Accelerated Publications

A Substrate–Cofactor Free Radical Intermediate in the Reaction Mechanism of Copper Amine Oxidase†

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Received October 7, 1991; Revised Manuscript Received November 18, 1991

ABSTRACT: Reduction of copper amine oxidase with substrate led to the appearance of a free radical which can be detected in anaerobiosis by ESR and optical spectroscopy. The origin of this radical was examined through studies of the semiquinones of 6-hydroxydopamine, an analogue of the recently identified cofactor 6-hydroxydopa. The ESR spectrum of the 6-hydroxydopamine radical was too narrow to account for the enzyme radical signal; however, after spontaneous reaction with primary amines the hyperfine splittings and spectral width obtained by modulation broadening became very similar to those observed for the oxidase radical species. This effect was ascribed to covalent binding of a nitrogen atom directly to the aromatic ring structure, suggesting that the amine oxidase radical is an amino-6-hydroxydopa semiquinone. Identical ESR spectra were obtained using the amines putrescine, cadaverine, *p*-[(dimethylamino)methyl]benzylamine, and ethylenediamine; these oxidase substrates gave identical enzyme radical spectra as well. The interaction between cofactor and substrate was proved unambiguously by the technique of isotopic labeling: addition of [$^{15}N_2$]ethylenediamine instead of the normal ^{14}N-labeled compound changed the ESR spectra of both the enzyme radical and its 6-hydroxydopamine counterpart. The results were confirmed by optical spectroscopy measurements; 6-hydroxydopamine and oxidized 6-hydroxydopamine gave spectra identical to those of reduced and oxidized amine oxidase, respectively. The 6-hydroxydopamine radical showed a sharp peak at 440 nm; upon addition of amines the maximum shifted to 460 nm, as found for the enzyme. It is proposed that copper amine oxidase represents the first example of a mixed substrate–cofactor radical within the family of tyrosine radical enzymes.

The presence of an organic cofactor in diamine oxidases, later characterized as copper amine oxidases, has been known for 50 years (Zeller, 1942), but the nature of this cofactor has been subject of much controversy. It was once thought by many authors to be pyridoxal phosphate, involved in a hitherto unknown redox reaction (Mondovì, 1985). The subsequent discovery of pyrroloquinolinequinone (PQQ[1] or methoxatin) as a prosthetic group in some bacterial dehydrogenases led to a series of studies reporting its presence also in amine oxidases (Ameyama et al., 1984; Lobenstein-Verbeek et al., 1984; Duine et al., 1987). However, a recent structural characterization of bovine plasma amine oxidase provided strong evidence that this enzyme contained an unusual amino acid, 6-hydroxydopa, with a functional role at the active site (Janes et al., 1990); the previous claims for the presence of PQQ were explained by cyclization of 6-hydroxydopa derivatives during isolation. This surprising finding has greatly stimulated the development of amine oxidase research, resulting in a burst of reports on structural and mechanistic aspects of the catalytic cycle (Barry et al., 1990; Kumazawa et al., 1990; Bellelli et al., 1991; Brown et al., 1991; Coleman et al., 1991; Dooley et al., 1990, 1991; Greenaway et al., 1991; Hartmann & Klinman, 1991; Janes & Klinman, 1991).

The organic cofactor had previously been suggested to be the origin of a free radical which could be detected by ESR spectroscopy when copper amine oxidase was reduced by a substrate (Finazzi-Agrò et al., 1984; Dooley et al., 1987). It had also been noticed that enzyme reduction caused a decrease in the Cu(II) ESR signal (Mondovì et al., 1969; Dooley et al., 1987). Only very recently, however, the formation of a Cu(I)–semiquinone state was demonstrated for a range of copper amine oxidases from different sources (Dooley et al., 1991). From the ESR spectra the radicals were assumed to be identical in all cases, but no spectral interpretation was given, nor was the involvement of 6-hydroxydopa shown.

We have used 6-hydroxydopamine as a cofactor analogue and characterized the semiquinone radicals generated in the presence of amines. A striking similarity is found between the radical formed in copper amine oxidases and that of an amino derivative of 6-hydroxydopamine.

Experimental Procedures

6-Hydroxydopamine (2,4,5-trihydroxyphenethylamine) and 6-hydroxydopa (2,4,5-trihydroxyphenylalanine) were obtained from Sigma (St. Louis, MO), and [$^{15}N_2$]ethylenediamine (98% ^{15}N isotope) was from Icon (Summit, NJ). DABA was synthesized according to the method of Bardsley et al. (1972). Amine oxidase was purified from lentil (*Lens culinaris*) seedlings as previously described (Floris et al., 1983).

