Biochemical and Biophysical Research Communications **274,** 32–36 (2000)
doi:10.1006/bbrc.2000.3087, available online at http://www.idealibrary.com on IDEAL®

Glutathione Transferase M2-2 Catalyzes Conjugation of Dopamine and Dopa *o*-Quinones

Alexies Dagnino-Subiabre,* Bruce K. Cassels,† Sofia Baez,‡ Ann-Sofie Johansson,§ Bengt Mannervik,§ and Juan Segura-Aguilar*

**Programme of Molecular and Clinical Pharmacology, ICBM, Faculty of Medicine, University of Chile, Independencia 1027, Casilla 70000, Santiago-7, Chile; †Department of Chemistry, Faculty of Sciences, University of Chile, Casilla 653, Santiago, Chile; §Department of Biochemistry, Biomedical Center, Uppsala University, Box 576, SE-751 23 Uppsala, Sweden; and ‡Unit of Biochemical Toxicology, Wallenberg Laboratory, Stockholm University, SE-10691 Stockholm, Sweden*

Received June 14, 2000

Human glutathione transferase M2-2 prevents the formation of neurotoxic aminochrome and dopachrome by catalyzing the conjugation of dopamine and dopa *o*-quinone with glutathione. NMR analysis of dopamine and dopa *o*-quinone-glutathione conjugates revealed that the addition of glutathione was at C-5 to form 5-*S*-glutathionyl-dopamine and 5-*S*-glutathionyl-dopa, respectively. Both conjugates were found to be resistant to oxidation by biological oxidizing agents such as O_2, H_2O_2, and $O_2^{\bullet-}$, and the glutathione transferase-catalyzed reaction can therefore serve a neuroprotective antioxidant function. © 2000 Academic Press

Key Words: **dopamine; dopa; glutathione transferase; *o*-quinones; aminochrome; dopachrome; 5-*S*-cysteinyldopa; 5-*S*-cysteinyldopamine; neuromelanin.**

It is now generally accepted that free radicals are involved in the degenerative processes affecting the nigro-striatal system of Parkinson's disease (PD) patients, although the exact mechanism of neurodegeneration *in vivo* is still unknown. One possible source of free radicals may be the redox shuttling between semiquinones and oxidized forms of dopamine and dopa such as aminochrome and dopachrome, respectively (1, 2). One-electron reduction of aminochrome to leukoaminochrome *o*-semiquinone radical has been proposed as the reaction responsible for the degeneration of dopaminergic neurons in PD (1, 2). Dopamine and dopa have been reported to be oxidized to aminochrome and dopachrome by oxygen, transition metals and peroxynitrite radicals (3–6). Furthermore, several enzymes have been reported to also catalyze the oxidation of dopamine and dopa to aminochrome, e.g. prostaglandin H synthase (7), xanthine oxidase (8), and several forms of cytochrome P450, especially CYP 1A2 (9). The present investigation demonstrates a new glutathione (GSH) conjugation reaction of dopamine and dopa *o*-quinones catalyzed by human glutathione transferase M2-2, which may have physiological significance since this conjugation prevents redox cycling and the formation of aminochrome and dopachrome.

MATERIALS AND METHODS

Chemicals

DL-Dopa, dopamine, tyrosinase from mushroom (EC 1.14.18.1), xanthine, xantine oxidase from buttermilk (EC 1.13.22), NADH, NADPH, and GSH were purchased from Sigma Chemical Co. (St. Louis, MO). Synthesis of aminochrome and dopachrome was performed as described previously (3, 10).

Preparation of Enzymes

Human glutathione transferase M2-2 (for nomenclature see Ref. 11) was expressed and purified as previously described (12). Protein determination was performed according to the method of Bradford (13).

Assay Conditions

Conjugation of dopamine and dopa o-quinone with GSH. The oxidation of dopamine and dopa was catalyzed by tyrosinase and the incubation mixture contained 0.1 M sodium phosphate, pH 6.5, 1 mM GSH, 5 μg GST M2-2 and 200 μM dopamine or dopa at 30°C. The reaction was started by addition of 10 μg tyrosinase and the oxidation was allowed to proceed for 10 min. Absorbance was monitored at 475 nm (dopamine) or 480 nm (dopa).

