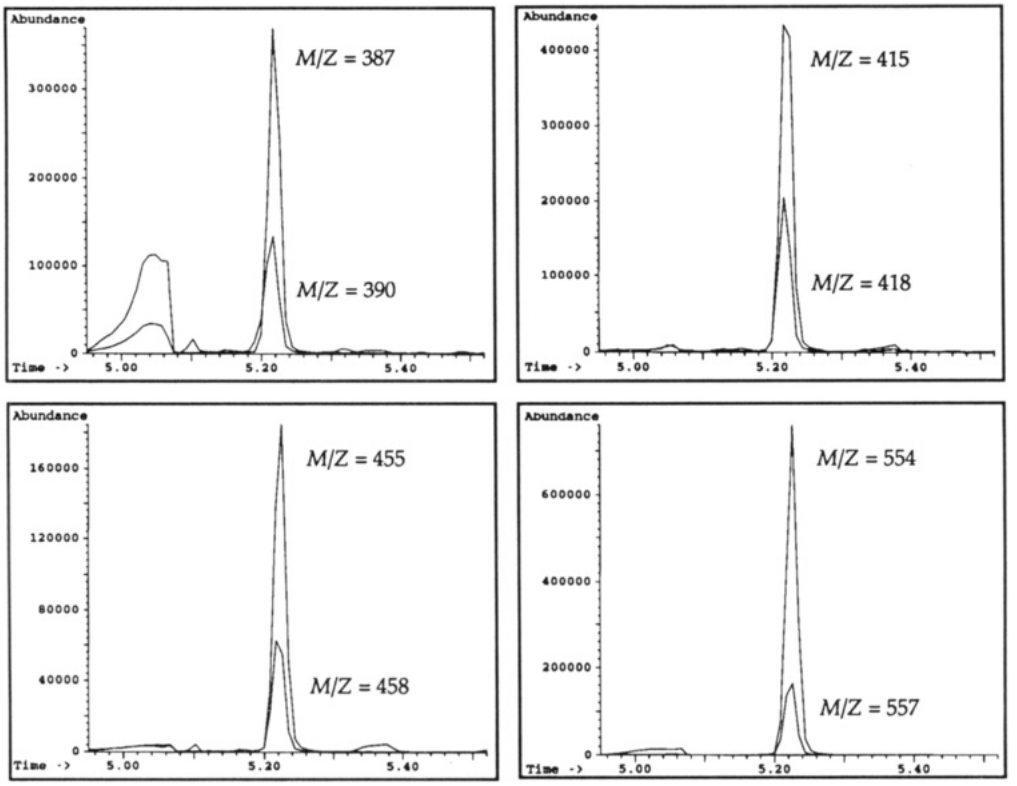
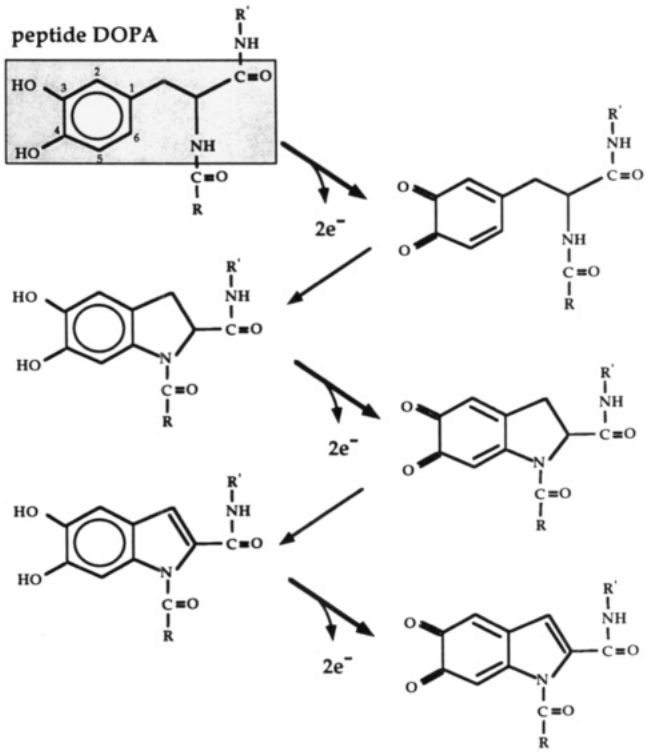


FIGURE 6: Selected ion chromatograms for each of the ions monitored in one hydrolyzed and derivatized insulin sample (1 mg/mL) after 460 Gy of γ radiation with oxygen gassing. Ions are displayed as four sets, each set containing an ion derived from the isotopomer standard and the corresponding ion for the unlabeled DOPA. (Ions monitored: group 1, 387/390; group 2, 415/418; group 3, 455/458; group 4, 554/557). The retention time for authentic DOPA derivatized and run under identical conditions was 5.22 min. Quantification of DOPA was based on the relative areas for each ion pair (see Materials and Methods). The values obtained are 387/390 = 2.75 ng/mL, 415/418 = 2.75 ng/mL, 455/458 = 2.91 ng/mL; 554/557 = 2.45, and mean ion pair value = 2.73 ng of DOPA/mL of irradiated insulin solution. The DOPA concentration determined by HPLC fluorometric analysis was 2.4 ng/mL. This result is representative of several.

Scheme I: Putative Reaction Pathway for Oxidation of Protein-Bound DOPA



capable of regenerating the catechol from the quinone is provided in our systems. Oxidation of the catechol (DOPA) to the corresponding quinone would be expected only to reduce, at most, two cytochrome *c* molecules. Thus, further oxidation of the PB-DOPA is presumably involved, such as has been described for the oxidation of free tyrosine to melanin (Rodriguez-Lopez et al., 1992). With PB-DOPA the amide portion of the DOPA is part of a peptide bond but is still able to undergo a 1,4 addition reaction where the amide nitrogen carries out a nucleophilic attack on C-6 of the phenol ring to give an indole-quinol (Scheme I). Such oxidation of this structure will reduce a further two cytochrome *c* molecules, which after another 1,4 addition reaction could be further oxidized. Thus these mechanisms alone, as shown in Scheme I, could explain the reduction of 6 mol of cytochrome *c* by 1 mol of PD-DOPA. It is not yet clear how the remaining reductant activity is generated.

Free DOPA and other reactive quinones have been implicated in a number of disease states (Ben-Shachar et al., 1991; O'Brien, 1991), and it is known that some extracellular adhesive proteins exploit potentially reactive PB-DOPA (Waite et al., 1992). If radical damage does give rise to PB-DOPA in vivo, this moiety may then play a significant role in the propagation of pathological radical damage: first, by being stable enough to diffuse from the site of the initial radical-generating event and thus transmit the radical damage to new locations; and second, by being able to promote further radical-generating reaction by replenishing the levels of reduced metals available to carry out Haber–Weiss type reactions. In vivo, the PB-DOPA may be able to undergo repeated catechol–quinone–catechol redox cycles by catalyzed reactions with cellular components such as ascorbate and glutathione, generating further radicals, as has been described with benzene quinone (Subrahmanyam et al., 1991). This would result in an amplification of the radical-damaging event that originally

generated the PB-DOPA.

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