Ascorbate Depletion as a Consequence of Product Recycling during Dopamine β-Monooxygenase Catalyzed Selenoxidation†

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ABSTRACT: The competence of dopamine β-monooxygenase (DBM) to process selenide substrates was investigated, in anticipation that the expected selenoxide products would exhibit unique reactivity and redox properties. The prototypical selenide phenyl 2-aminoethyl selenide (PAESe) was synthesized and shown to be a substrate for DBM with the characteristic e/O_2 ratio of 2:1 for monooxygenation. The kinetic parameters for oxygenation of PAESe were found to be similar to those for the DBM-catalyzed sulfoxidation of the cognate sulfide phenyl 2-aminoethyl sulfide [May, S. W., & Phillips, R. S. (1980) *J. Am. Chem. Soc. 102*, 5981–5983], and selenoxidation was stimulated by fumarate in a manner similar to other well-characterized DBM monooxygenation reactions. Identification of phenyl 2-aminoethyl selenoxide (PAESeO) as the enzymatic product was accomplished by the demonstration of coincident elution of authentic PAESeO with the enzymatic product in three significantly different HPLC systems. PAESeO was found to oxidize ascorbic acid with the concomitant and stoichiometric reduction of PAESeO back to the selenide, PAESe. As a consequence of this nonenzymatic reaction, ascorbate-supported DBM turnover was prematurely terminated under standard assay conditions due to depletion of reducted ascorbate. The kinetics of the redox reaction between PAESeO and ascorbate were investigated with a spectrophotometric assay of ascorbate at 300 nm, and a second-order rate constant of 3.4 M^{-1} s^{-1} was determined at pH 5.0, 25 °C. Spectrophotometric assay of cytochrome *c* (cyt *c*) reduction at 550 nm during the oxidation of ascorbate by PAESeO demonstrated that no cyt *c* trappable semidehydroascorbate was produced in this nonenzymatic reaction. Experiments with chromaffin granule ghosts showed a 70–80% conversion of reduced ascorbate to dehydroascorbate upon incubation of ascorbate-containing ghosts with PAESe, with a nearly stoichiometric recovery of total ascorbate. If electron equivalents utilized by DBM in vivo arise solely from the recycling of vesicular semidehydroascorbate, as has been hypothesized, the cyclic oxidation and rereduction of selenium-containing DBM substrate analogues at the expense of ascorbate may provide an unique approach to the modulation of DBM activity in vivo, thus allowing modification of adrenergic activity for clinical purposes.

Dopamine β-monooxygenase (DBM,[1] EC 1.14.17.1), a copper-containing monooxygenase present in mammalian tissues (Levin & Kaufman, 1961; Friedman & Kaufman, 1965, 1966), catalyzes the benzylic hydroxylation of dopamine to norepinephrine and thus plays a key role in neurotransmitter interconversion (Van der Schoot & Creveling, 1965; Skotland & Ljones, 1979; Rosenberg & Lovenberg, 1980; Villafranca, 1981). Although DBM is traditionally viewed as a "specific hydroxylase", previous work in our laboratory has demonstrated several new kinetically facile monooxygenase activities for DBM: stereoselective sulfoxidation of phenyl aminoalkyl sulfides (May & Phillips, 1980; May et al., 1981b), oxygenative ketonization of benzylic *S* alcohols (May et al., 1981a, 1982), epoxidation of properly designed olefinic substrates (May et al., 1983; Padgette et al., 1985a), and oxidative N-dealkylation of a variety of molecules with benzylic amine functionalities (Padgette et al., 1984a, 1985a,b). In addition, DBM-catalyzed oxidative ketonization of β-halophenethylamines has been reported (Klinman & Krueger, 1982; Mangold & Klinman, 1984), and alkyne oxidation has been reported by both us and Villafranca and co-workers (Colombo et al., 1984; Padgette et al., 1985a). However, DBM is still considerably more selective than the relatively nonspecific cytochrome P-450 and microsomal flavin monooxygenases.

Of primary interest in our ongoing studies on designing new substrates and inhibitors for DBM is the possibility that such compounds may be of therapeutic value. Thus, we have examined cardiovascular effects elicited by the sulfide substrates and have found potent antihypertensive activities in SHR (Herman et al., 1982, 1983; Pollock et al., 1983, 1984, 1985; Padgette et al., 1984b). While the precise mechanism of the antihypertensive activity of these compounds is under active investigation, results on hand are consistent with a depletion of NE arising from competition with DA for DBM-catalyzed oxygenation. Similarly, we have recognized the pharmacological potential of DBM-targeted benzylic *S* alcohols as ketonizable prodrugs (May et al., 1981a), as well as the potential of modulating catecholamine levels via suicide inhibitors of DBM (Padgette et al., 1985a).

As an extension of this approach, we recently turned our attention to selenides as potential DBM substrates. We anticipated that DBM would carry out selenoxidation with high facility, comparable to that exhibited with sulfide substrates, but recognized that the expected selenoxide products should exhibit unique reactivity and redox properties (Reich, 1978). We report herein the first demonstration of facile selen-

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[1] Abbreviations: $ASCH_2$, fully reduced ascorbic acid; cyt *c*, cytochrome *c*; DA, dopamine; DBM, dopamine β-monooxygenase; EDTA, ethylenediaminetetraacetate monosodium salt; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-pressure liquid chromatography; HPLC-EC, high-pressure liquid chromatography with electrochemical detection; NE, norepinephrine; PE, Potter–Elvehjem homogenizer; PAES, phenyl 2-aminoethyl sulfide; PAESO, phenyl 2-aminoethyl sulfoxide; PAESe, phenyl 2-aminoethyl selenide; PAESeO, phenyl 2-aminoethyl selenoxide; RT, retention time; SHR, spontaneously hypertensive rats; Tris, tris(hydroxymethyl)aminomethane.