† The work was supported by the Italian Research Council, CNR Project Chimica Fine II.

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[1] Abbreviations: DABA, *p*-[(dimethylamino)methyl]benzylamine; PQQ, pyrroloquinolinequinone; Tris, tris(hydroxymethyl)aminomethane.

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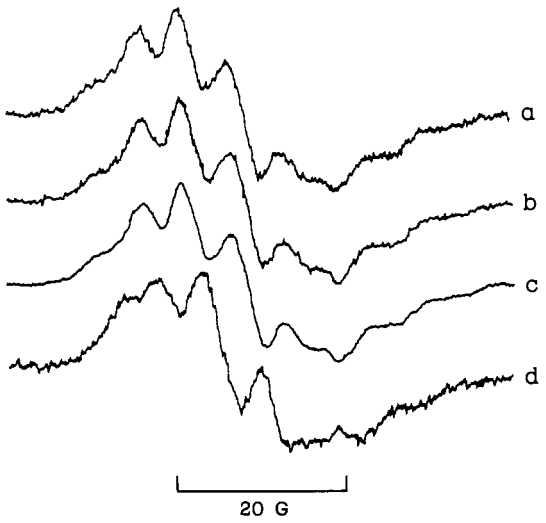

FIGURE 1: ESR spectra of the amine oxidase free radical generated in anaerobiosis by addition of different substrates. Samples contained 20 mg/mL enzyme in 20 mM Tris buffer, pH 7.4, and the following substrates (10–30 mM): ethylenediamine (a), putrescine (b), cadaverine (c), [^{15}N]ethylenediamine (d). A total of 24–60 scans were accumulated; the spectra are drawn to the same size to facilitate comparison of spectral line shapes.

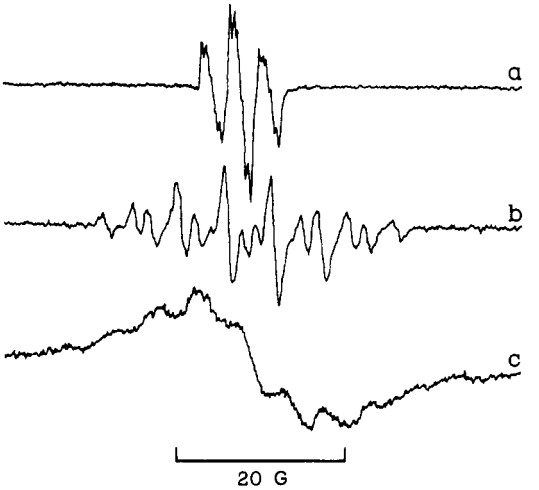

FIGURE 2: ESR spectra of radicals generated spontaneously in 100 mM potassium carbonate buffer, pH 12.0, by 1 mM 6-hydroxydopamine (a) or 1–2 mM oxidized 6-hydroxydopamine + 10 mM ethylenediamine (b and c). Spectrum c is the same as spectrum b but recorded using a modulation amplitude of 8.3 G. Spectra are shown at different amplification levels to emphasize spectral changes.

Stock solutions of 100 mM 6-hydroxydopa or 6-hydroxydopamine in 10 mM oxygen-free HCl were prepared fresh and kept under nitrogen in Thunberg cells, from which aliquots could be withdrawn anaerobically. Samples were prepared in either 20 mM Tris-HCl buffer, pH 7.4, or 100 mM potassium carbonate buffer at pH 12.0; control measurements were carried out using 20 mM potassium phosphate, pH 7.4, or 100 mM sodium borate, pH 12.0, respectively, to ascertain that the choice of buffer did not influence the observed ESR signals. Samples were mixed under nitrogen in septum-stoppered Thunberg cells to which necessary additions could be made anaerobically with a syringe. Oxidized 6-hydroxydopamine was prepared by exposure of diluted solutions in buffer to air for 4–24 h, with subsequent degassing and storage under nitrogen.

