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Myoglobin Modification by Enzyme-Generated Dopamine Reactive Species

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Dedicated to Professor Jan Reedijk on occasion of his 65th birthday

Abstract: The generation of reactive quinone species (DAQ) from oxidation of dopamine (DA) is involved in neurodegenerative pathologies like Parkinson's disease (A. Borta, G. U. Höglinger, *J. Neurochem.* **2007**, *100*, 587–595). The oxidation of DA to DAQ can occur either in a single two-electron process or in two consecutive one-electron steps, through semiquinone radicals, giving rise to different patterns of reactions. The former type of reaction can be promoted by tyrosinase, the latter by peroxidases in the presence of H_2O_2, which can be formed under oxidative stress conditions. Both enzymes were employed for the characterization of the thiol–catechol adducts formed by reaction of DA and cysteine or glutathione, and for the identification of specific amino acid residues modified by DAQs in two representative target proteins, human and horse heart myoglobin. Our results indicate that the cysteinyl–DA adducts are formed from the same quinone intermediate independently of the mechanism of DA oxidation, and that the hallmark of a radical mechanism is the formation of the cystine dimer. The reactivity of quinone species also controls the DA-promoted derivatization of histidine residues in proteins. However, for the modification of the cysteine residue in human myoglobin, a radical intramolecular mechanism has been proposed, in which the protein acts both as the catalyst and target of the reaction. Most importantly, the modification of myoglobins through DAQ linkages, and in particular by DA oligomers, has dramatic effects on their stability, as it induces protein unfolding and incorporation into insoluble melanic precipitates.

Keywords: dopamines · melanins · myoglobin · protein modifications · quinones

Introduction

Catecholamines, such as the neurotransmitter dopamine (DA) and its precursor L-dopa, and oxidative stress are mutually implicated in neurodegeneration. DA-induced neurotoxicity is in particular involved in the pathogenesis of Parkinson's disease (PD), a degenerative neurological disorder characterized by hypokinesia, rigidity, and tremor.[1–4] Oxidative stress has long been linked to the neuronal cell death associated with various neurodegenerative pathologies, such as Alzheimer's disease, PD, and amyotrophic lateral sclerosis, but it is still unclear whether oxidative stress is a major cause or merely a consequence of neurodegeneration.[5] PD, in particular, is characterized by high levels of DA in dopaminergic neurons of the substantia nigra (SN), reduced antioxidant capacity due to glutathione (GSH) deficiency,[6–8] and by an increase in iron concentration in the SN.[9–11]

In the brain, tyrosinase (Ty) may enzymatically oxidize excess amounts of DA to form melanin, thus preventing the slow progression of cell damage induced by the reactive oxygen species (ROS) generated by the competing autoxidation of DA.[12–14] Two types of melanin pigments are produced in mammals, the normal black-to-brown eumelanins and the reddish-brown pheomelanins. Both eumelanin and pheomelanin are generated from dopaquinone through a series of redox reactions, but pheomelanin also needs the presence of thiols (such as cysteine or GSH), as the units of pheomelanins (benzothiazines) are formed from addition reactions of cysteine to dopaquinone.[15] Furthermore, the quinone formed upon oxidation of DA, dopamine quinone (DAQ), forms an addition product with cysteine (cysteinyl–DA).

The potential relevance of the oxidation products of DA and cysteinyl–DA to PD prompted several groups to characterize their structure, the metabolic pathways, and their toxicity.[16–24] The initial products from the reactions of cysteine

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with catecholamine quinones generated with a variety of methods (electrochemically,[20] enzymatically with Ty/O_2[21] or peroxidase/H_2O_2,[22] with a system generating $O_2^{\bullet-}$ and H_2O_2,[23] or with Fe^{II}/H_2O_2[24]) have been shown to be the 2-*S*- and 5-*S*-cysteinyl–catecholamine (always the predominant product), and the 2-*S*,5-*S*-dicysteinyl–catecholamine conjugates.

The cytotoxicity of catechols has been ascribed to covalent binding of the quinones to various proteins, giving rise to pathological modifications.[25–33] DAQ modification of target proteins can apparently induce changes in their structures and properties, aggregation, precipitation, and in some instances promote cell death. Actually, the accumulation of aberrant or misfolded proteins, protofibril formation, and deposits in Lewy bodies in the brain is one important feature in PD.[12,34,35] Quinones are reactive towards a variety of nucleophiles, including the amino group of DA or L-dopa and the side chains of many amino acids. Most amino acids add relatively slowly to quinones, except for cysteine, which not only favorably competes with intracyclization of the amino group in DAQ, but adds to this quinone three orders of magnitude faster than the side chain amino group of other amino acids.[36] The reactivity of various quinones with the cysteine residues of bovine serum albumin, alcohol dehydrogenase, isocitrate dehydrogenase,[37,38] and the human DA transporter[39] have been reported.

The oxidation of DA to DAQ is a two-electron process and it may occur either in a single step or in two consecutive one-electron oxidations. In the latter case, a reactive semiquinone radical intermediate is formed, which may give rise to a different pattern of reactions with respect to DAQ. In this context, it is interesting that a simpler pattern of glutathionyl–catechin adducts was obtained by using peroxidase and H_2O_2 with respect to tyrosinase and O_2.[40] Since a variety of oxidants of biological relevance are able to oxidize DA, it would be important to assess whether the various oxidants may lead to different products in DA reactions. Here we report on the analysis of the thiol–catechol adducts obtained when lactoperoxidase (LPO), in the presence of H_2O_2, or Ty are used for the oxidation of DA. The kinetic analysis of the peroxidase-catalyzed oxidation of DA and thiols leads to the proposal of a reaction pathway for the generation of the thiol–catechol conjugates. In the case of tyrosinase the activity is limited to DA, whereas thiols like cysteine are inhibitors of the enzyme.[41] The reactive species generated by the two enzyme systems have been also studied for their capability to modify amino acid residues in proteins. To this end, two representative globular proteins were used as targets, human myoglobin (HMb) and horse heart myoglobin (hhMb), with the aim of investigating the competitive occurrence of modification at cysteinyl, histidyl, or lysyl residues, since only HMb contains a cysteine residue (Cys110). Mbs have been chosen as target proteins for their relatively small size, compact structure, and the extensively accumulated biochemical and biophysical characterization data, which includes a large number of X-ray crystal structures.[42] In addition, the modification of these proteins by reactive oxygen and nitrogen species has been extensively studied previously by our group, and this allows us to apply an established protocol for protein fragmentation and analysis of the modification sites.[43,44]

Results

Catalytic activity of LPO and the Mbs in catechol oxidation: LPO, hhMb, and HMb, in the presence of hydrogen peroxide, catalyze the oxidation of DA and L-dopa to the corresponding quinones, which rapidly evolve to cyclic compounds (aminochrome and dopachrome, respectively) with a characteristic absorption band at $\lambda = 476$ nm. The reaction rates depend on both the H_2O_2 and catechol concentration. Under H_2O_2 saturating conditions, the protein activity depends on the catechol concentration through Michaelis–Menten-type behavior. From data analysis, the following kinetic parameters were obtained: the catalytic rate constant (k_{cat}), the Michaelis–Menten constant (K_M), and k_{cat}/K_M; the data are collected in Table 1. As expected, LPO exhibits higher catalytic activity than hhMb and HMb. HMb is much more effective than hhMb in discriminating between DA and L-dopa. This behavior depends entirely upon a strong difference in the K_M values (≈ 270 and ≈ 1 mM for DA and L-dopa, respectively). As a result, the catalytic efficiency of HMb at low catechol concentration (expressed by the parameter k_{cat}/K_M) is also significantly higher in the oxidation of L-dopa with respect to the oxidation of DA.

Table 1. Steady-state kinetic parameters for the LPO-, hhMb-, and HMb-dependent oxidation of DA and L-dopa by H_2O_2 in 200 mM phosphate buffer (pH 7.5) at 25 °C.[a]

Protein	Substrate	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [$M^{-1}s^{-1}$]
LPO	DA	3.6 ± 0.5	2010 ± 90	$(5.6 \pm 0.6) \times 10^5$
LPO	L-dopa	n.d.	n.d.	$(1.0 \pm 0.2) \times 10^4$
hhMb	DA	46 ± 5	1.42 ± 0.05	31 ± 3
hhMb	L-dopa	22 ± 5	0.98 ± 0.11	44 ± 5
HMb	DA	≈ 270	≈ 5.1	19.4 ± 0.9
HMb	L-dopa	1.06 ± 0.07	0.60 ± 0.01	570 ± 30

[a] n.d. = not determined.