Determination of conjugate stability. The stability of the conjugate in the presence of biological oxidants was measured by exposing a 100 μM solution of the conjugate to dioxygen, superoxide radicals or hydrogen peroxide for 30 min. The incubation mixture contained 100 μM dopamine, 1 mM GSH, 5 μg GST M2-2 and 4 μg tyrosinase in 0.1 M sodium phosphate, pH 6.5, at 30°C. Superoxide radicals and hydrogen peroxide were produced enzymatically by the xanthine/xanthine oxidase system, containing 1 mM xanthine and 0.2 U xanthine oxidase in 0.1 M potassium phosphate pH, 7.4. The conju-

0006-291X/00 $35.00

Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.

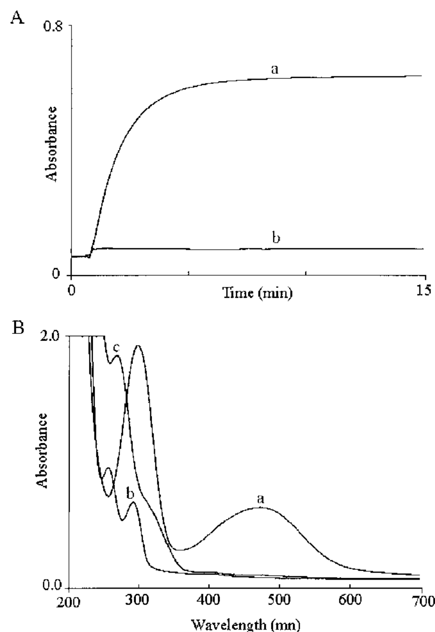


FIG. 1. Dopamine *o*-quinone GSH conjugation catalyzed by glutathione transferase M2-2. (A) Oxidation of dopamine to aminochrome catalyzed by tyrosinase and its prevention by GSH conjugation. The reaction was monitored at 475 nm. The oxidation of 200 μM dopamine at pH 6.5 and 30°C was catalyzed by 10 μg tyrosinase (a). The conjugation of the dopamine *o*-quinone (formed by the action of tyrosinase) was performed in the presence of 1 mM GSH, and 5 μg GST M2-2 (b). (B) Absorption spectrum of the GSH conjugate. The absorption spectrum of 5-*S*-glutathionyldopamine (b) is compared with those of aminochrome (a) and the oxidized form of 5-*S*-glutathionyl dopamine (c). Experimental procedures are described in the experimental section.

gates (100 μM) were oxidized by 1 mM Mn^{3+}-pyrophosphate complex to *o*-quinone conjugate to be used as control.

NMR Studies

For the structural analysis of the GSH conjugate of dopamine or dopa *o*-quinone, 300 μM dopamine, 1 mM GSH and 5 μg GST M2-2 in 1 ml 0.1 M sodium phosphate (pH 6.5) in deuterium oxide were incubated with 10 μg tyrosinase. The ^{1}H NMR spectra of the reaction mixtures was recorded using a Bruker AMX 300 instrument, operating at 300.13 MHz, with suppression of the HDO signal (17408 scans).

RESULTS

Glutathione Conjugation of Dopamine o-Quinone Catalyzed by GST M2-2

Cyclization of dopamine *o*-quinone to aminochrome (Fig. 1A, trace a), resulting from oxidation of dopamine catalyzed by tyrosinase, was completely inhibited in the presence of glutathione transferase M2-2 and GSH (Fig. 1A, trace b). The inhibition of cyclization of dopamine *o*-quinone was due to the formation of a GSH conjugate. The absorption spectrum of the dopamine *o*-quinone GSH conjugate exhibited two peaks at 257 and 292 nm (Fig. 1B, spectrum b). The spectrum of the oxidized dopamine *o*-quinone GSH conjugate, obtained by oxidation with Mn^{3+}-pyrophosphate complex, has one absorption maximum at 267 nm and shoulders at 306 and near 380 nm (Fig. 2, spectrum c) and lacks the characteristic quinone peak at 480 nm. The spectral differences are also evident by comparing the color of the solutions of the oxidized dopamine *o*-quinone GSH conjugate (pale yellow) and of the oxidized aminochrome-GSH conjugate (blue).