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oxidation activity for DBM and on the ability of enzymatically generated selenoxide products to undergo recycling back to the selenide at the expense of reduced ascorbate. Since reduced ascorbate is the physiological reductant essential to DBM activity in the chromaffin vesicle (Daniels et al., 1983), these results establish the basis for a novel approach to modulating NE levels that may allow the design of novel therapeutic agents.

Materials and Methods

Dopamine β-monooxygenase was isolated and purified from bovine adrenals as described previously (May et al., 1981a) and exhibited a specific activity of 9.9–33 units/mg. Tyramine hydrochloride, ascorbic acid, sodium fumarate, catalase (C-10), superoxide dismutase, cytochrome *c*, and ascorbate oxidase were obtained from Sigma Chemical Co. Catalase obtained from Boehringer Mannheim was also used. Diphenyl diselenide and 2-methyl-2-oxazoline were obtained from Aldrich Chemical Co. All other chemicals and solvents were obtained from standard commercial sources and were of the highest quality obtainable.

Synthesis of Phenyl 2-Aminoethyl Selenide. Phenylselenol was synthesized by hypophosphorous acid reduction of diphenyl diselenide in a modification of the method of Gunther (1966): bp 29.0 °C (1.4 mmHg); ^{1}H NMR ($CDCl_3$) δ 1.54 (s, 1 H), 7.35 (m, 5 H). The phenylselenol obtained was reacted with 2-methyl-2-oxazoline as in the method of Wehrmeister (1963) to yield *N*-acetyl-PAESe as shiny white plates after recrystallization from benzene: mp 93.7–94.5 °C; ^{1}H NMR ($CDCl_3$) δ 1.90 (s, 3 H), 3.08 (m, 2 H), 3.57 (m, 2 H, J = 6 Hz), 6.11 (br s, 1 H), 7.34 (m, 3 H), 7.78 (m, 2 H); mass spectrum, m/e 243 (M^+). The acetamide was hydrolyzed by refluxing overnight in 6 M HCl. The solution was then basified, extracted with chloroform, and dried over potassium carbonate, and the solvent was removed in vacuo to yield the free amine of PAESe as a clear yellow liquid. The addition of HCl to an EtOH/Et_2O solution of the free amine yielded the hydrochloride salt, which was recrystallized from EtOH/Et_2O to give long white crystals of PAESe (39% overall yield): mp 149.2–150.7 °C, loss of crystal structure at 131 °C; ^{1}H NMR (D_2O) δ 3.21 (s, 4 H), 7.58 (m, 5 H); mass spectrum, m/e 201 (M^+). Anal. Calcd for $C_8H_{12}ClNSe$: C, 40.61; H, 5.12; N, 5.92. Found: C, 40.73; H, 5.11; N, 5.90.

Synthesis of Phenyl 2-Aminoethyl Selenoxide (PAESeO). Hydrogen peroxide oxidation of PAESe (Sharpless et al., 1973) was followed to completion by reverse-phase HPLC, excess H_2O_2 was destroyed with catalase, and the reaction mixture was lyophilized to yield the selenoxide product, which was recrystallized from MeOH/Et_2O to give small white crystals of PAESeO in 78% yield: mp 101.2–101.7 °C (dec) (gas liberated); ^{1}H NMR (D_2O) δ 3.41 (m, 4 H), 7.66 (m, 5 H); IR (Nujol mull) strong Se=O stretch at 818 cm^{-1}. Anal. Calcd for $C_8H_{12}ClNOSe$: C, 38.03; H, 4.80; N, 5.55. Found: C, 37.96; H, 4.87; N, 5.43.

DBM Assay and Determination of Kinetic Parameters. DBM activity was measured as described previously (May et al., 1981b), with the exception that 0.5 μM $CuSO_4$ was used with 0.1 mg/mL Boehringer Mannheim catalase. Kinetic constants were obtained by computer fit to the hyperbolic form of the Michaelis–Menten equation to obtain V_{max} and K_m (Cleland, 1967), and for the determination of k_{cat}, the molecular weight of DBM was taken to be 290 000 (Rosenberg & Lovenberg, 1980). Determination of the specific activity of the enzyme during each kinetic experiment, with 10 mM tyramine as substrate under standard assay conditions, allowed normalization to an arbitrary specific activity of 14.7 units/mg for DBM. Normalized k_{cat} values were thus obtained by multiplying the calculated k_{cat} values by a scaling factor.

Determination of Electron to Oxygen Stoichiometry. The spectrophotometric assay for ferricyanide formation at 420 nm (ϵ = 1100 M^{-1} cm^{-1}), with ferrocyanide as the electron donor for DBM, was used to measure the electron consumption during DBM turnover as described previously (May et al., 1981b).

Polarographic determinations of the oxygen consumption for the ferrocyanide-supported DBM oxygenation of PAESe, with the same assay solution, were run in parallel with the electron consumption experiments. The electron to oxygen stoichiometry for the enzymatic reaction was determined by the comparison of the initial rate of ferrocyanide oxidation to the initial rate of oxygen consumption and by the comparison of ferrocyanide oxidation to oxygen consumption over a fixed interval of time for individual pairs of DBM assays.

Identification of the Product of DBM Oxygenation of PAESe. HPLC of the enzymatic reaction product under standard reverse-phase conditions was accomplished with a 25 cm × 4.6 mm Excalibar C-18 reverse-phase column (Spherisorb ODS II, 5 μm) and a mobile phase of 50% MeOH and 50% 0.1 M ammonium phosphate, pH 6.0 (v/v), at a flow rate of 1.50 mL/min with UV detection at 236 nm. The Excalibar column was also used with the above mobile phase containing 5 mM octanesulfonate in the ammonium phosphate buffer under otherwise identical conditions. For cation-exchange HPLC using a 25 cm × 4.6 mm column packed with 5-μm Nucleosil SCX cation-exchange resin at a flow rate of 1.0 mL/min, the mobile-phase composition was as follows: solvent A, 33% MeOH and 67% 0.05 M $NH_4H_2PO_4$ (v/v); solvent B, 33% MeOH, 67% 0.05 M $NH_4H_2PO_4$, and 1.0 M NH_4Cl (v/v). The sample was injected into a 90% solvent A–10% solvent B mobile phase, and after a 2-min load period, a 10-min 10–100% solvent B salt gradient was initiated. Following completion of the gradient, a 100% solvent B mobile phase was run for 4 min.