To understand if this difference in selectivity between the Mbs were connected to the mode of protein–substrate interaction, ^{1}H NMR spectroscopy relaxation time measurements were performed for both DA and L-dopa in the presence of hhMb or HMb. In these experiments, the paramagnetic contribution to relaxation by the high-spin Fe^{3+} center of the protein can be exploited to get an estimate of the distances of the protons of bound catechol from the iron atom.[45] The data show that the effect of the Mbs on the relaxation of the catechol protons is negligible: assuming an electron relaxation time (τ_s) of 5×10^{-11} s,[45] the iron–proton distances for the Mb-bound catechols was evaluated to be above 11 Å in all cases. This suggests that DA and L-dopa cannot enter into the distal cavity of the protein and approach the heme; therefore, substrate discrimination likely depends on specific

electrostatic or polar interactions of the catechols with residues on the protein surface.

Reaction of LPO and hhMb compound II derivatives with *N*-acetylcysteine and DA: The reaction of heme proteins with hydrogen peroxide yields an unstable compound I intermediate that rapidly evolves to an appreciably longer-lived compound II intermediate, the reduction of which constitutes the rate-limiting step of the peroxidase catalytic cycle. The reduction of the compound II intermediate LPO–Fe^{IV}=O to the resting, met form LPO–Fe^{III} by *N*-acetyl-L-cysteine or DA was followed spectrophotometrically through the shift of the Soret band under pseudo-first-order conditions; with *N*-acetyl-L-cysteine (cys), the observed rate constant (k_{obs}) was linearly dependent on the substrate concentration (Figure 1) according to Equation (1):

$$k_{obs} = k_{II}[cys] + b \quad (1)$$

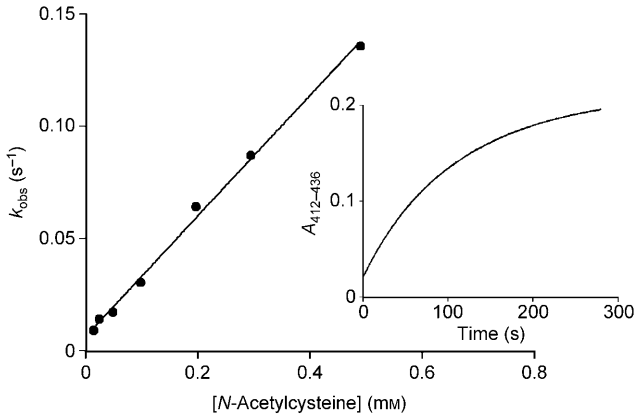


Figure 1. Dependence of the pseudo-first-order rate constant for reduction of LPO compound II (3.7 μM) to LPO–Fe^{III} on *N*-acetylcysteine concentration in 0.2 M phosphate buffer (pH 7.5) at 25 °C. The inset shows, as an example, the absorbance changes (λ = 412–436 nm) with time in the experiment with *N*-acetylcysteine (0.015 mM).

in which the nonzero value for the intercept on the *y* axis (*b*) is due to self-reduction of LPO–Fe^{IV}=O to the met form. Fitting of the experimental data to Equation (1) gave the rate constant $k_{II} = (268 \pm 7)\ M^{-1}s^{-1}$ for the reduction of LPO compound II with *N*-acetyl-L-cysteine.

In the presence of DA, the reduction of LPO compound II is very fast; with the amount of DA required by the pseudo-first-order conditions, the reaction was over in less than 1 s. Therefore, only an estimate of the rate constant k_{II} could be obtained, approximately $2.5 \times 10^5\ M^{-1}s^{-1}$, that is, in the range of the k_{cat}/K_M parameter obtained from steady-state studies (Table 1). A comparison of the k_{II} values shows that the reaction of the LPO compound II species with catechol is by far more efficient than that with *N*-acetyl-L-cysteine.

The reactivity of the compound II derivative of the Mbs was studied for hhMb, as it is known that HMb in the presence of H_2O_2 (and in the absence of substrates) does not form a stable compound II but undergoes homodimer formation through cross-linking between two cysteine residues.[46] Therefore, HMb is not suitable for mechanistic analysis. In the reduction of hhMb compound II to the met form, the spectral changes for hhMb–Fe^{IV}=O reduction by either DA or *N*-acetyl-L-cysteine with time followed first-order behavior. The observed rate constants depend on the substrate concentration with a saturating behavior, and can be described by Equation (2):

$$k_{obs} = \frac{k_{max}[S]}{K_D + [S]} + b \quad (2)$$

in which K_D represents the dissociation constant of the hhMb–Fe^{IV}=O/substrate (S) (i.e., DA or *N*-acetyl-L-cysteine) complex, and k_{max} is the first-order decay constant of the ferryl complex to the met form. Also in this case, to take into account the self-decay of the compound II intermediate, the *b* constant was introduced into Equation (2). Fitting of the experimental data to Equation (2) gave the kinetic parameters reported in Table 2.

Regarding the reduction of hhMb compound II by DA, the high efficiency of the reaction precludes the possibility of obtaining rate data employing [DA] > 8 mM (above which the reaction finishes in the mixing time), thus making the estimate of k_{max} unreliable. Therefore, the only reliable parameter obtained from the reduction of hhMb–Fe^{IV}=O by DA is the ratio $k_{max}/K_D = 32\ M^{-1}s^{-1}$, which is very similar to the k_{cat}/K_M value obtained from steady-state studies (Table 1). Moreover, from the comparison of the curves and the kinetic parameters obtained with dopamine and *N*-acetyl-L-cysteine (reported in Figure 2 and Table 2, respectively), it emerges that also for myoglobin (as for LPO) the reaction of the compound II species with catechol is by far more efficient than that with cysteine.

Identification of cysteinyl–DA and glutathionyl–DA conjugates: The Cys–dopa conjugates were isolated and characterized by reacting L-dopa with Cys in the presence of tyrosinase and O_2,[21] which oxidize the catechol to quinone in a two-electron process. The adducts formed were 2-*S*-cysteinyl–L-dopa, 5-*S*-cysteinyl–L-dopa (most abundant), and 2-*S*-5-*S*-dicysteinyl–L-dopa. This product pattern is similar to that

Table 2. Kinetic parameters for LPO–Fe^{IV}=O and hhMb–Fe^{IV}=O reduction by DA and *N*-acetylcysteine in 200 mM phosphate buffer (pH 7.5) at 25 °C.

Protein	Substrate	K_D [mM]	k_{max} [s^{-1}]	k_{max}/K_D [$M^{-1}s^{-1}$]	k_{II} [$M^{-1}s^{-1}$]
LPO	DA	–	–	–	$\approx 2.5 \times 10^5$
LPO	*N*-acetyl-L-cysteine	–	–	–	268 ± 7
hhMb	DA	19 ± 9	0.6 ± 0.2	32 ± 4	–
hhMb	*N*-acetyl-L-cysteine	< 2	0.025 ± 0.003	≈ 15	–

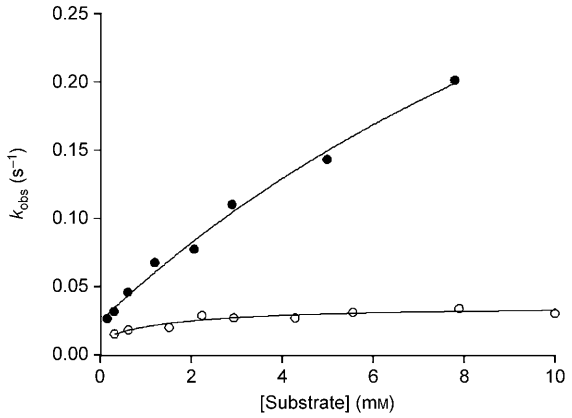


Figure 2. Dependence of the pseudo-first-order rate constant for reduction of hhMb compound II (3.7 μM) to hhMb–Fe^{III} on DA (●) or *N*-acetylcysteine (○) concentration in 0.2 M phosphate buffer (pH 7.5) at 25 °C.