Conjugation of Dopa o-Quinone with Glutathione

The possibility that GST M2-2 catalyzes the conjugation of dopa *o*-quinone was also investigated. No formation of dopachrome was observed when dopa was oxidized by tyrosinase in the presence of GST M2-2 and GST, suggesting that dopa *o*-quinone was conjugated with GSH as in the case of dopamine *o*-quinone. Dopa *o*-quinone-GSH exhibited an absorption spectrum similar to that of dopamine *o*-quinone-GSH with two maxima at 257 and 292 nm (Fig. 2B, spectrum a). The absorption spectrum of the oxidized dopa *o*-quinone-GSH was also similar to that of dopamine *o*-quinone-GSH, when it was oxidized by Mn^{3+}-pyrophosphate complex, exhibiting a maximum at 273 nm and shoulders at 308 and 381 nm (Fig. 2B, spectrum b).

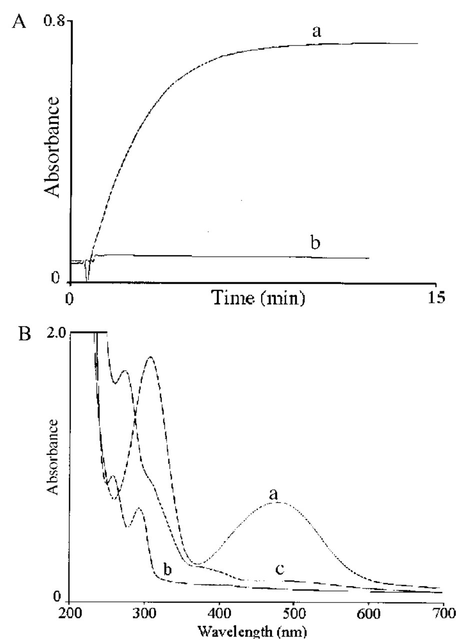


FIG. 2. Dopa *o*-quinone GSH conjugation catalyzed by glutathione transferase M2-2. (A) The formation of dopachrome (a) catalyzed by tyrosinase was monitored at 480 nm. Conjugation of dopa *o*-quinone catalyzed by GST M2-2 prevents cyclization of dopa *o*-quinone (b). (B) The absorption spectrum of dopachrome (a), 5-*S*-glutathionyldopa (b) and 5-*S*-glutathionyldopa *o*-quinone (c). Experimental procedures are described under Materials and Methods.

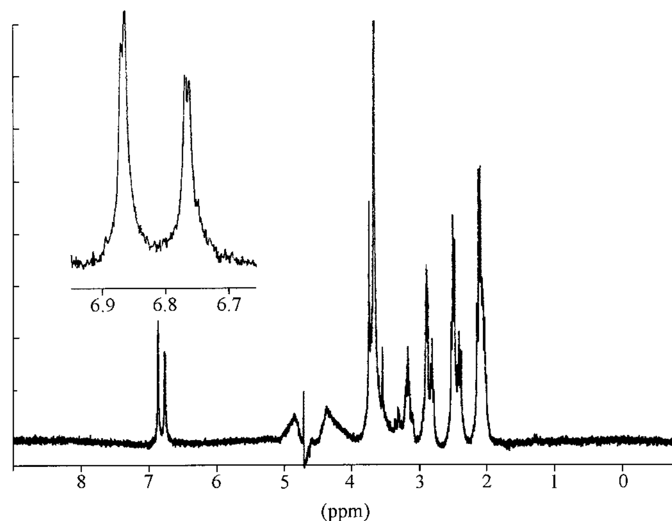
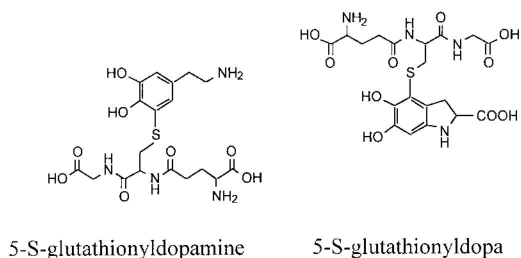


FIG. 3. ^{1}H NMR spectrum (300 MHz) of 5-*S*-glutathionyldopamine in D_2O in the presence of excess GSH. Inset: aromatic proton region.