Quantitation of Ascorbate and PAESe in DBM Reaction Mixtures. The fully reduced ascorbate ($ASCH_2$) remaining in reaction mixtures was analyzed by HPLC-EC at 0.60 V (BAS LC4B) according to the procedure of Diliberto et al. (1983). Identification of $ASCH_2$ in the mixtures was achieved by reverse-phase ion-pair chromatography with an Alltech C-18 column. The mobile phase (pH 4.8) consisted of 10 mM sodium acetate buffer, 0.13 mM EDTA, and 5 mM tetrabutylammonium chloride.

The PAESe remaining in reaction mixtures was analyzed by HPLC with UV detection at 236 nm. Identification of PAESe in the mixtures was accomplished by reverse-phase chromatography with an Alltech C-18 column and a mobile phase composed of 40% MeOH, 60% 0.1 M $NH_4H_2PO_4$, and 5 mM sodium octyl sulfate, pH 6.0 (v/v).

Oxidation of Ascorbate by PAESeO. Reverse-phase HPLC was used to quantitate the oxidation–reduction reaction between PAESeO and ascorbate. Linear standard curves were obtained from peak height vs. concentration plots for both PAESe and PAESeO. Aliquots of 20 mM PAESeO and 20 mM ascorbate were mixed, and HPLC samples were taken for direct injection. The concentrations of selenoxide and selenide at each reaction time were determined from their respective standard curves, and authentic PAESe was used to verify the identity of the reduced product peak.

The kinetics of ascorbate oxidation by PAESeO were determined from a spectrophotometric assay of ascorbate depletion at 300 nm (Skotland & Ljones, 1980; Mattok, 1965).

Ascorbate solutions were prepared in deoxygenated 0.127 M NaOAc buffer, pH 5.0, and the extinction coefficient at 300 nm was determined for each solution prior to its use. The redox reaction was initiated by the addition of an aliquot of PAESeO to 3.00 mL of the ascorbate solution in a 1.00-cm cuvette thermostated at 25 °C, and the decrease in absorbance at 300 nm was monitored against a blank containing only the acetate buffer. A value for a second-order rate constant was determined from the initial rates of each of 12 reactions, and for four of the reactions k was also determined graphically from plots of ln $[b(a-x)/a(b-x)]$ vs. time (Frost & Pearson, 1953).

For the attempted trapping of semidehydroascorbate during the oxidation of ascorbate by PAESeO, the spectrophotometric assay of cytochrome c reduction described by Yamazaki (1962) and used by Skotland and Ljones (1980) was employed. Ascorbate, final concentration 0.104 mM, was added to a solution of 0.017 mM cyt c in 0.063 M NaOAc, pH 5.00, 25 °C, and the addition of either ascorbate oxidase to 0.24 unit/mL or PAESeO to a final concentration of 0.0514 mM was used to initiate the enzymatic or selenoxide-dependent oxidation of ascorbate.

Quantitation of ascorbate, PAESeO, PAESe, and cyt c reduction during the oxidation of ascorbate by PAESeO was accomplished during a spectrophotometric assay of cyt c reduction at 550 nm (ϵ = 22000 M^{-1} cm^{-1}), 25 °C. To a 1.00-mL sample of 8.0×10^{-5} M cyt c in 0.127 M NaOAc, pH 5.00, was added ascorbate, final concentration 3.35×10^{-4} M, and PAESeO, final concentration 5.05×10^{-4} M. The absorbance at 550 nm was monitored until it no longer increased (22–23 min), during which time three 20-μL samples were taken and injected directly onto a C-18 reverse-phase HPLC column (described above) with a mobile phase of 50% MeOH and 50% 0.1 M KH_2PO_4 (v/v), at a flow rate of 1.00 mL/min. The absorbance of the HPLC eluent was monitored at 236 nm. The peak heights of ascorbate (RT 2.96 min), PAESeO (RT 5.52 min), and PAESe (RT 9.08 min) were compared to standard curves obtained under identical conditions for the determination of the concentration of each of the components. The amount of cyt c that had been reduced at the time of obtaining each HPLC sample was determined from the absorbance at 550 nm in the spectrophotometric assay.

Preparation of Chromaffin Granule Ghosts. Chromaffin granules were prepared according to the method of Kirschner (1962), with slight modification. Chromaffin granule ghosts were prepared according to the method of Njus and Radda (1979) except that catalase (0.05 mg/mL) was included to minimize DBM inactivation. In the final step of the procedure, 10-mL samples of the ghost preparation were layered on discontinuous density gradients over 5 mL of 15% Ficoll and 85% 0.26 M sucrose/10 mM Hepes, pH 7.0 (w/w), and 10 mL of 0.4 M sucrose/10 mM Hepes, pH 7.0. After centrifugation at 90000g for 30 min at 4 °C, a band of ghosts was collected at the Ficoll–sucrose interface.

Ghost aliquots (0.2–0.4 mg of protein) as prepared above, either containing 10 mM $ASCH_2$ or not, were incubated at 37 °C with 10 mM PAESe in an incubation medium containing 0.26 M sucrose/10 mM Hepes, pH 7.0, 5 mM ATP, 2.5 mM $MgSO_4$, and 15 μg of ascorbate oxidase. At the termination of the incubation, the reaction mix was layered on 5 mL of 0.4 M sucrose/10 mM Hepes and centrifuged at 36000g for 15 min to recover the ghost fraction. The supernatants above the pelleted ghosts were carefully removed, the tubes were swabbed dry, 0.2 mL of Tris–phosphate, pH 7.0, was added to the pellet, and a homogeneous solution was effected with the aid of a 0.5-mL Teflon–glass PE homogenizer. After the determination of recovered ghost protein, an aliquot of concentrated $HClO_4$ was added to make the homogenate 0.1 M in $HClO_4$ and the precipitated protein removed by filtration. This acidic extract was stored at −70 °C prior to HPLC analysis.