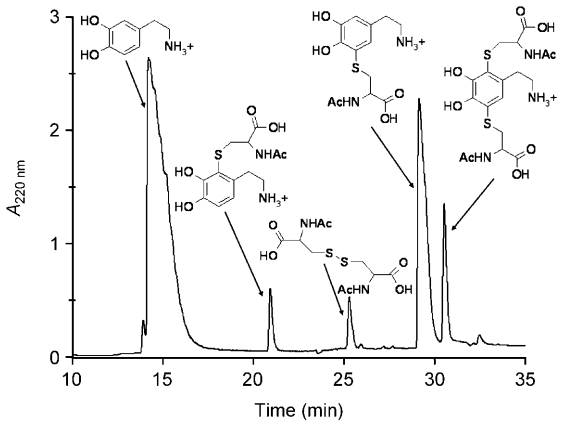


Figure 3. HPLC elution profile, with absorbance reading at $\lambda = 220$ nm, for the LPO (8×10^{-8} M) catalyzed reaction of *N*-acetylcysteine (20 mM) with DA (30 mM) in the presence of H_2O_2 (5 mM) in 200 mM phosphate buffer, pH 7.5, at room temperature. The assignment of the peaks is shown.

obtained by generating DAQ electrochemically from dopamine,[20] whereas the 5-*S*-isomer was the only detectable adduct when DA was oxidized by a system generating $O_2^{\bullet-}$ and H_2O_2 (as a model of oxidative stress).[23] With these last two methods, catechol oxidation may occur through a one-electron oxidation process leading to a transient dopamine semiquinone radical, which then dismutates in solution to DAQ and DA. To investigate the reactivity of the DA semiquinone radical, *N*-acetyl-L-cysteine was employed, in which the protected amino group prevents the cyclization of cysteinyl–DA conjugates to generate benzothiazines.[47] The semiquinone radical was generated by LPO in the presence of H_2O_2, which is very efficient in this reaction.[48,49] The products of the enzymatic reaction were separated by using HPLC and characterized by using MS and NMR spectroscopy (with ^1H and ^1H-^1H DQF-COSY spectra). Besides the peak of unreacted DA, four major compounds were isolated, corresponding to 2-*S*-*N*-acetyl-L-cysteinyl–DA, 5-*S*-*N*-acetyl-L-cysteinyl–DA (the predominant product), 2-*S*-5-*S*-di-*N*-acetyl-L-cysteinyl–DA, and *N*-acetyl-L-cystine; no significant amount of 6-*S*-*N*-acetyl-L-cysteinyl–DA was detected. An analogous mixture of products was reported for the horseradish peroxidase catalyzed oxidation of L-dopa in the presence of Cys.[22] Figure 3 shows the HPLC profile, together with the assignment of the peaks to the various products. The spectroscopic characterization for all the products is reported in Table S1 in the Supporting Information; for the cysteinyl–DA conjugates, the data are similar to those reported previously by Xu et al.[20]

A product pattern of Cys–DA adducts similar to that obtained with LPO, but with lower yields, was obtained with the Mbs, indicating a similar mechanism for the Mb and peroxidase-mediated formation of the adducts. Also, when the reaction of *N*-acetyl-L-cysteine with DA was carried out in the presence of Ty, under the same experimental conditions as before but without H_2O_2, the products and relative yields were similar, except for *N*-acetyl-L-cystine, which in this case was formed only in trace amounts by autoxidation of cysteine (data not shown). Thus, the only marker for DA semiquinone formation is the cystine dimer, whereas the Cys–DA adducts are formed from the same quinone intermediate independently of the mechanism of DA oxidation.

With the aim of modeling the reactivity of the Cys residue in a peptide environment, the tripeptide glutathione (GSH) was taken into consideration. When GSH was reacted with LPO-generated dopamine semiquinone and quinone species, the mixture of DA conjugates contained 2-*S*-glutathionyl–DA, 5-*S*-glutathionyl–DA (see Figure S3 in the Supporting Information for its ^1H-^1H DQF-COSY spectrum), 2-*S*-5-*S*-diglutathionyl–DA, and the glutathionyl dimer (Table S2 in the Supporting Information), which are analogous to the products derived from the reaction of *N*-acetyl-L-cysteine, but also 6-*S*-glutathionyl–DA, which was obtained in a significant amount to allow its NMR spectroscopic and MS characterization. Also Ito et al.[50] reported, for L-dopa conjugates obtained by Ty oxidation, that the yield of 6-*S*-glutathionyl–L-dopa is much higher than that of the 6-*S*-cysteinyl–L-dopa adduct. The complete spectroscopic characterization of the 2-*S*- and 6-*S*-glutathionyl–dopamine conjugates is reported here for the first time, whereas the other adducts have been previously characterized.[51]

Also in this case, for the reaction of GSH with DA in the presence of Ty, under the same experimental conditions as with LPO/H_2O_2, the same products with similar relative yields were obtained, except for the absence of the glutathionyl dimer. Thus, the latter compound is in vitro a marker of the occurrence of dopamine semiquinone radicals.

Histidinyl–DA and lysyl–DA conjugates: Besides cysteine, histidine can also form addition products with quinones, but with lower efficiency (the reaction of DAQ with *N*-acetylcysteine is estimated to be at least 10^6 times faster than that of *N*-acetylhistidine) and a different regioselectivity.[52,53] By operating under the same conditions as those described pre-

viously for cysteine in the presence of LPO and H_2O_2, and also with different concentrations of the reagents, both histidine and *N*-α-acetylhistidine gave only the DA oxidation products, which indicated that the reaction of imidazole with the quinone is too slow to compete with the internal cyclization of DAQ and the consequent oligomerization. The same absence of nucleophilic reactivity towards enzyme-generated DAQ species, at least under the relatively mild conditions employed here, was observed for lysine. These results agree with those obtained by Whitehead et al.[39] regarding the reactivity of the electrochemically generated DA quinone with cysteine, lysine, and histidine (at pH 7.4); also in that case DAQ was found to react only with cysteine. Products from the reaction of histidine with quinones were obtained when *N*-acetyldopamine was employed,[53] in which the amino protecting group prevents the faster DAQ cyclization reaction.[36]

Modification of HMb and hhMb by reactive DA quinones: Although histidine (His) and lysine (Lys) as free amino acids exhibit very low reactivity against DAQ and dopamine semiquinone, their reactivity in a protein could be different due to the influence of the protein environment. To assess this point, the derivatization of Cys, His, and Lys residues in the Mbs by the reactive quinone species generated from DA was investigated. The modifications undergone by HMb and hhMb were analyzed by using HPLC–ESI-MS/MS upon reacting DA (1 mM) and Mb (6×10^{-5} M) under various conditions: 1) in the presence of H_2O_2 alone (0.3 mM), in which the Mbs act both as a source and a target of the quinones; 2) in the presence of H_2O_2 (0.3 mM) and LPO (8×10^{-8} M); and 3) in the presence of Ty (8×10^{-8} M). In the second and third cases, the high reactivity of LPO[54] and Ty[55,56] with DA make the contribution of the Mbs self-promoted derivatization negligible. In these experiments, the reactants (H_2O_2 and DA) were added to the solutions divided into small aliquots; in this way, the concentration of the reactive quinone or semiquinone species was always kept very low, with the aim of simulating a pathophysiological condition.[57,58] Moreover, it is worth noting that no derivatization was observed for the fraction of HMb or hhMb left in solution when these proteins were reacted with higher concentrations of H_2O_2 (up to 3 mM concentration); this may indicate that under forcing conditions the proteins were incorporated into the melanic precipitate that is formed in the reactions (see below).

The modifications undergone by the proteins were identified by tandem MS studies on the polypeptide fragments resulting from tryptic digestion of the apomyoglobin (apoMb) derivatives. The data are reported in Table 3, together with the percentage of derivatization obtained from the integration of the peaks in the chromatograms with extracted ion current (EIC). The HPLC–ESI-MS/MS analysis with the SEQUEST algorithm showed the presence of two types of derivatization in the Mbs. In HMb, the Cys110 residue in the 103–118 peptide was found modified with a DA moiety, with a mass increase of 151 amu with respect to the unmodified peptide. The MS/MS spectrum of this modified peptide is shown in Figure 4. In the case of hhMb, the modification is observed at the histidine residues His81 and/or His82 in the 79–87 peptide, with an increment of 302 amu with respect to the molecular weight of the unmodified peptide. In the latter case, the MS/MS data are consistent both with the addition of one DA molecule to each His or a DA dimer to a single His.

Table 3. Percentage derivatization of Mb residues (Cys110 in HMb, His81 and/or His82 in hhMb derivatives, respectively) upon modification of the proteins with DA in the presence of the catalytic systems indicated, operating in 200 mM phosphate buffer (pH 7.5). The percentage derivatization of each modified residue was calculated from the HPLC–MS/MS traces by considering the ratio of the ion current of the modified peptide relative to the total ion current of the same peptide (in both the unmodified and modified form).

Protein target	Catalytic system	Cys110 +151 amu [%]	His81/His82 +302 amu [%]
HMb	HMb/H_2O_2	30	0
HMb	LPO/H_2O_2	5	0
HMb	Ty/O_2	0	0
hhMb	$hhMb/H_2O_2$	[a]	≈3
hhMb	LPO/H_2O_2	[a]	≈2
hhMb	Ty/O_2	[a]	≈2

[a] Cys110 is absent in hhMb.