Determination of GSH-Conjugate Structures by NMR

The ^{1}H NMR spectra of the conjugates showed clear changes in the aromatic proton resonance pattern, from the ABM system of dopamine and dopa to a meta-coupled AB system (δ 6.77 and 6.87 ppm, J = 1.8 Hz for dopamine, Fig. 3, and δ 6.74 and 6.85 ppm, J = 1.9 Hz, for dopa, not shown), in agreement with monosubstitution of the dopamine and dopa molecules at C5. A concomitant downfield shift of the S—CH_2 multiplet of GSH (δ 2.87 to about 3.1 ppm, with a splitting pattern consistent with the magnetic nonequivalence of the two CH_2 protons) was observed. This downfield shift is expected as a result of the magnetic anisotropic effect of the aromatic ring on the S—CH_2 group of the conjugate (chemical shifts for Ar—S—CH_2 groups lie typically in the 3.0–3.2 ppm range). Finally, the multiplet at 2.48 ppm assigned to the CH_2 group at the gamma position of the glutamyl residue of GSH is shifted slightly upfield to 2.4 ppm, suggesting that this group prefers a position in close proximity to the aromatic ring in the reaction product. Taken together, these results lead to the conclusion that the products of GSH conjugation of dopamine and dopa *o*-quinone catalyzed by GST M2-2 are 5-S-glutathionyldopamine and 5-S-glutathionyldopa, respectively:



Stability of 5-S-Glutathionyl Conjugates

The stability of 5-S-glutathionyldopamine and 5-S-glutathionyldopa in the presence of biological oxidizing agents such as dioxygen, hydrogen peroxide and superoxide radical was also studied by monitoring for possible spectral changes. No oxidation of 5-S-glutathionyldopamine or 5-S-glutathionyldopa was observed when the conjugates were incubated for 20 min in air, or with the xanthine/xanthine oxidase system in the presence or absence of 20 U superoxide dismutase during 20 min (data not shown).

DISCUSSION

The finding that GST M2-2 catalyzes the GSH conjugation of dopamine and dopa *o*-quinones to 5-S-glutathionyldopamine and 5-S-glutathionyldopa, thereby preventing the formation of aminochrome and dopachrome, suggests that this enzyme may play a neuroprotective role in the human brain (Fig. 4). Oxidation of dopamine and dopa to their corresponding *o*-quinones and their one-electron reduction to *o*-semi-