Absorption spectra were recorded at room temperature with a Jasco Model 650 spectrophotometer or a Perkin-Elmer Lambda 9 instrument. ESR measurements were done at room temperature with a Bruker ESP 300 instrument using an X-band high-sensitivity TM_{110}-type cavity. Approximately 100 μL of sample was drawn into a thin-walled flat glass capillary (Pedersen & Cox, 1988), and the capillary was sealed with plasticine; oxygen diffusion into the samples during the measurements was found to be negligible. Spectra were typically recorded using 0.5–1.0-G modulation, 10-mW microwave power, and scan times of 40 s for each 100-G sweep; routinely 24 or more spectra were accumulated to obtain a suitable signal to noise ratio. Overmodulated spectra were obtained using 8.3-G modulation and 20-mW power; these spectra are not completely similar to the spectra of immobilized radicals, partly because the anisotropic component of bound radical motion cannot be "simulated" in this way and partly due to the limited range of instrumental modulation settings available.

Results

Incubation of amine oxidase with various amine substrates under anaerobic conditions led to the appearance of an ESR signal centered around $g = 2.0$, indicating the generation of a free radical intermediate (Figure 1a–c). The characteristic hyperfine splitting showed the radical to be identical to the one previously observed for the cyanide–enzyme complex (Finazzi-Agrò et al., 1984). Interestingly, the presence of cyanide did not affect the size of the signal (not shown); cyanide is assumed to stabilize the Cu(I)–semiquinone complex (Dooley et al., 1991). When efficient substrates such as putrescine or DABA were used, the radicals could easily be obtained even with air-saturated samples; in fact, the catalytic activity of the oxidase will consume all the available oxygen within a few minutes.

The semiquinone of 6-hydroxydopamine was examined for its ability to mimic the enzyme radical. Although a small radical ESR signal could be seen at physiological pH values, the semiquinone was too unstable for practical purposes; thus all samples were prepared at alkaline pH where a large signal was readily obtained. The 6-hydroxydopamine semiquinone formation, dependent on trace amounts of oxygen and metal ions (Floyd & Wiseman, 1979), could be monitored by its typical spectrum (Figure 2a), as reported previously (Borg et al., 1978; Floyd & Wiseman, 1979). Complete immobilization of the radical by freezing or "simulated" immobilization by overmodulation did not broaden the signal significantly (total width 8.7 G) but caused an almost complete loss of the hyperfine structure (results not shown). The narrow 6-hydroxydopamine radical signal could be broadened by addition of amines (Figure 2b), indicating the spontaneous formation of an amine–6-hydroxydopamine derivative, with a nitrogen covalently bound to the quinone ring. This amine adduct was only formed from oxidized 6-hydroxydopamine; it could not be detected in samples of the reduced compound until oxygen was introduced by mixing. Unfortunately, it was not possible to broaden these radical spectra through immobilization with glycerol, polyvinylpyrrolidone, or similar compounds, due to the rapid disappearance of the radical signals (results not shown). Incubation of 6-hydroxydopamine with albumin led to the appearance of a small ESR signal with partially unresolved hyperfine structure (not shown). This indicated that oxidized 6-hydroxydopamine could react directly with albumin, like many other quinone compounds (Webb, 1966; Kalyanaraman et al., 1987). Attempts to immobilize the radical by freezing resulted in complete disappearance of the ESR signal. However, even though it was not experimentally feasible to immobilize the semiquinone in a controlled manner, it was possible to achieve a good "simulation" of the

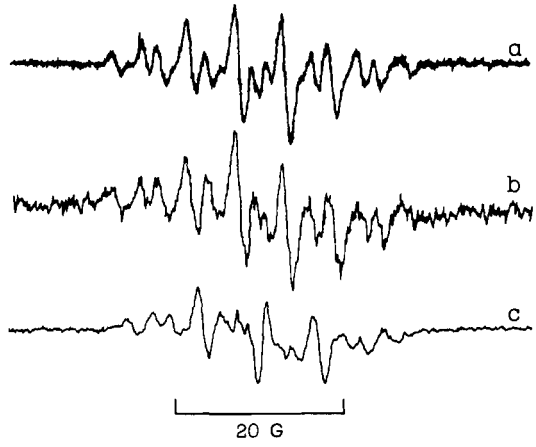

FIGURE 3: ESR spectra of 6-hydroxydopamine amine adduct radicals. Samples were prepared anaerobically containing 1 mM 6-hydroxydopamine in 20 mM Tris buffer, pH 7.4, and 10 mM ethylenediamine (a), 30 mM putrescine (b), or 10 mM [^{15}N]ethylenediamine (c). Spectra identical to (a) and (b) could be observed using cadaverine or DABA. Each spectrum is the sum of 24 accumulations.

broadened amine–6-hydroxydopamine ESR signal by simply overmodulating the spectrum (Figure 2c). The splitting pattern obtained became remarkably similar to the one found for the amine oxidase radicals.