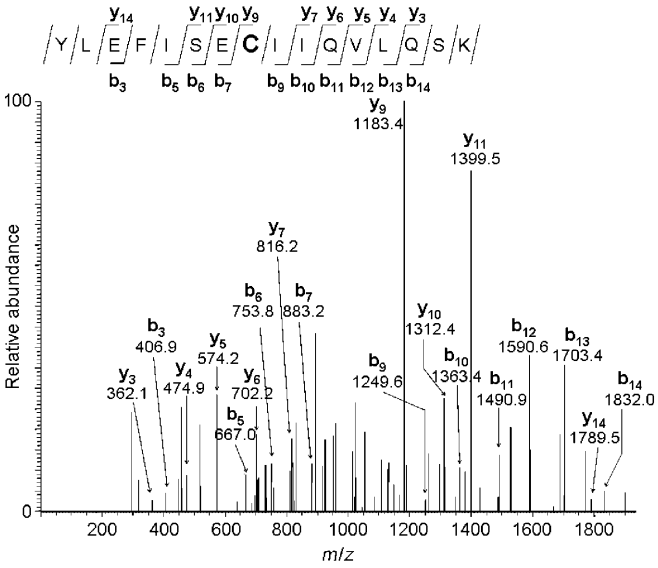


Figure 4. MS/MS spectrum of the *m*/*z* 1033.1 peak (mass of 2064.3 amu) assigned to the 103–118 peptide of HMb in a double-charged state containing the Cys110–DA adduct. The assignment of the y and b ion series are shown. Above the spectrum, the sequence of the 103–118 peptide is shown with the modified residue in bold and with the summary of the y and b ions found in the spectrum.

It has been reported that the oxidation of DA by peroxidase/H_2O_2 (but not by Ty/O_2) could also lead to the neurotoxin 6-hydroxydopamine, the toxicity of which is related to the susceptibility to nucleophilic attack by the protein residues to the corresponding quinone.[59] Nevertheless, in our

conditions we never observed this type of protein derivatization, which would correspond to the presence of a modified HMb or hhMb peptide with a mass increment of 167 amu with respect to the unmodified peptide.

The greater reactivity of Cys relative to His residues appears both from the absence of His modification in HMb and by the much larger amount of Cys110 derivatization in HMb with respect to the His81/His82 derivatization in hhMb. Interestingly, His modification is not observed in HMb, possibly because Cys110 in HMb protects His residues from the reaction with the quinone species. Regarding the amount of Cys110–DA formed in the various experiments, the endogenous derivatization promoted by HMb appears to be the most efficient mechanism, as Cys110–DA is formed in much higher yield (30%) than in the presence of LPO as external catalyst (5%). On the other hand, at least under the mild conditions employed here, Ty does not appear to promote the formation of a DA adduct of the Cys residue to any appreciable extent, possibly because the competing melanization process is faster.

The reactivity of the His81/His82 residues in hhMb towards DAQ appears to be different from the above-discussed Cys reactivity in HMb, since a low (<5% yield) and comparable extent of derivatization was obtained in all the conditions (hhMb/H_2O_2, LPO/H_2O_2, or Ty/O_2).

Tandem MS analysis of the protein/melanic precipitate: The formation of a dark brown precipitate was observed in the reaction mixtures obtained upon modification of both HMb and hhMb in the presence of reactive DAQ species, whatever their source (Mb/H_2O_2, LPO/H_2O_2, or Ty/O_2). The amount of insoluble material increased by dialyzing the apoMb derivatives before subjecting them to tryptic fragmentation. The dark color of the precipitate, both before and after heme extraction from the Mbs, indicates the presence of a melanin-type DA polymer. Upon melanin formation, the oligomers of reactive quinones may covalently link the proteins, thus producing insoluble melanin–protein conjugates. For this reason, the precipitates collected from the reaction mixtures were also subjected to peptic, tryptic, or consecutive peptic–tryptic digestion, and the resulting polypeptide fragments were analyzed by using HPLC–ESI-MS/MS. For both the HMb and hhMb derivatives, the peptides resulting from each proteolytic treatment cover the complete Mbs sequence and, together with the unmodified peptides, also the modification at Cys110 (+151 amu) in HMb and at His81/His82 (+302 amu) in hhMb were observed. Numerous attempts were performed with the aim of identifying other possible derivatization sites; in particular, the SEQUEST algorithm was applied to the search of peptide fragments containing histidine or lysine residues modified with up to eight DA moieties per protein molecule. Actually, the hhMb/melanin precipitates obtained both in the absence and in the presence of LPO, and proteolyzed with pepsin followed by trypsin, clearly revealed the presence of the 80–96 peptide with a mass increment of 1359 amu (i.e., nine DA moieties) with respect to the corresponding unmodified peptide. The MS/MS spectra are consistent with the addition of five DA units at the His81/His82 cluster and four DA units at the His93 residue (see Figure S4 in the Supporting Information for its MS/MS spectrum). It is likely that other multiderivatized sites exist in the protein–melanic deposits, even though their detection may be complicated.

Guanidine hydrochloride (Gdn-HCl) denaturation assay: The unfolding midpoint $[Gdn\text{-}HCl]_0$ and thermodynamic parameters obtained for Gdn-HCl-induced denaturation of native Mbs and the Mbs resulting from covalent DA modification are reported in Table 4. In the case of hhMb, the derivatization of the His81/His82 residues with two DA molecules, even at low levels (≈ 3%, Table 3), appreciably affects the stability of the protein, lowering both the free energy change for conversion of native to unfolded protein in the absence of denaturant (ΔG°_{N-U}) and the solvent-exposed surface area ($-m$). These results indicate that the modification of polar residues (i.e., histidine) make the protein less sensitive to a hydrophilic denaturant like Gdn-HCl, as confirmed also by the higher Gdn-HCl concentration causing 50% denaturation ($[Gdn\text{-}HCl]_0$) obtained in the case of modified hhMb with respect to the native protein (Table 4).

Table 4. Thermodynamic parameters for unfolding induced by Gdn-HCl (ΔG°_{N-U} and $-m$) and Gdn-HCl concentration causing 50% denaturation ($[Gdn\text{-}HCl]_0$) of native and modified Mbs (labeled Mbs*, and obtained by reaction of the proteins with 1 mM DA and 0.3 mM H_2O_2), in 200 mM phosphate buffer (pH 6.0) at 25°C.

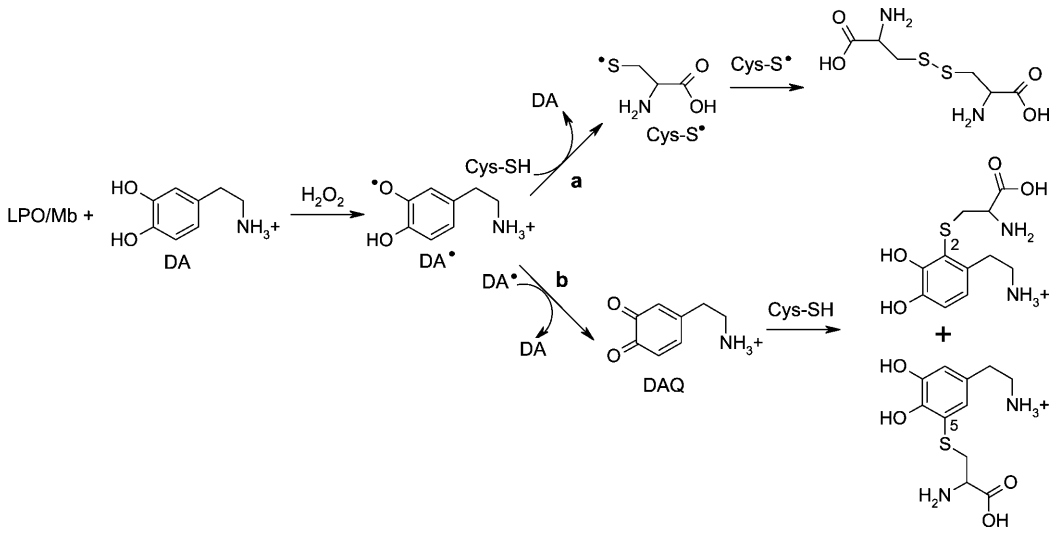
Protein	ΔG°_{N-U} [kcal mol^{-1}]	$-m$ [kcal mol^{-1} M^{-1}]	$[Gdn\text{-}HCl]_0$ [M]
hhMb	6.17 ± 0.09	6.05 ± 0.09	1.028 ± 0.003
hhMb*	5.54 ± 0.04	5.31 ± 0.04	1.039 ± 0.002
HMb	5.17 ± 0.06	5.04 ± 0.06	1.030 ± 0.003
HMb*	n.d.[a]	n.d.[a]	n.d.[a]

[a] The experimental data of protein absorbance versus [Gdn-HCl] show a complex pseudosigmoidal behavior that cannot be fitted with the equation involving the denaturation of a single protein.