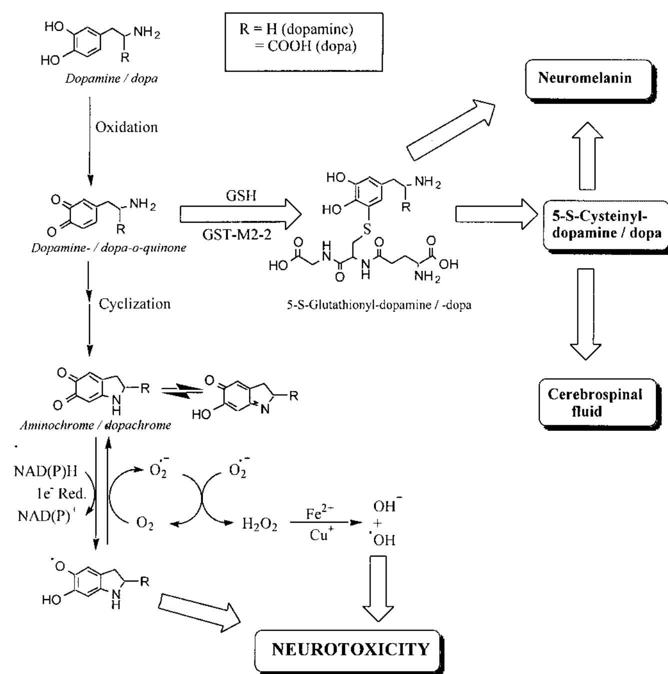


FIG. 4. Proposed antioxidant and neuroprotective conjugation of dopamine and dopa *o*-quinone catalyzed by GST M2-2. Glutathione transferase M2-2 prevents the formation of aminochrome and dopachrome by conjugating the *o*-quinones of dopamine and dopa to 5-*S*-glutathionyldopamine and 5-*S*-glutathionyldopa, respectively, which can be excreted to the cerebrospinal fluid or can undergo polymerization to form neuromelanin. Aminochrome and dopachrome can be reduced by one-electron quinone reductase to leukoaminochrome- or leukodopachrome *o*-semiquinone radicals which may result in redox cycling, oxidative stress and the degeneration of dopaminergic neurons.

quinone radicals does not seem to be the reaction responsible of the degenerative processes in dopaminergic system. Instead, the cyclized o-quinones aminochrome and dopachrome appear to give rise to toxicity. One-electron reduction of aminochrome to leukoaminochrome *o*-semiquinone radical has been proposed to be the reaction responsible for the neurodegenerative processes occurring in the dopaminergic system (1, 2). Recently, it has been reported that aminochrome is toxic in a mouse cerebral cortex cell line (14). A very significant difference in the reactivity of dopamine *o*-semiquinone and leukoaminochrome *o*-semiquinone radical has been reported (2). The high reactivity of leukoaminochrome *o*-semiquinone radical was demonstrated by ESR studies where the dopamine *o*-semiquinone stability in the presence of Zn^{2+} contrast with the high reactivity and instability of leukoaminochrome *o*-semiquinone (2). Leukoaminochrome *o*-semiquinone radical is an extremely reactive metabolite (2) that is subject to reoxidation in the presence of oxygen and initiates a redox cycling process, reducing dioxygen to superoxide radicals (1). It is interesting to note that very small amounts of aminochrome may produce large quantities of reactive oxygen species due to this cycling process, depleting NADH and/or NADPH. Depletion of NADPH will prevent the reduction of GSSG to GSH by glutathione reductase, thereby compromising one of the main antioxidant systems in the cell. In addition, depletion of NADH will inhibit the formation of ATP coupled to the mitochondrial electron transport chain. Another way to induce toxicity is to promote formation of hydroxyl radical ($^{\bullet}OH$), one of the most harmful free radicals. Hydrogen peroxide, which can be obtained by dismutation of superoxide radicals, may give rise to the hydroxyl radical in the presence of metal ions such as Fe^{2+} and Cu^{+}. In addition, leukoaminochrome *o*-semiquinone is a radical that by itself can react with nucleophilic molecules, such as RNA, DNA and proteins or induce lipid peroxidation and inactivation of enzymes by oxidation of essential thiol groups. Furthermore, superoxide dismutase and catalase have been reported to play a prooxidant role during one-electron reduction of aminochrome by increasing the autoxidation rate of leukoaminochrome *o*-semiquinone radical (1).

The physiological relevance of GSH conjugation of *o*-quinones derived from catecholamines depends upon the existence of these metabolites *in vivo.* The conjugate 5-S-glutathionyldopamine is the precursor of 5-S-cysteinyldopamine. It has been demonstrated that 5-S-glutathionyldopamine is rapidly metabolized in rat brain to 5-S-cysteinyldopamine in reactions mediated by gamma-glutamyl transpeptidase and dipeptidase (15). Therefore, it seems plausible that 5-S-glutathionyldopamine can also be converted by gamma-glutamyl transpeptidase and dipeptidase in human brain to form 5-S-cysteinyldopamine, which can be excreted from the cell. Indeed, 5-S-cysteinyldopamine has been detected in cerebrospinal fluid of PD patients and control subjects by using HPLC (16). In the human brain 5-S-cysteinyldopamine has been identified in dopamine-rich brain regions such as caudate nucleus, putamen, globus pallidus and substantia nigra (17). In addition to excretion, a pathway of 5-S-cysteinyldopamine removal is its incorporation into neuromelanin. 5-S-Cysteinyldopamine has been reported to be the main source of the pheomelanin moiety of human neuromelanin isolated from substantia nigra (18). The physiological relevance of GSH conjugation of dopamine *o*-quinone to 5-S-glutathionyldopamine as a neuroprotective reaction is supported by the finding that formation of 5-glutathionyl- and 5-S-cysteinyl-dopamine prevented dopamine-mediated DNA damage in a dose-dependent manner (19).