The fully reduced ascorbate ($ASCH_2$) present in the ghost-derived volumes was analyzed by HPLC-EC exactly as described before. Dehydroascorbate was determined by the difference in $ASCH_2$ content before and after reduction with an amount of dithiothreitol equal to ca. 10 times the amount of total $ASCH_2$ plus dehydroascorbate.

Results

DBM Oxygenation of Phenyl 2-Aminoethyl Selenide (PAESe). Incubation of PAESe with purified DBM under standard reaction conditions with ascorbate as the reducing agent resulted in an enzyme-dependent consumption of oxygen. Elimination of either the reducing agent or the substrate resulted in no oxygen consumption, which was suggestive of DBM-catalyzed monooxygenation of PAESe. The expected electron to oxygen stoichiometry of 2:1, characteristic of monooxygenases, was demonstrated by a comparison of (a) the initial rates of ferrocyanide-supported oxygen consumption to ferrocyanide oxidation and (b) the total amount of oxygen consumed to the total amount of ferrocyanide oxidized over a fixed interval of time. In control experiments with the known DBM substrates, tyramine and phenyl 2-aminoethyl sulfide (PAES), the same electron to oxygen stoichiometry was obtained. The oxygenation of PAESe by DBM was also stimulated by fumarate in a manner similar to that for other well-characterized DBM monooxygenation reactions (May et al., 1981b).

Kinetic constants for the ascorbate-supported DBM monooxygenation of PAESe were obtained with standard kinetic assay techniques (see Materials and Methods). The K_m and k_{cat} for PAESe were found to be 11 mM and 41 s^{-1}, respectively. These parameters are similar to those for PAES (K_m = 14.3 mM and k_{cat} = 57 s^{-1}), the slightly lower value of k_{cat}/K_m being a result of both a lower K_m and a decrease in the turnover number, k_{cat}. Both heteroatom oxygenations are kinetically more facile than oxygen insertion into the benzylic C–H bond of the cognate phenylalkylamine, 3-phenylpropylamine. We note that kinetic parameters reported here were obtained at a single oxygen concentration and thus do not represent true limiting values. However, they do reflect the kinetic situation under atmospheric conditions and thus are relevant to an assessment of the possible pharmacological utility of these compounds.

Identification of the Product of DBM Oxygenation of PAESe. Because of the similar oxidative chemistry of sulfides and selenides (Riech, 1978) and in view of our previous demonstration that the sulfoxide product is produced stoichiometrically from PAES by DBM (May & Phillips, 1980), we expected that the product of DBM oxygenation of the analogous selenium compound would be phenyl 2-aminoethyl selenoxide (PAESeO). Therefore, authentic selenoxide, PAESeO, was synthesized and used to develop three distinct HPLC systems capable of detecting production of the selenoxide product in an enzymatic reaction mixture. The first HPLC assay utilized reverse-phase chromatography, while the second HPLC assay used ion-pairing reverse-phase chromatography. Cation-exchange HPLC was yet a third independent method for the identification of PAESeO in the enzymatic reaction mixture. In all three systems, the appearance of a

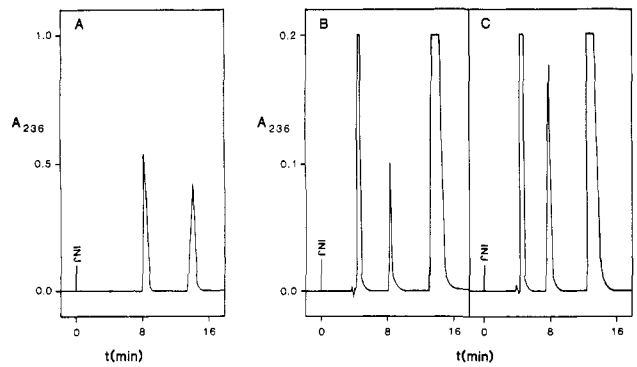

FIGURE 1: Identification of PAESeO as the product of DBM oxygenation of PAESe. Cation-exchange HPLC using a Nucleosil SCX column: solvent A is 33% MeOH and 67% 0.05 M $NH_4H_2PO_4$ (v/v); solvent B is 33% MeOH, 67% 0.05 M $NH_4H_2PO_4$, and 1.0 M NH_4Cl (v/v); the flow rate was 1.00 mL/min. The sample was injected with a 90% solvent A–10% solvent B (v/v) mobile phase, and after a 2-min load period, a 10-min 10–100% (v/v) solvent B salt gradient was initiated. Following completion of the gradient, 100% solvent B was held for 4 min. (Panel A) Chromatogram of a mixture of PAESe and authentic PAESeO; the selenoxide elutes first at a retention time of 8 min. (Panel B) Chromatogram of DBM reaction mixture, with 27 mM PAESe and 1.7 mM $K_4Fe(CN)_6$ as the electron donor after a reaction time of 6 h. (Panel C) Chromatogram of the same sample as in (B) after spiking with authentic PAESeO.

product peak with the same retention time as that of the authentic selenoxide was observed as the enzymatic reaction proceeded (see Figure 1). Similarly, these HPLC systems were used to show that spiking of the enzymatic reaction mixture with the authentic selenoxide increased the height of the product peak without changing its retention time. The peak corresponding to the enzymatically produced PAESeO did not appear in reaction mixtures that did not contain either DBM, a reducing agent, or the substrate PAESe.