A more considerable effect on protein stability is induced by DA derivatization of the Cys110 residue in HMb. Actually, the presence of a significant fraction of modified protein in the case of HMb reacted with DA/H_2O_2 (≈ 30%, Table 3) significantly alters the curve of absorbance versus [Gdn-HCl]. The graph does not show a single sigmoid and therefore could not be fitted with the equation corresponding to a simple two-state model employed for native HMb.

Discussion

Mechanism of the catalytic formation of cysteinyl–DA conjugates: The high reactivity of tyrosinase in the oxidation of catechols like DA and L-dopa[55,56] requires the formation of the quinone followed by the nucleophilic addition of the −SH group for the cysteinyl–catechol conjugates, obtained from the reaction of catechols with *N*-acetylcysteine (or GSH). In the case of peroxidases, the situation is complicat-



Scheme 1. Mechanism for the LPO/Mb-promoted formation of cystine (path **a**) and monocysteinyl–DA conjugates (path **b**).

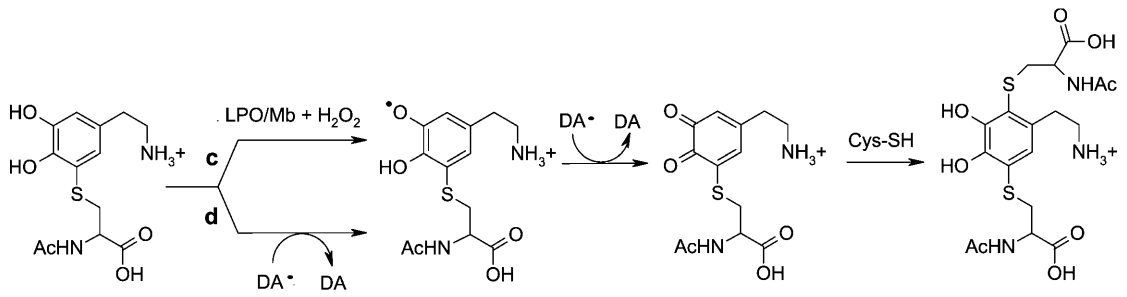
ed since the enzyme-active species can react with both the thiol and the catechol and with the latter substrate both the quinone and semiquinone species are formed in the solution. The kinetic studies on the activity of the LPO or Mb intermediates and the analysis of the composition of the product mixture obtained from the reaction of DA with *N*-acetylcysteine, or GSH, in the presence of LPO or Mb/H_2O_2, in comparison with those obtained with Ty, supports the reaction mechanism reported in Scheme 1.

In particular, as the second reduction step in the peroxidase catalytic cycle[60], involving the compound II intermediate, is generally the slow step, we determined the rate constants for the reduction of the LPO–Fe^{IV}=O intermediate by DA and *N*-acetylcysteine. The rate constants (k_{II}) obtained for the reaction of *N*-acetylcysteine and DA, 268 and $2.5\times10^5\,M^{-1}s^{-1}$, respectively, indicate that when both of these substrates are present in solution, the peroxidase will strongly prefer the reaction with catechol, which will be oxidized to the semiquinone radical (DA•). As an example, with [DA] = 18 μM, the LPO–Fe^{IV}=O species is converted into the native form in less than 1 s, whereas with cysteine at a similar concentration (15 μM) the reaction requires 400–500 s (inset in Figure 1). In the case of myoglobin, the preference for the oxidation of DA with respect to cysteine is not so striking, but at the concentration of the two substrates employed in the formation of the cysteinyl–DA conjugates ([DA] = 30 mM and [*N*-acetylcysteine] = 20 mM), it is possible to evaluate from the data in Table 2 that only a minor fraction (≈6%) of hhMb–Fe^{IV}=O is reduced by cysteine rather than by DA. The higher DA reactivity could also be evaluated from the extrapolation of the curves in Figure 2 at high substrate concentration.

The DA• radical generated from the one-electron oxidation of dopamine by the LPO or Mb compound I and II, can either disproportionate, thus generating DAQ (path **b**, Scheme 1), or oxidize a cysteine molecule to a cysteinyl radical (path **a**). In the former case, the nucleophilic attack of the cysteinyl thiol group on the electron-deficient quinone leads to the monocysteinyl adducts (2-*S*-Cys–DA, 5-*S*-Cys–DA, or 2-*S*-GSH–DA, 5-*S*-GSH–DA, and 6-*S*-GSH–DA). Conversely, the cystine dimer is generated by a coupling reaction of two cysteinyl radicals.

Another possible pathway for the generation of the cysteinyl–dopamine adducts is the coupling between semiquinone and cysteinyl radicals. Nevertheless, such a reaction can be excluded by considering the similar product composition for the Cys–DA adducts obtained by one-electron oxidation (catalyzed by LPO or Mb) and two-electron oxidation (catalyzed by Ty). The product distribution is controlled by the reactivity of DAQ with cysteine, both for the LPO/Mb-promoted and the Ty-promoted reactions, and not by the reactivity of the semiquinone species. The formation of the cystine dimer in the former reaction is significant, since it can be considered the hallmark of a radical mechanism, which is also that supposed to occur under oxidative stress conditions. Regarding the regioselectivity of the nucleophilic attack, the preference for the C5 position of the quinone ring has been explained to result from an intramolecular base-catalyzed Michael 1,6-addition, based on the formation of a hydrogen bond between the carbonyl group at the C4 position and the thiol proton.[61,62]

The dicysteinyl–DA conjugates (2-*S*-5-*S*-diCys/GSH–DA) can be obtained by reaction of cysteine with the 5-*S*-Cys–DAQ adduct (or 2-*S*-Cys–DAQ, see below) generated from the reaction between the DA and 5-*S*-Cys–DA semiquinones, according to Scheme 2. The latter species is derived from the direct one-electron oxidation of 5-*S*-Cys–DA by LPO/Mb compounds I and II (path **c**), or by the DA•-mediated oxidation of 5-*S*-Cys–DA (path **d**). Actually, dopamine and cysteinyl–dopamine compete for the reaction with LPO/Mb reactive species, the former being favored by its higher concentration (path **d**) and the latter by its lower redox potential[20] (path **c**).



Scheme 2. Mechanism for the LPO/Mb-promoted formation of the dicysteinyl–DA conjugate through protein-active species (path **c**) and DA• (path **d**).

Scheme 2 shows the formation of 2-*S*-5-*S*-diCys–DA starting from 5-*S*-Cys–DA, since this is the predominant monocysteinyl adduct in the reaction mixture, but the dicysteinyl conjugate could also be obtained from 2-*S*-Cys–DA according to an analogous reaction pathway.

The similarity in product pattern observed when GSH is used instead of *N*-acetylcysteine indicates that Schemes 1 and 2 can be extended to cysteine-containing peptides such as GSH and proteins, and also explains the generation of the mono- and diglutathionyl conjugates (2-*S*-glutathionyl–dopamine, 5-*S*-glutathionyl–dopamine, 6-*S*-glutathionyl–dopamine, and 2-*S*-5-*S*-diglutathionyl–dopamine, respectively). It is worth noting that a key role has been attributed to GSH in neuron degeneration; its translocation from glial cells (where it is largely synthesized)[63] into the cytoplasm of dopaminergic cell bodies in the SN, increases in response to chronic brain insult.[16,64] Interestingly, the glutathionyl–DA adducts, resulting from the scavenging of DAQ by GSH, are hydrolyzed to cysteinyl–DA adducts by intraneuronal peptidase enzymes.[57,65,66] The possibility that GSH, rather than cysteine, is the precursor of cysteinyl–DA is supported by the higher GSH concentration in the brain with respect to cysteine.[57,67] A neuroprotective role of GSH has been also suggested since it can act as a DAQ scavenger.[68]

Myoglobin modification by DA reactive species—the initial steps: Under oxidative stress conditions, where relatively high levels of hydrogen peroxide are produced, activation of heme proteins and in particular peroxidases can occur. In the presence of catecholamines, such as DA, the consequent DAQ reaction with target proteins can induce changes in their structures and properties, with pathological implications.[25–33]

The reactive species formed upon oxidation of DA can diffuse into the solution but also react with the protein responsible for their formation. The Mbs are good targets for the investigation of the capability of the DA-generated reactive species of modifying amino acid residues of the heme protein generating them since, due to their limited reactivity, they act both as the catalyst and substrate. In particular, HMb contains several histidines and lysines, and a single cysteine, which is expected to be the most reactive residue towards quinone species.[52] The high Cys reactivity has been confirmed by our results, since Cys110 was modified to a significant extent in the HMb self-promoted reaction (Table 3). It is interesting to note that with the enzymes used to promote DA oxidation, LPO is less efficient than HMb itself in the DA derivatization of cysteine, and that Ty does not promote Cys110 derivatization to any detectable extent. This could indicate a different reactivity of the HMb cysteine residue in the one-electron and two-electron oxidation pathways. Although the three-dimensional structure of wild-type HMb is not available, a reference structure for localizing the residues subjected to modifications is the crystal structure of the HMb mutant Lys45Arg/Cys110Ala (Figure 5).[69] In particular, Cys110 is not directly exposed to the protein surface, therefore its modification probably indicates that the active species, that is, DA• or DAQ, diffuses from outside the distal cavity (where DA binds to the Mbs according to our NMR spectroscopy relaxation-time results) inside the protein. According to the data reported in Table 3, the diffusion of the semiquinone species to the site of derivatization is more efficient, in particular when generated by the same HMb protein molecule that also contains the target residue of the derivatization.