The amines putrescine, cadaverine, ethylenediamine, and DABA all gave rise to the same amine–6-hydroxydopamine radical spectrum (Figure 3), and consequently, their modulation-broadened spectra were identical, in agreement with the results in Figure 1. These radicals were found to be stable also at neutral pH, in contrast to the 6-hydroxydopamine semiquinone, and their decay curves in anaerobiosis were very similar to those found for the amine oxidase radicals (data not shown). A final proof for the presence of substrate nitrogen in the radical structure was achieved through the use of ^{15}N-labeled ethylenediamine; isotopic labeling changed both the enzyme radical spectrum (Figure 1d) and the corresponding ethylenediamine–6-hydroxydopamine radical (Figure 3c).

Further evidence for the similarity between 6-hydroxydopamine and the cofactor of amine oxidase was obtained by optical spectroscopy (Figure 4). The absorption spectrum of oxidized 6-hydroxydopamine was very similar to the spectrum of the oxidized enzyme, with a well-defined band at 270 nm and a characteristic absorption centered around 490 nm. The latter disappears upon reduction of oxidized 6-hydroxydopamine with amine substrates, whereas the band in the ultraviolet region is shifted to 290 nm (not shown). Reduction of amine oxidase with substrates causes a similar disappearance of the 490-nm band (Figure 4); the changes in the ultraviolet part of the spectrum are obscured by the strong protein absorption at 280 nm.

In samples of 6-hydroxydopamine with trace amounts of oxygen present, the semiquinone was formed, showing two characteristic bands with maxima at 420 and 439 nm (Figure 5a) (Steenken & Neta, 1982). In the presence of amines, the two bands shifted toward 440 and 460 nm (Figure 5b), in almost complete coincidence with the peaks found for the enzyme radical species (Figure 5c). Similar radical spectra have been published for several plant and mammalian oxidases (Hill & Mann, 1964; Rinaldi et al., 1984; Dooley et al., 1987).

DISCUSSION

Strong evidence for the presence of 6-hydroxydopa in copper amine oxidases has been provided by NMR and mass spectrometry (Janes et al., 1990) and by resonance Raman spectroscopy (Brown et al., 1991). The results obtained in the

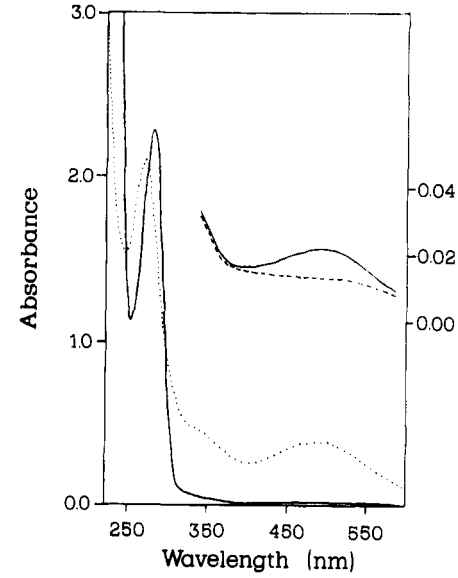

FIGURE 4: Optical absorption spectra of lentil copper amine oxidase and 6-hydroxydopamine. Oxidized (resting) enzyme (solid line) is compared with oxidized 6-hydroxydopamine (dotted line). The peak at 490 nm is shown at an expanded scale for the oxidized (full line) and the reduced enzyme (dashed line). The oxidase concentration was 2.2 mg/mL; reduction was obtained by addition of 1 mM putrescine to an air-saturated sample; 6-hydroxydopamine (0.1 mM) was completely oxidized by exposure to air for 4 h.