As shown in Figure 2, panel A, enzymatic production of PAESeO was linear with respect to time in the ferrocyanide-supported DBM oxygenation of PAESe. In contrast, linearity of product formation with respect to time was not observed in the ascorbate-supported oxygenation reaction, and this suggested a complicating factor. Comparison of the rates of oxygen consumption showed that while the ascorbate-supported oxygenation was initially more than an order of magnitude more rapid, DBM activity was maintained for a much shorter period of time than in the ferrocyanide-supported reaction. The loss of DBM activity when ascorbate was used as the reducing agent was investigated, and it was found that neither the addition of $CuSO_4$ nor additional aliquots of DBM were able to restore enzymatic oxygen consumption. The loss of DBM activity was not due to a pH effect, as the pH of the inactivated system was found to be unchanged. The addition of an aliquot of the inactive assay solution had no inhibitory effect on the observed oxygen consumption rate of a fresh DBM–PAESe incubation mixture as would be expected if an inhibitory species were building up in solution. The addition of neither superoxide dismutase nor catalase to the inactive DBM system resulted in a measurable increase in dissolved oxygen concentration. In contrast, when ascorbate was added to an assay solution that had lost activity, the result was a complete return of enzymatic activity.

Figure 2, panel B, illustrates the time courses of DBM/PAESe reaction cycles under conditions of excess ascorbate, limiting PAESe, and reaction solutions preequilibrated with 100% oxygen. In each cycle, the reaction was allowed to proceed until oxygen consumption ceased, and samples of the reaction mixture were withdrawn for HPLC and HPLC-EC analyses. It is clearly evident from this figure that each cycle

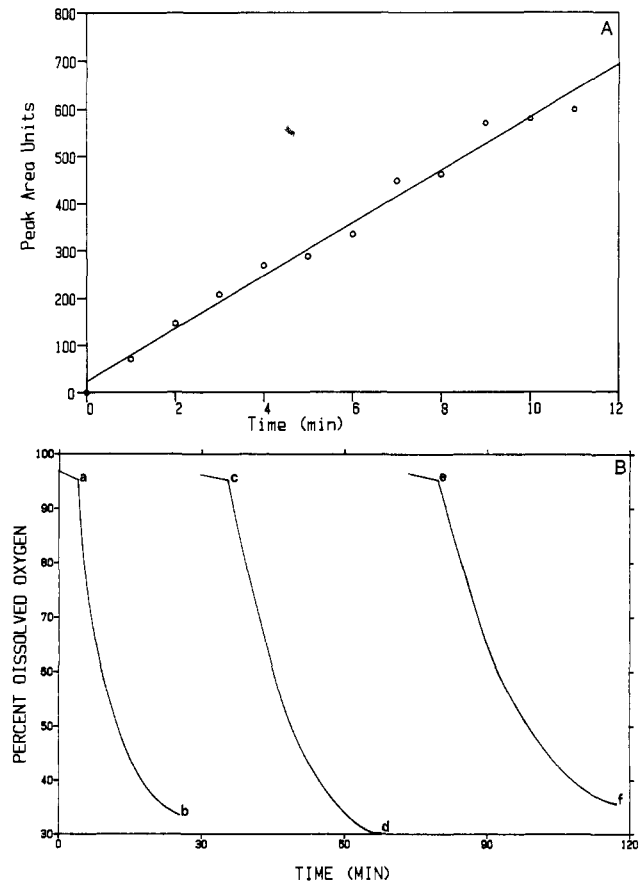

FIGURE 2: (Panel A) Formation of PAESeO with respect to time with ferrocyanide as the electron donor. The initial concentrations of PAESe and $K_4Fe(CN)_6$ in the DBM reaction mixture were 20 and 1.8 mM, respectively. The peak area of PAESeO was determined from chromatograms of the DBM reaction mixture obtained on a C-18 reverse-phase HPLC column with a mobile phase of 50% MeOH and 50% 0.1 M $NH_4H_2PO_4$ and 5 mM sodium octyl sulfate, pH 6.0 (v/v), and with a flow rate of 1.25 mL/min. Deviation from linearity at the lower reaction times corresponds to poor resolution of the product peak at such low concentrations. (Panel B) Time courses of repeated ascorbate depletion cycles. Reaction mixture contained 1.03 mM ascorbate and other components of the standard DBM assay mixture and had been preequilibrated with 100% oxygen (ca. 1.1 mM) for 10 min. (Point a) Reaction was initiated by addition of PAESe to a final concentration of 0.253 mM. (Point b) The concentrations of oxygen and ascorbate remaining were 0.53 and 0.03 mM, respectively. The mixture was then reequilibrated with 100% oxygen. (Point c) Reaction was reinitiated by addition of ascorbate to a final concentration of 1.0 mM. (Point d) The concentration of oxygen remaining was 0.47 mM, while ascorbate was undetectable. The mixture was then reequilibrated with 100% oxygen. (Point e) Reaction was reinitiated by addition of ascorbate to a final concentration of 1.0 mM. (Point f) The concentrations of oxygen and PAESe remaining were 0.56 and 0.124 mM, respectively. Ascorbate again was undetectable.

terminated prior to oxygen depletion, that after reequilibration with oxygen no turnover occurred prior to recharging with ascorbate, and that repeated cycling after each ascorbate addition proceeded to virtually the same end point. Analysis of the reaction mixtures at the termination points showed that PAESe sufficient to support oxygenation was still present, while ascorbate levels were near zero (see Figure 2, panel B legend). It is important to note that the amounts of ascorbate and oxygen consumed in each cycle were approximately 4 and 2 times, respectively, the amounts of each required for DBM oxygenation of all of the PAESe present initially. These results are consistent with an ascorbate-dependent recycling of the selenoxide product back to the selenide, with this process continuing until all the ascorbate is depleted. In order to