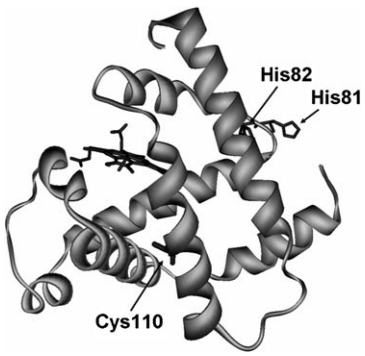
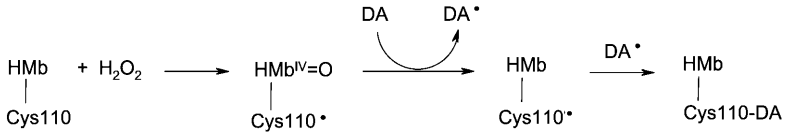


Figure 5. Structure of the Lys45Arg/Cys110Ala mutant of HMb.[69] The disposition of the side chains of Cys110, Hys81, and His82 present in the wild-type protein are shown.

Another possibility is that the Cys110 residue is oxidized to the cysteine radical upon reaction of HMb with H_2O_2.[44,70] In this case, Cys110• will be formed by the HMb active species analogue of compound I of peroxidases. This

yields an HMb compound II-like species that in turn reacts with a DA molecule oxidizing it to semiquinone. The coupling between DA$^{\bullet}$ and Cys110$^{\bullet}$ radicals gives rise to the observed HMb–DA adduct (Scheme 3). The DA$^{\bullet}$ radical may approach the thiol radical from the active site of HMb or from the bulk of the solution in an intermolecular process. This hypothesis outlines a completely different mechanism for the HMb Cys110–DA adduct formation with respect to that formed with free cysteine. In this case, in fact, the reaction occurs through radical coupling, whereas the previous mechanism consists of a nucleophilic addition of the thiol to the DA–quinone. Some indication of the actual mechanism could be deduced from the regiochemistry of the Cys–DA covalent linking in the protein. The MS/MS fragmentation pattern of the DA-modified peptide 103–118 shown in Figure 4 does not allow one to distinguish between the possible isomers (2-*S*, 5-*S*, or 6-*S*). However, the presence of a single peptide containing the DA modification on Cys110 in the HPLC chromatogram seems to indicate that only one of the possible Cys–DA isomers is present as the major or only species in the modified protein, possibly at C5 of the aromatic ring, which is the principal site of attack with the free amino acid with both nucleophilic or radical mechanisms.[20]



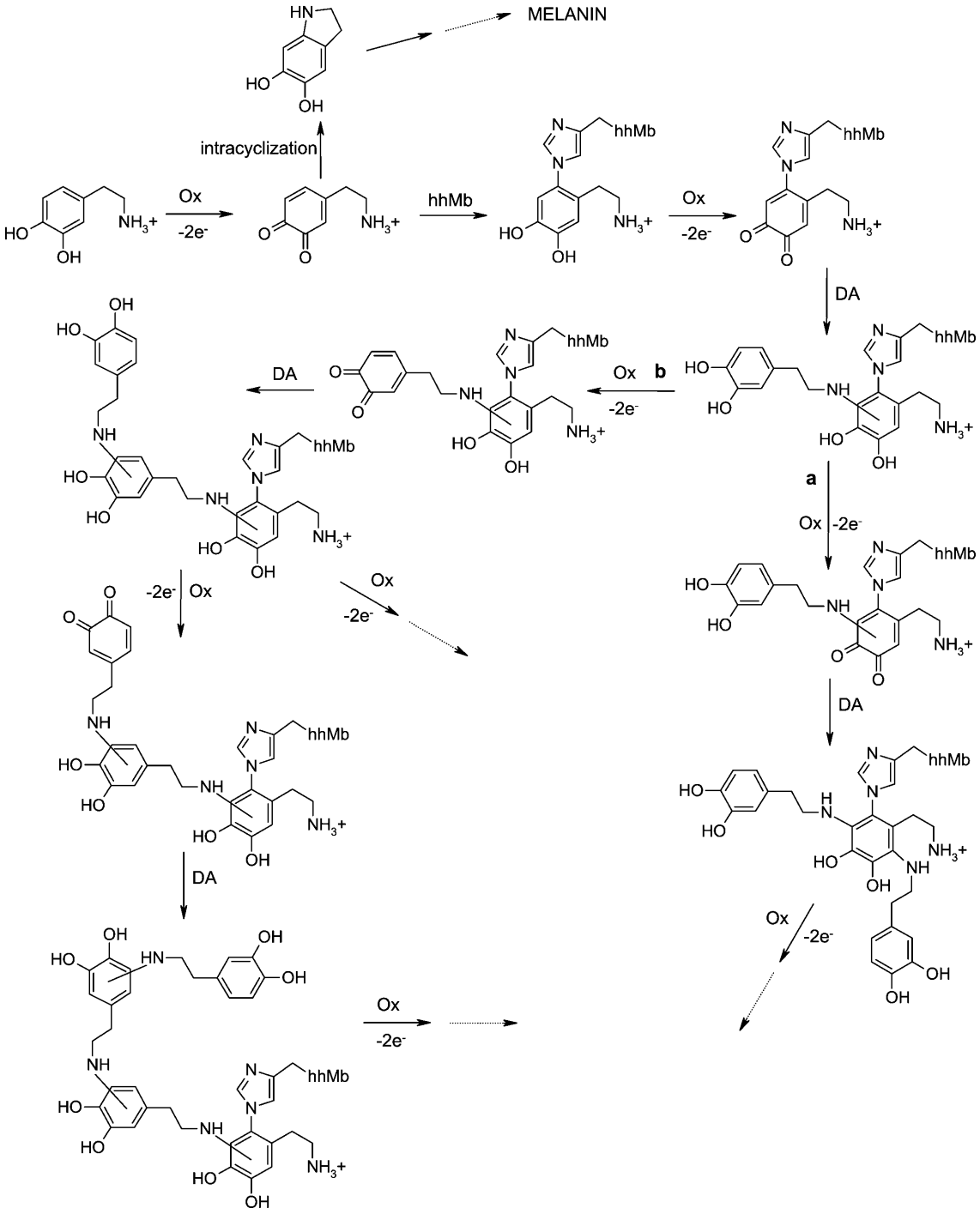
Scheme 3. Radical mechanism for the HMb self-promoted formation of the Cys110–DA adduct.

The reduced extent of modification of Cys110 produced by LPO/H_2O_2 (Table 3) may be related to the fraction of hydrogen peroxide that, instead of giving rise to compound I of the peroxidase, reacts with HMb to give the thiol radical. In fact, although the rate constant for the formation of LPO compound I is several orders of magnitude larger[71,72] than that of Mb,[73] the concentration of the latter protein in solution is much larger (thousands fold). The relatively high yield observed for Cys110 self-modification by HMb may be seen as a protecting effect of the protein when it acts in "side reactions" such as that generating the undesirable DAQ reactive species. The scavenging effect of Cys110 is also in agreement with the lack of His modification in HMb.

With hhMb it is possible to analyze the reactivity of histidine residues, as cysteine is absent. Whereas upon treatment of free histidine with the DAQ-generating systems no hystidinyl–DA conjugates were formed, hhMb DA derivatization occurred at H81 and/or H82 residues even under mild conditions. This indicates that the hhMb environment can influence the reactivity of histidines, and enhance it. The effect could be derived from a lowering of the pK_a of the imidazole residue, which is induced by the protein residues in the local environment through their (partial) positive charge and polarity; this is suggested by the fact that modification occurs at a His cluster. Regarding the regiochemistry of the nucleophilic attack, we could extend the results obtained from the free amino acid–catechol adduct to the protein system by considering the C6 position of the DA aromatic ring[53] as the main derivatization site. The reactivity of His residues of hhMb towards DAQ species differs from that of Cys in HMb in that a similar extent of His81/His82 modification is obtained in the former case with all of the DAQ-generating protein systems (Table 3), which indicates an intermolecular mechanism of derivatization. Thus, in this process the reactive DAQ species is generated by a protein molecule different from that containing the histidine targets of modification.