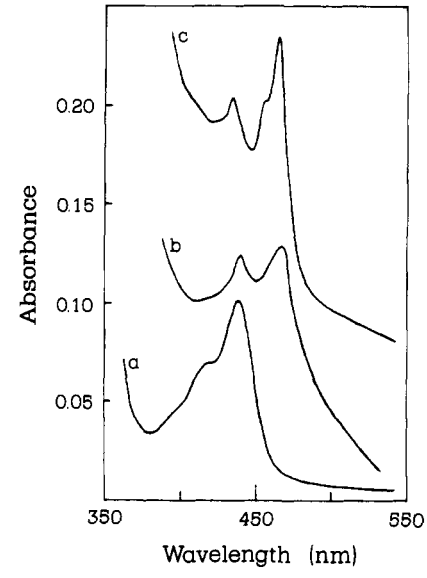

FIGURE 5: Visible absorption spectra of semiquinone radicals: 0.5 mM 6-hydroxydopamine in 100 mM potassium carbonate, pH 12, with trace amounts of air (a); 1.3 mM oxidized 6-hydroxydopamine + 2 mM ethylenediamine in 20 mM Tris, pH 7.4, under nitrogen atmosphere (b); 3.2 mg/mL copper amine oxidase + 1 mM putrescine in 20 mM Tris, pH 7.4, under nitrogen atmosphere (c). Spectrum b was recorded with 1.1 mM oxidized 6-hydroxydopamine in the reference cuvette. Notice the difference in scales compared to Figure 4.

present work with optical spectroscopy and ESR measurements confirm the role of 6-hydroxydopa as a common cofactor in the catalytic cycle. The perfect matching of enzyme and model compound absorption spectra, together with the evidence obtained for the radical species, seems to rule out PQQ, in agreement with the presently prevailing opinion (Kumazawa et al., 1990; Klinman et al., 1991).

The application of immobilized model compounds to simulate protein-bound radicals has been used successfully in studies of the photosystem II Y_D radical (Barry et al., 1990); the tyrosine radicals per se can account for the features of the

photosystem II radical species. In contrast, the 6-hydroxydopamine semiquinone can only mimic the amine oxidase radical after formation of an amine adduct. The isotopic labeling experiments leave little doubt that a similar intermediate state occurs during enzyme catalysis. It is intriguing that the strategy of ^{15}N-labeled substrate was tried out more than 20 years ago (Mondovì et al., 1967) in an attempt to detect hyperfine splitting in the Cu(II) ESR signal. But these measurements were made at liquid nitrogen temperatures where the radical is not seen; the cryolability of the amine oxidase radical was recently demonstrated by Dooley et al. (1991).

In this work we have used 6-hydroxydopamine as a suitable analogue of the 6-hydroxydopa cofactor. The use of 6-hydroxydopa itself was also tested, but this compound is extremely unstable due to fast intramolecular ring closure (Chapman et al., 1970). In fact neither ESR nor optical spectra were similar to those of the enzyme (results not shown). In comparison, 6-hydroxydopamine is relatively stable and oxidizes more slowly to a stable quinone with very little tendency to polymerize (Chapman et al., 1970). The ESR spectra of the 6-hydroxydopamine radical measured under high-resolution conditions gave splitting constants identical to those previously reported (Floyd & Wiseman, 1979); no other radicals could be detected. It should be remembered that in the enzyme the carboxyl and amino groups of 6-hydroxydopa are incorporated in the polypeptide backbone structure and thus are not available for cyclization reactions. 6-Hydroxydopamine conserves the aromatic ring structure which determines the main spectroscopic features observed in ESR and optical spectroscopy. It therefore seems appropriate to use 6-hydroxydopamine as a model in studies on the enzyme cofactor.

Although several reaction schemes of the copper amine oxidase catalytic mechanism have been presented in recent publications, only two include a free radical intermediate (Dooley et al., 1991; Bellelli et al., 1991). The catalytic mechanism is generally described in terms of a transamination reaction with the formation of a Schiff base, a model which has persisted since the original reaction scheme based on a pyridoxal phosphate cofactor. However, quinones are known to undergo 1,4-addition of primary amines (Webb, 1966); a similar reaction of the cofactor would of course lead to a completely different mechanism. So far there is little solid evidence supporting either hypothesis , but it is interesting that the sharp absorption band at 460 nm is typical of *p*-semiquinones (Steenken & Neta, 1982). From high-resolution ESR spectra of the 6-hydroxydopamine amine adduct radical, it should be possible to determine the exact point of attack of the amine on the quinone ring and in this way establish the strcture of the amine–cofactor intermediate.

In conclusion, our results give spectroscopic evidence for the function of 6-hydroxydopa as the prosthetic group in copper amine oxidases. Furthermore, it is shown that an amine–cofactor radical is generated during the catalytic cycle. Copper amine oxidase can be considered a member of the tyrosine radical protein family, which so far encompasses ribonucleotide reductase, prostaglandin H synthase, photosystem II, and galactose oxidase. It was recently discovered that galactose oxidase forms an intramolecular cysteine–tyrosine radical species (Ito et al., 1991), but copper amine oxidase represents the first example of a substrate–cofactor radical protein.