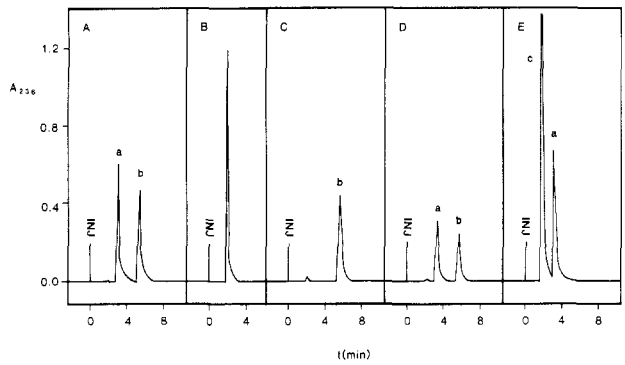

FIGURE 3: Oxidation of ascorbate by PAESeO. HPLC chromatograms were obtained with a C-18 reverse-phase column with a mobile phase of 50% MeOH and 50% 0.1 M $NH_4H_2PO_4$ (v/v) at a flow rate of 1.50 mL/min. The reactions were run in water at room temperature and followed by HPLC to completion. (A) Chromatogram of a mixture of 10 mM PAESe (b) and 10 mM PAESeO (a). (B) Chromatogram of 10 mM ascorbate. (C) Reaction of 10 mM PAESeO and 10 mM ascorbate after 60 min: (b) PAESe. (D) Reaction of 10 mM PAESeO and 5 mM ascorbate after 24 min: (a) PAESeO; (b) PAESe. (E) Reaction of 10 mM PAESeO and 10 mM $K_4Fe(CN)_6$ after 64 min: (c) $K_4Fe(CN)_6$; (a) PAESeO.

confirm this hypothesis, experiments identical with those of Figure 2B were carried out with PAES as the substrate. As expected, the first reaction cycle terminated cleanly upon depletion of the limiting amount of PAES present, and addition of a fresh aliquot of $ASCH_2$ did not restart the reaction. HPLC and HPLC-EC analyses of the reaction mixture at the termination point showed that, while stoichiometric formation of the sulfoxide product (PAESO), stoichiometric consumption of oxygen, and total depletion of substrate had occurred, $ASCH_2$ was still present, with only an amount corresponding to that required for normal substrate turnover having been consumed. These results were precisely those expected for normal DBM turnover and contrast sharply with our observations for PAESe. Thus, we initiated an investigation of the stability of PAESeO in the presence of ascorbate.

Oxidation of Ascorbate by PAESeO. Since selenoxides are known to be mild oxidizing agents (Reich, 1978) and ascorbate has been shown to be oxidized by diphenyl selenoxide to dehydroascorbate (Perina et al., 1973), the ability of PAESeO to oxidize ascorbate was explored. HPLC was used to show that the authentic selenoxide was stable at room temperature in the standard DBM reaction mixture, which did not contain either DBM or ascorbate. Upon the addition of ascorbate to a final concentration of 10 mM, PAESeO disappeared from the HPLC chromatogram and a peak with the same retention time as PAESe appeared. As can be seen in Figure 3, when a mixture of 10 mM PAESeO and 5 mM ascorbate in water was allowed to react for 24 min at room temperature, HPLC analysis demonstrated that the concentration of PAESeO was diminished by 50% at the expense of ascorbate and that a corresponding amount of PAESe was formed. Similarly, the reaction of 10 mM PAESeO and 10 mM ascorbate for 60 min resulted in the complete depletion of the selenoxide and the appearance of a PAESe peak, the height of which corresponded to 9.2 mM PAESe. These results demonstrate that PAESeO is stoichiometrically reduced to PAESe by ascorbate. In constrast to the reduction of PAESeO by ascorbate, the reaction of 10 mM PAESeO and 10 mM potassium ferrocyanide for 64 min caused neither the appearance of any PAESe nor the loss of any PAESeO. These results are consistent with PAESeO possessing a reduction potential at pH 5.0 between that of ferrocyanide (E = 0.420 V) and ascorbate (E = 0.110 V), from Iyanagi et al. (1985).

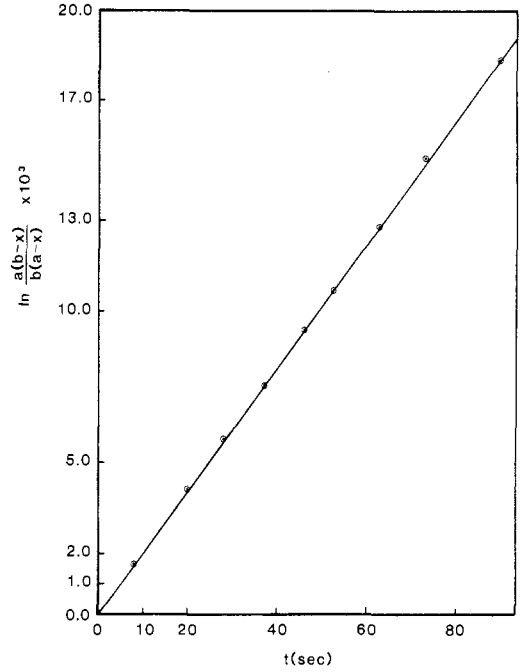

FIGURE 4: Kinetics of ascorbate oxidation by PAESeO. PAESeO, final concentration 1.66 mM, was added to a 1.75 mM solution of ascorbate in deoxygenated 0.127 M sodium acetate buffer, pH 5.0. The depletion of ascorbate was followed spectrophotometrically at 300 nm and 25 °C against a blank containing only the acetate buffer.

The kinetics of the oxidation–reduction reaction between PAESeO and ascorbate were investigated by a spectrophotometric assay of ascorbate depletion at 300 nm (Skotland & Ljones, 1980; Mattock, 1980). The rate of oxidation of ascorbate showed a first-order dependence on both the concentration of ascorbate and the concentration of selenoxide in 0.127 M NaOAc buffer, pH 5.0, and an average value of 3.4 M^{-1} s^{-1} for the second-order rate constant (25.0 °C) was calculated from the initial rates of 12 reactions of various substrate concentrations. Plots of ln $[b(a - x)/a(b - x)]$ vs. time were linear (Figure 4) and corroborated the value of the second-order rate constant determined from initial rates. Under pseudo-first-order conditions with an ascorbate concentration of 10 mM, which corresponds to the ascorbate level in our standard DBM assay solutions, these kinetic values yield a $t_{1/2}$ of 20 s^{-1} for PAESeO at 25 °C and pH 5.0.