Myoglobin modification by DA reactive species—protein–melanic conjugates: Modification of proteins through DAQ linkages has dramatic effects on their stability, as it may induce unfolding and protein precipitation. Even at early stages, the reaction of quinone species with protein residues depresses the thermodynamic stability of the protein (Table 4), but further reactions lead to more dramatic consequences. We systematically observed that the modification of HMb or hhMb by reactive DAQ species occurs with the concomitant formation of a dark melanic precipitate. Also, the oligomers formed as intermediates in the process of melanin formation are reactive species that may react with protein residues. The observation of Mb peptides in the tryptic fragments from treatment of the protein–melanic precipitate indicates that Mb molecules are incorporated into the precipitate. The inability to control these side reactions and the precipitation of the protein prevents the full detection of the protein residues modified by DAQ species. In addition, an extensive modification of protein residues masks the recognition sites to the proteolytic enzymes, thus preventing fragmentation of the protein and thorough analysis of the modification sites. Actually, the HPLC–MS/MS analysis always showed the His81/His82-modified peptide in the hhMb–melanin precipitates; it was also found left in solution. Since this modification by itself does not induce the protein precipitation (as deduced from the unfolding studies), we can presume that what causes hhMb precipitation is the cross-linking between the protein and DAQ oligomers. In fact, proteolytic digestion of hhMb–melanin precipitate showed the peculiar modification of the His81/His82 cluster with five DA units and the His93 residue with a 4-DA oligomer. We can speculate that the derivatization of the His81/His82 cluster promotes the unfolding of the protein and this facilitates the attack of dopamine oligomers to other protein residues and causes the incorporation of the protein into the precipitate. In this respect, it is worth noting that His93 is the heme proximal ligand in the native protein and therefore it can become accessible only upon unfolding of hhMb.

Scheme 4 describes a possible mechanism for the formation of adducts between histidine residues and DA oligo-



Scheme 4. Proposed mechanism for the formation of the adducts of histidine–DA oligomers in hhMb.

mers. The process starts with the initial addition of DAQ to a histidine residue. Since His–DA, like Cys–DA,[20] is more readily oxidized to quinone than DA itself, the protein-bound DA can be an end product but also a potential electron-donor agent, as similarly proposed by Akagawa et al. for the precipitation of the Cys–DA modified proteins.[13] For hhMb and other proteins lacking cysteine it is necessary to evoke the reaction between histidine residues and DA oligomers as most likely responsible for the formation of protein–melanic conjugates. It should be also appreciated that DAQ undergoes a fast intramolecular cyclization and that this reaction reduces its half-life in solution and competes with the addition of an external nucleophilic group. But in the oxidized form of His–DA the cyclization is prevented, as the imidazole moiety blocks the C6 position of the quinone ring, thus allowing the addition of the amino group of another DA molecule. Therefore, in the mechanism proposed in Scheme 4, the formation of the growing DA oli-

gomeric chain occurs in several steps, in which the oxidation of the catechol species bound to the protein yields quinone derivatives that are unable to give internal cyclization. The oxidation is then followed by the addition of a DA molecule, instead of DAQ, to the modified protein. This type of nucleophilic addition by DA amino groups to the quinone species is in agreement with the constant mass increment of 151 amu observed in tandem MS studies, which corresponds to the addition of entire DA units. In the mechanism reported in Scheme 4, both the oxidation of the catechol ring of the DA molecule directly connected to the histidine residue and the terminal DA, according to path **a** and path **b**, respectively, have been considered. The terminal arrows reported in the scheme indicate that the DA oligomerization process can proceed with the oxidation of every catechol moiety present in the growing protein–melanic conjugate. Furthermore, besides the reaction of the DA amino group, the nucleophilic addition of DA hydroxyl groups to the quinone form of the growing adduct, which would lead to the formation of ether bonds, also cannot be excluded as a further modification pathway.

Conclusion

The present study has shown that, notwithstanding the high reactivity of the catechol-derived reactive species (semiquinones and quinones), only specific amino acid residues are modified in the proteins. Their surface exposure and the local environment in the protein seems to be a key factor ruling the reactivity. The extent of protein modification also depends upon the system generating the reactive quinone species, and whether the mechanism is of radical type and involves intra- or intermolecular processes. Most importantly, when the derivatization becomes extensive it induces protein precipitation and its incorporation into the melanic precipitate. We are currently trying to set up new protocols for the fragmentation and analysis of the insoluble protein–melanic conjugates, as this type of investigation represents a fundamental step in the understanding of the nature, composition, and mechanism of formation of neuromelanins and other protective neuronal pigments.[74] We are currently extending the investigation of the effect of dopamine modification to another protein of the globin family, human neuroglobin, a protein that is expressed in the brain, and contains three cysteine residues, but the function of which is still unclear.[75]

Experimental Section

Reagents: All buffer solutions were prepared with deionized Milli-Q water. Hydrogen peroxide (30% solution), dopamine hydrochloride, L-dopa, *N*-acetyl-L-cysteine, *N*-α-acetyl-L-histidine, L-histidine, *N*-α-acetyl-L-lysine, reduced L-glutathione, guanidine hydrochloride, trypsin, pepsin, horse heart Mb, and mushroom tyrosinase were obtained from Sigma. The other reagents were obtained at the best grade available. The concentration of hydrogen peroxide solutions was controlled by monitoring the formation of the 2,2′-azinobis(3-ethylbenzothizoline-6-sulfonic acid) (ABTS) radical cation according to a standard enzymatic method.[76] Lactoperoxidase was purified from bovine milk as previously described;[77] recombinant human Mb was expressed and purified as previously reported.[44] In all the experiments the Mbs were utilized in their met form. All spectrophotometric measurements were performed on a Hewlett Packard HP 8452A diode array spectrophotometer.

Kinetic studies of catechol oxidation: The kinetic experiments were carried out in 200 mM phosphate buffer, pH 7.5, using a quartz cuvette with path length of 1 cm, kept at (25.0±0.1) °C by using a thermostat, and equipped with a magnetic stirrer. The initial solution containing the protein (LPO, hhMb, or HMb) and variable substrate (DA or L-dopa) concentrations (final volume 1600 μL) was obtained by mixing solutions of appropriate concentration of the reagents in the buffer. The reaction was started by the addition of the H_2O_2 solution and was followed during the initial 10–15 s by monitoring the absorbance change at $\lambda = 476$ nm for both the substrates (the λ_{max} of both dopaminechrome (DAC) and dopachrome (dopaC)). The conversion of the rate data from absorbance per second into molarity per second was done by using the extinction coefficients of DAC and dopaC at $\lambda = 476$ nm ($\varepsilon = 3300$ and $3600\,M^{-1}cm^{-1}$, respectively).[78] The kinetic parameters were obtained by fitting the plots of experimental rates at different catechol concentrations to the Michaelis–Menten equation.

For each substrate, the rate dependence on the reactant concentrations was studied through two steps: 1) finding a suitable [H_2O_2] that maximizes the rate but avoids unwanted excess of the oxidant, and then using this [H_2O_2] for step 2) in which the dependence of the rate versus [catechol] was studied. The LPO and hhMb (or HMb) concentrations were 0.01 and 1 μM, respectively, while the concentrations of the other reactants were as follows: with LPO and DA, [H_2O_2] = 0.53 mM, [DA] = 5–50 mM; with LPO and L-dopa, [H_2O_2] = 0.53 mM, [L-dopa] = 2–40 mM; with hhMb and DA, [H_2O_2] = 36 mM, [DA] = 5–250 mM; with hhMb and L-dopa, [H_2O_2] = 36 mM, [L-dopa] = 2–33 mM; with HMb and DA, [H_2O_2] = 136 mM, [DA] = 0.7–30 mM; with HMb and L-dopa, [H_2O_2] = 136 mM, [L-dopa] = 0.6–25 mM.

The reaction rates observed for the noncatalytic reaction, that is, in the absence of the protein, or without hydrogen peroxide are completely negligible.