One-electron reduction of dopachrome catalyzed by NADPH cytochrome P450 reductase has also been reported to generate a redox cycle with concomitant reduction of oxygen to superoxide radical (20). The question is whether L-dopa can be oxidized to dopachrome during the years of treatment of PD patients with high concentrations of L-dopa. The possibility that oxidative-reductive pathways of L-dopa/L-dopamine occur *in vivo* in brain is supported by the finding that cysteinyl adducts, such as 5-cysteinyldopa, have been reported to be present in rat, guinea pig and human brain (21, 22). 5-S-Glutathionyldopa is the precursor of 5-S-cysteinyldopa in reactions catalyzed by gamma-glutamyl transpeptidase and N-acetyltransferase. Further evidence for the occurrence and stability of 5-cysteinyldopa in humans is its excretion in the urine of patients with melanoma (23).

We propose that GSH conjugation of the *o*-quinones of dopamine and dopa is an important antioxidant and neuroprotective reaction, which may have relevance in the prevention of degenerative processes of the dopaminergic system in human brain.

ACKNOWLEDGMENTS

This work was supported by FONDECYT Grant No 1990622, DID (University of Chile), Tore Nilson Foundation for Medical Research, the Swedish Cancer Society, and in part by the Presidential Chair in Science for BKC, Chile, 1996. BKC acknowledges a generous gift of equipment from the Alexander von Humboldt Foundation (Germany).

REFERENCES

1. Baez, S., Linderson, Y., and Segura-Aguilar, J. (1995) Superoxide dismutase and catalase enhance autoxidation during one-electrón reduction of aminochrome by NADPH cytochrome P450 reductasa. *Biochem. Mol. Med.* **54,** 12–18.
2. Segura-Aguilar, J., Metodiewa, D., and Welch, C. J. (1998) Metabolic activation of dopamine *o*-quinones to o-semiquinonas by NADPH cytochrome P450 reductasa may play an important role in oxidative stress and apoptotic effects. *Biochim. Biophys. Acta* **1381,** 1–6.