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Affinity Purification of Functional Receptors for *Escherichia coli* Heat-Stable Enterotoxin from Rat Intestine†

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Received September 27, 1991; Revised Manuscript Received November 15, 1991

ABSTRACT: Active receptors for *Escherichia coli* heat-stable enterotoxin (ST) were partially purified by ligand-affinity chromatography. The affinity column was prepared by coupling ST to biotin derivatized with an extended N-hydroxysuccinylated spacer arm prior to binding to monomeric avidin immobilized on agarose. Detergent extracts of rat intestinal mucosa membranes were quantitatively depleted of ST binding activity when chromatographed on this affinity matrix. Biotinylated ST-receptor complexes were eluted from affinity columns with 2 mM biotin and these complexes quantitatively dissociated with bile salts. Using this technique, functional ST receptors were purified maximally about 2000-fold, with about 3% of the total activity in crude extracts recovered in these purified preparations. Analysis of affinity-purified preparations by polyacrylamide gel electrophoresis and silver staining demonstrated a major protein subunit of 74 kDa. Affinity cross-linking of these preparations to ^{125}I-ST demonstrated specific labeling predominantly of the 74-kDa subunit. In addition, lower amounts of labeled ST were incorporated into subunits of 164 and 45 kDa, confirming the heterogeneous nature of ST receptors. Purified receptors bound ST in a concentration-dependent fashion, with an IC_{50} of 10^{-9} M. These studies demonstrate that ligand-affinity chromatography can be employed to purify ST receptors. The availability of purified receptors will facilitate further studies of mechanisms underlying ST-induced intestinal secretion.

Escherichia coli heat-stable enterotoxin (ST)[1] is a low molecular weight peptide which induces secretory diarrhea in humans and animals (Giannella, 1981; Levine et al., 1986; Moon, 1978). It is a major etiologic agent causing travelers diarrhea and endemic diarrhea in developing countries, the leading cause of pediatric morbidity and mortality worldwide (Giannella, 1981; Levine et al., 1986; Moon, 1978). The mechanisms by which ST alters intestinal fluid and electrolyte transport remain unclear. Previous studies demonstrated that the initial step mediating ST activity involves binding of toxin to specific protein receptors located on brush border membranes of intestinal mucosal cells (Giannella et al., 1983; Frantz et al., 1984; Dreyfus & Robertson, 1984; Kuno et al., 1986; Cohen et al., 1987). ST-receptor interaction activates particulate guanylate cyclase, resulting in intracellular cGMP accumulation (Field et al., 1978; Dreyfus & Robertson, 1984; Kuno et al., 1986; Waldman et al., 1986; Waldman & Murad, 1987; Huott et al., 1988; Hugues & Waldman, 1991). This cascade has been suggested to mediate the alterations in fluid and electrolyte transport resulting in diarrhea (Field et al., 1978; Hughes et al., 1978; Dreyfus & Robertson, 1984; Kuno et al., 1986; Waldman et al., 1986; Waldman & Murad, 1987; Huott et al., 1988). Data suggest that ST receptors are structurally and functionally heterogeneous and the mechanisms by which they induce diarrhea complex. Covalent cross-linking of radiolabeled ST to intestinal mucosal membranes results in the specific incorporation of label into protein subunits of different molecular weights (Kuno et al., 1986; Gariepy & Schoolnik, 1986; Ivens et al., 1989; Thompson & Giannella, 1990). Also, ST-receptor populations with a high

† This work was supported, in part, by a PMA Foundation Research Starter Grant. M.H. was supported, in part, by funds from the CNRS, France, NATO, and the Philippe Foundation. M.R.C. was the recipient of a PMA Foundation Medical Student Research Fellowship Pharmacology-Clinical Pharmacology. S.A.W. was the recipient of a PMA Faculty Development Award in Pharmacology and Toxicology.

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[1] Abbreviations: BCA, bicinchoninic acid; DSS, disuccinimidyl suberate; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; cGMP, guanosine cyclic 3',5'-phosphate; NHS-LC-biotin, *N*-hydroxysuccinimide-long-chain-biotin; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ST, *Escherichia coli* heat-stable enterotoxin.

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