Reactions of LPO−Fe^{IV}=O and hhMb−Fe^{IV}=O with *N*-acetylcysteine or DA: The LPO and hhMb compound II intermediates were prepared by incubating the proteins (3.7 μM in 200 mM phosphate buffer, pH 7.5) with H_2O_2 (2 equiv) for about 15 min, until the Soret band shifted from 412 to 430 nm in the case of LPO, and from 410 to 420 nm in the case of hhMb, and stabilized at the final wavelengths. The reduction of LPO−Fe^{IV}=O to LPO−Fe^{III}, and hhMb−Fe^{IV}=O to hhMb−Fe^{III}, after the addition of the reducing substrate, was followed spectrophotometrically by recording the variation of absorbance at 412 and 436 nm, and at 410 and 428 nm, respectively, at (25.0±0.1) °C.

The reaction of LPO−Fe^{IV}=O with DA was carried out by adding the substrate at 18 μM final concentration; in the case of *N*-acetylcysteine, different substrate concentrations (from 0.015 to 4.7 mM) were employed. The reactions of hhMb−Fe^{IV}=O with both DA and *N*-acetylcysteine were studied by employing different substrate concentrations, from 0.15 to 7.8 mM for DA, and from 0.31 to 10 mM for *N*-acetylcysteine.

NMR spectroscopy relaxation measurements: The effect of the addition of variable amounts of hhMb or HMb (0–200 μM) on the T_1 relaxation time for the protons of both DA (40 mM) and L-dopa (40 mM), in deuterated 0.2 M sodium phosphate buffer pD 7.5, was determined at 25 °C with a Bruker AVANCE 400 NMR spectrometer operating at 400.13 MHz proton resonance, by using the standard inversion recovery method.[79] To eliminate interference by metal impurities, a small amount of ethylenediaminetetraacetic acid (EDTA) was added to the solutions.

HPLC analysis of amino acid–DA and GSH–DA conjugates: The product mixtures derived from the reaction of amino acids (*N*-acetyl-L-cysteine, *N*-α-acetyl-L-histidine, L-histidine, and *N*-α-acetyl-L-lysine) and GSH with the DA–oxidation products generated by LPO/H_2O_2, Mb/H_2O_2, or Ty/O_2 were obtained by allowing the reaction to occur for 10 min at room temperature in 200 mM phosphate buffer pH 7.5. The

concentration of the reactants were as follows: 1) using *N*-acetyl-L-cysteine or GSH as nucleophiles, with LPO: [nucleophile] = 20 mM, [DA] = 30 mM, [LPO] = 8×10^{-8} M, [H_2O_2] = 5 mM (divided into 5 aliquots of 1 mM each, added every 5 min); with Mbs: [nucleophile] = 20 mM, [DA] = 30 mM, [Mb] = 2×10^{-6} M, [H_2O_2] = 5 mM (divided into 5 aliquots of 1 mM each, added every 5 min); with Ty: [nucleophile] = 20 mM, [DA] = 30 mM, [Ty] = 1×10^{-8} M; 2) in the case of *N*-α-acetyl-L-histidine, L-histidine, or *N*-α-acetyl-L-lysine several runs were performed using DA (30 mM), and changing the reagent concentrations as follows: [nucleophile] = 20–40 mM, [LPO] = 8×10^{-8} M, [H_2O_2] = 1–25 mM (divided into 5 aliquots, with additions every 5 min). The products were analyzed by HPLC by using a Jasco MD-1510 instrument with spectrophotometric diode array detection equipped with a Supelcosil LC18 reverse-phase semipreparative column (5 μm, 250 × 10 mm). Elution was carried out starting with 0.1% trifluoroacetic acid (TFA) in water for 5 min, followed by a linear gradient, in 55 min, to 20% acetonitrile in water containing 0.1% TFA. The flow rate was 5 mL min^{-1}. Spectrophotometric detection of the eluate was performed in the range 200–600 nm. The retention times of the products obtained with *N*-acetyl-L-cysteine and GSH are reported in Tables S1 and S2 in the Supporting Information, respectively; no derivatization of *N*-α-acetyl-L-histidine, histidine, or *N*-α-acetyl-L-lysine was observed.

MS and NMR spectroscopic characterization of cysteinyl and glutathionyl derivatives of DA: The products eluted from HPLC were collected, taken to dryness, and analyzed by NMR spectroscopy and MS. ^{1}H NMR spectra of the compounds dissolved in D_2O were recorded at 25 °C on a Bruker AVANCE 400 spectrometer, operating at 9.37 T; ^{1}H resonances were assigned through the ^{1}H and ^{1}H-^{1}H DQF-COSY spectra.

Electrospray ionization MS spectra were acquired by using an LCQ ADV MAX ion-trap mass spectrometer equipped with an ESI ion source and controlled by Xcalibur software 1.3 (Thermo-Finnigan, San Jose, CA, USA). ESI experiments were carried out in positive-ion mode under the following constant instrumental conditions: source voltage 5.0 kV, capillary voltage 46 V, capillary temperature 210 °C, tube lens voltage 55 V.

Modification of the Mbs by quinone species and tandem MS analysis of protein fragments: Samples of modified HMb and hhMb were prepared by adding DA (1 mM) and the following reagents to the protein solutions (6×10^{-5} M, in 50 mM phosphate buffer, pH 7.5): a) for the Mb/H_2O_2 catalytic system: 0.3 mM H_2O_2; b) for the LPO/H_2O_2 catalytic system: 8×10^{-8} M LPO, 0.3 mM H_2O_2; c) for the Ty/O_2 catalytic system: 1×10^{-8} M Ty. The solutions of DA, H_2O_2, and Ty were added in 5 aliquots; after the last addition, the proteins were allowed to react at room temperature for 10 min.

For the analysis of protein fragments, the modified HMb and hhMb derivatives (and, for comparison purposes, the unmodified proteins) (about 1 mg) were transformed into the apoproteins by the standard hydrochloric acid/2-butanone method.[80] After overnight dialysis against 20 mM ammonium bicarbonate buffer at pH 8.0, the melanic precipitates were separated by centrifugation from the apoMb derivatives, washed three times with water (to retain only the covalent melanin–protein conjugates in the precipitate), acidified to pH 3 with HCl, washed again with water, and subsequently resuspended in 20 mM ammonium bicarbonate buffer (pH 8.0) for proteolysis with trypsin, and 20 mM phosphate buffer (pH 2.5) for proteolysis with pepsin, respectively. All the digestions were performed at 37 °C with the protease (trypsin or pepsin) 1:50 (w/w) for 3 h and overnight for the apoproteins and melanic residues, respectively. The samples of HMb derivatives were further reacted with 5 mM dithiothreitol for 15 min at 37 °C prior to the HPLC–MS/MS analysis, since Cys-containing peptides generally couple during digestion. The reduction of the disulfide bonds was necessary for the quantification of the unreacted cysteine residues in HMb derivatives.

LC–MS and LC–MS/MS data were obtained by using the LCQ ADV MAX ion-trap mass spectrometer with the instrumental conditions reported in the previous section. The system was run in automated LC–MS/MS mode and using a Surveyor HPLC system (Thermo Finnigan, San Jose, CA, USA) equipped with a BioBasic C18 column (5 μm, 150 × 2.1 mm). The elution was performed by using 0.1% HCOOH in distilled water (solvent A) and 0.1% HCOOH in acetonitrile (solvent B), with a flow rate of 0.2 mL min^{-1}; elution started with 98% solvent A for 5 min, followed by a linear gradient from 98 to 55% A in 65 min. MS/MS spectra obtained by collision-induced dissociation (CID) were performed with an isolation width of 2 Th (m/z); the activation amplitude was around 35% of the ejection radiofrequency (RF) amplitude of the instrument. For the analysis of protein fragments derived from Mb derivatives, the mass spectrometer was set such that one full MS scan was followed by using ZoomScan mode and an MS/MS scan on the most intense ion from the MS spectrum. To identify the modified residues, the acquired MS/MS spectra were automatically searched against a protein database for human or horse heart Mb using the SEQUEST algorithm incorporated into Bioworks 3.1 (ThermoFinnigan, San Jose, CA (USA)).

Guanidine hydrochloride denaturation assay: The stability to denaturation of the modified HMb and hhMb derivatives, which were obtained by reaction of the proteins (≈5 μM), with 1 mM DA and 0.3 mM H_2O_2, and, for comparison purposes, of the unmodified proteins, were determined in 200 mM phosphate buffer (pH 6.0) by monitoring the absorbance variation of the Soret band of the proteins upon addition of increasing amounts (up to 1.7 M final concentration) of a 8 M guanidine hydrochloride solution in the same buffer. Data were corrected for dilution by the Gdn–HCl addition. The Gdn–HCl concentration at 50% unfolding of the proteins and the thermodynamic parameters for denaturation were evaluated from the absorbance versus denaturant concentration curves according to a standard method.[81]

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