The 1:1 stoichiometry of the oxidation of ascorbate by PAESeO is consistent with either a one-electron or a two-electron process, due to the rapid dismutation of semidehydroascorbate. Furthermore, mechanisms can be envisioned for either case that account for the first-order dependence of the rate of the reaction on ascorbate and selenoxide. In order to determine whether or not semidehydroascorbate was a trappable intermediate during the oxidation of ascorbate by PAESeO, cyt c was used as a radical trapping reagent. Yamazaki (1962) has previously characterized the reaction of cyt c with semidehydroascorbate, generated by the oxidation of ascorbate by ascorbate oxidase, and shown that while cyt c reacts slowly with ascorbate itself, the reactivity of semidehydroascorbate in reducing cyt c is orders of magnitude greater. Figure 5 shows that the addition of ascorbate oxidase (final concentration 0.24 unit/mL) to a solution of 0.104 mM ascorbate and 0.017 mM cyt c in 0.063 M NaOAc, pH 5.0, 25 °C, caused a rapid increase in the rate of cyt c reduction as monitored spectrophotometrically at 550 nm. In contrast, the addition of PAESeO (final concentration 0.514 mM) under identical conditions resulted in no observable selenoxide-dependent reduction of cyt c (Figure 5).

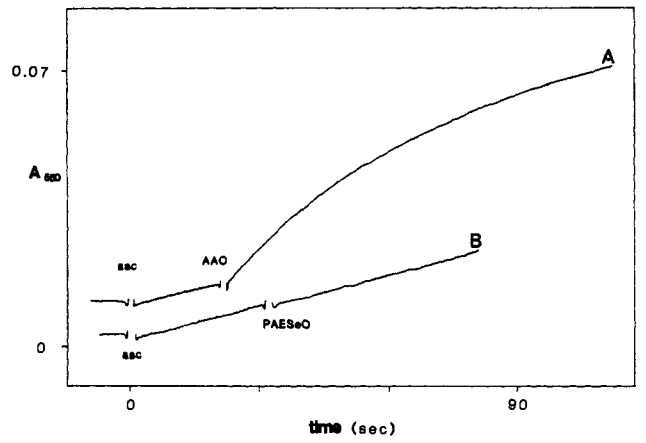

FIGURE 5: Reduction of cytochrome *c* by semidehydroascorbate. Ascorbate, final concentration 1.04×10^{-4} M, was added to a solution of 1.7×10^{-5} M in 0.063 M NaOAc, pH 5.00, 25 °C. (A) Enzymatic generation of semidehydroascorbate with 0.24 unit/mL ascorbate oxidase; (B) addition of PAESeO to a final concentration of 5.14×10^{-4} M.

Additional experiments were carried out wherein the concentrations of ascorbate, PAESeO, PAESe, and cyt *c* were quantitated during the oxidation of ascorbate by PAESeO (Figure 6). As the reduction of cyt *c* was monitored spectrophotometrically, samples of the reaction mixture were chromatographed by reverse-phase HPLC to quantitate ascorbate, PAESeO, and PAESe. The concentrations of the reduction products, cyt *c* (Fe^{2+}) and PAESe, increase as ascorbate is oxidized. When expressed as electrons found per ascorbate oxidized, an average value of 2.2 ± 0.2 is obtained. It is clear from Figure 6 that the background rate of cyt *c* reduction by ascorbate is minor as the oxidation of ascorbate paralleled the disappearance of PAESeO. After 5 min, the concentration of reduced cyt *c* increased very little while the oxidation of ascorbate and the concomitant reduction of PAESeO to PAESe continued at relatively rapid rates. Thus, these results demonstrate quantitatively that no cyt *c* trappable semidehydroascorbate is being produced during the oxidation of ascorbate by PAESeO. This result is consistent with the reduction potential of the PAESeO/PAESe couple being below that of the semidehydroascorbate/reduced ascorbate couple, which has been reported to be 400 mV at pH 6.0 (Iyanagi et al., 1985).

Ascorbate Depletion in Chromaffin Granule Ghosts. In order to examine the interaction of PAESe and ascorbate in ghost preparations, chromaffin granule ghosts, prepared to initially contain 10 mM ascorbic acid and 0.05 mg/mL catalase (which was added to minimize damage by ascorbate autoxidation), were incubated with externally applied PAESe for 30–60 min. A reduction of approximately 70–80% in the levels of $ASCH_2$ remaining within the ghosts was detected as compared to control incubations not containing PAESe. Moreover, it was demonstrated that this disappearance of $ASCH_2$ within the ghosts incubated with PAESe was the result of an oxidative process causing a concomitant appearance of the fully oxidized form of ascorbate, dehydroascorbate. The sum of unreacted $ASCH_2$ plus newly formed dehydroascorbate within the ghosts corresponded to 95% of total initial ascorbate. This indicates that depletion of $ASCH_2$ is not due to some degradative or diffusional process, and it also establishes that the majority of the ghosts survived these incubation and recovery protocols; the small loss of ascorbate noted (5%) is most likely a consequence of the known ability of dehydroascorbate but not $ASCH_2$ to diffuse through the ghost membrane (Tirrell & Westhead, 1979).