3. Segura-Aguilar, J., and Lind, C. (1989) On the mechanism of the Mn^{3+}-induced neurotoxicity of dopamine prevention of quinone-derived oxygen toxicity by DT-diaphorase and superoxide dismutase. *Chem.-Biol. Interact.* **72,** 309–324.
4. Hawley, M. D., Tatawawadi, S. V., Piekarski, S., and Adams, R. N. (1967) Electrochemical studies of the oxidation pathways of catecholamines. *J. Am. Chem. Soc.* **89,** 447–450.
5. Harrison, W. H., Whisler, W. W., and Hill, B. J. (1968) Catecholamine oxidation and ionization properties indicated from the H^- release, tritium exchange, and spectral changes which occur during ferricyanide oxidation. *Biochemistry* **7,** 3089–3093.
6. Daveu, C., Servy, C., Dendane, M., Marin, P., and Ducrocq, C. (1997) Oxidation and nitration of catecholamines by nitrogen oxides derived from nitric oxide. *Nitric Oxide* **3,** 234–243.
7. Hastings, T. G. (1995) Enzimatic oxidation of dopamine: The role of prostaglandin H synthase. *J. Neurochem.* **64,** 919–924.
8. Foppoli, C., Coccia, R., Cini, C., and Rosei, M. A. (1997) Catecholamines oxidation by xanthine oxidase. *Biochim. Biophys. Acta* **1334,** 200–206.
9. Segura-Aguilar, J. (1996) Peroxidase activity of liver microsomal vitamin D 25 hydroxylase catalyzes 25-hydroxylation of vitamin D_3 and oxidation of dopamine to aminochrome. *Biochem. Mol. Med.* **58,** 122–129.
10. Baez, S., Linderson, Y., and Segura-Aguilar, J. (1994) Superoxide dismutase and catalase prevent the formation of oxygen reactive species during reduction of cyclized dopa *ortho*-quinone by DT-diaphorase. *Chem.-Biol. Interact.* **93,** 103–116.
11. Mannervik, B., Awasthi, Y. C., Board, P. G., Hayes, J. D., Di Ilio, C., Ketterer, B., Listowsky, I., Morgenstern, R., Muramatsu, M., Pearson, W. R., Pickett, C. B., Sato, K., Widersten, M., and Wolf, C. R. (1992) Nomenclature for human glutathione transferases. *Biochem. J.* **282,** 305–306.
12. Johansson, A.-S., Bolton-Grob, R., and Mannervik, B. (1999) Use of silent mutations in cDNA encoding human glutathione transferase M2-2 for optimized expression in *Escherichia coli. Protein Expression Purif.* **17,** 105–112.
13. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72,** 248–254.
14. Arriagada, C., Dagnino-Subiabre, A., Caviedes, P., Martin-Armero, J., Caviedes, R., and Segura-Aguilar, J. (2000) Studies of aminochrome toxicity in a mouse derived neuronal cell line: is this toxicity mediated via glutamate transmission? *Amino Acids,* in press.
15. Shen, X. M., Xia, B., Wrona, M. Z., and Dryhurst, G. (1996) Synthesis, redox properties, in vivo formation, and neurobehavioral effects of N-acetylcysteinyl conjugates of dopamine: Possible metabolites of relevance to Parkinson's disease. *Chem. Res. Toxicol.* **9,** 1117–1126.
16. Cheng, F. C., Kuo, J. S., Chia, L. G., and Dryhurst, G. (1996) Elevated 5-S-cysteinyldopamine/homovanillic acid ratio and reduced homovanillic acid in cerebrospinal fluid: Possible markers for and potential insights into the pathoetiology of Parkinson's disease. *J. Neural. Transm.* **103,** 433–446.
17. Rosengren, E., Linder-Eliasson, E., and Carlsson, A. (1985) Detection of 5-S-cysteinyldopamine in human brain. *J. Neural. Transm.* **63,** 247–253.
18. Carstam, R., Brinck, C., Hindemith-Augustsson, A., Rorsman, H., and Rosengren, E. (1991) The neuromelanin of the human substantia nigra. *Biochim. Biophys. Acta* **1097,** 152–160.
19. Picklo, M. J., Amarnath, V., Graham, D. G., and Montine, T. J. (1999) Endogenous catechol thioethers may be pro-oxidant or antioxidant. *Free Radical Biol. Med.* **27,** 271–277.
20. Baez, S., and Segura-Aguilar, J. (1996) The role of superoxide dismutase and catalase on dopachrome reduction by NADPH cytochrome P450 reductase and DT-diaphorase. *In* Biennal Meeting International Society for Free Radical Research, p. 264, 1–5 October, Barcelona, Spain.
21. Fornstedt, B., Pileblad, E., and Carlsson, A. (1990) In vivo autoxidation of dopamine in guinea pig striatum increases with age. *J. Neurochem.* **55,** 655–659.
22. Carlsson, A., and Fornstedt, B. (1991) Catechol metabolites in the cerebrospinal fluid as possible markers in the early diagnosis of Parkinson's disease. *Neurology* **41,** 50–51.
23. Hasegawa, M., Takata, M., Hatta, N., Wakamatsu, K., Ito, S., and Takehara, K. (1997) Simultaneous measurement of serum 5-S-cysteinyldopa, circulating intercellular adhesion molecule-1 and soluble interleukin-2 receptor levels in Japanese patients with malignant melanoma. *Melanoma Res.* **7,** 243–251.