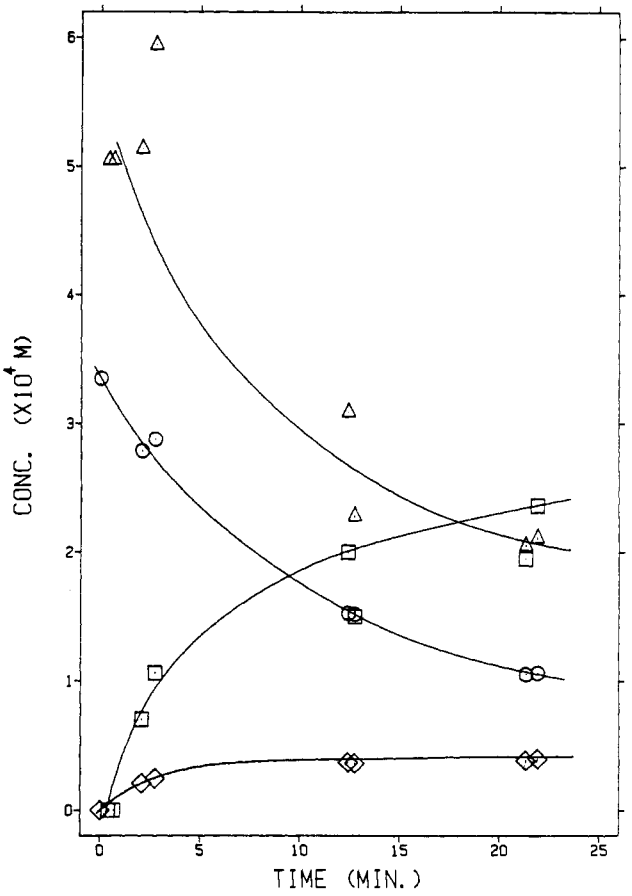

FIGURE 6: Time course of the formation of PAESe and reduced cyt *c* during the oxidation of ascorbate by PAESeO. Initial concentrations: ascorbate, 3.35×10^{-4} M; cyt *c*, 8.00×10^{-5} M; PAESeO, 5.05×10^{-4} M. The concentrations of ascorbate, PAESeO, and PAESe were determined from standard curves of HPLC peak heights, while the amount of cyt *c* reduced was determined from A_{550}. Triangle, PAESeO; circle, ascorbate; square, PAESe; diamond, reduced cyt *c*.

Discussion

The data presented herein represent the first demonstration that DBM is capable of oxygenating the benzylic selenium moiety. As expected for monooxygenations proceeding via the normal reductive oxygenation pathway for DBM, oxygenation of the prototypical selenide substrate PAESe exhibits an electron to oxygen stoichiometry of 2:1 and is stimulated by fumarate in a manner similar to other known DBM oxygenation reactions. Kinetic constants obtained for PAESe establish that DBM processes this selenide substrate with high facility, comparable to that exhibited with the cognate sulfide substrate phenyl 2-aminoethyl sulfide (May & Phillips, 1980; May et al., 1981b).

Identification of PAESeO as the enzymatic product was accomplished by the demonstration of the coincidence of authentic PAESeO with the enzymatic product in three significantly different HPLC systems. A C-18 reverse-phase HPLC column was used to resolve enzymatically produced PAESeO from the components of the DBM reaction mixture under standard mobile-phase conditions, and also when the mobile phase was modified with the ion-pairing reagent sodium octanesulfonate. The conditions of product resolution with sodium octanesulfonate resulted in an increase in the retention time by 26% over that of the standard reverse-phase system for both the synthetic and enzymatically produced PAESeO. The further demonstration of coelution of authentic PAESeO and enzymatically produced PAESeO on a cation-exchange

HPLC column confirms identification of the enzymatic product from DBM oxygenation of PAESe as the corresponding selenoxide, PAESeO. To our knowledge, neither PAESe nor PAESeO has been synthesized previously. We note that systematic efforts to extract the free amine of synthetic PAESeO from basic solutions were unsuccessful; thus, we were unable to isolate enzymatically produced selenoxide in pure form from enzymatic reaction mixtures. Similarly, attempts to identify the product of the DBM-catalyzed oxygenation of PAESe were complicated by the inability to obtain a characteristic mass spectrum for the authentic selenoxide PAESeO. The problem of thermal decomposition and rearrangement of selenium-containing organic compounds in the mass spectrometer are well-known (Agenas, 1973).

As stated in the introduction, our interest in selenide substrates for DBM was predicated on the expectation that the enzymatically produced selenoxides would exhibit unique redox properties, quite distinct from those of the products formed from other heteroatom substrates. The results reported here indeed demonstrate a striking aspect of the DBM-catalyzed oxygenation of PAESe to PAESeO—the ability of the selenoxide product to oxidize the physiological reducing agent ascorbate, with the concomitant and stoichiometric recycling of PAESeO back to PAESe. We have demonstrated that this nonenzymatic recycling reaction exhibits simple second-order kinetics, and we have shown a 1:1 stoichiometry between molecules of ascorbate oxidized and molecules of selenoxide reduced to selenide, thus accounting quantitatively for all the electrons transferred. Furthermore, we have shown that this recycling process gives rise to an in situ depletion of reduced ascorbate in DBM/selenide reaction mixtures, thus terminating DBM activity. As expected, enzymatic activity is restored when reducing equivalents are resupplied. Our results with ferrocyanide verify the conclusion that product recycling is solely responsible for the unusually fast termination of ascorbate-supported DBM selenoxidation activity. Ferrocyanide is incapable of recycling selenoxide to selenide; as a consequence, the ferrocyanide-supported selenoxidation activity of DBM does not terminate through this extraordinary depletion of reducing agent. This finding implies that the reduction potential for the PAESeO/PAESe couple is less than that of the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ couple (420 mV). It is known that the one-electron potential for the semidehydroascorbate/$ASCH_2$ couple is near 400 mV at pH 6.0 (Iyanagi et al., 1985), a result fully compatible with our inability to detect semidehydroascorbate.

On the basis of the kinetics of selenoxide reduction by reduced ascorbate, we estimate that under standard conditions of initial rate measurements for DBM, PAESeO has a half-life of about 20 s. The time course of selenoxide buildup is a complex function of rate and binding constants and is affected by time-dependent changes in ascorbate and oxygen concentrations. We note that although a kinetic expression for the time course has not been developed, addition of fresh reduced ascorbate to the terminated reaction solution results in a concomitant restoration of enzymatic activity and a total disappearance of selenoxide that had built up at the end of the previous reaction phase.

Taken together, the results reported here establish that oxygenation of PAESe by DBM and reduction of the enzymatic product PAESeO back to PAESe by ascorbate define a cyclic path of ascorbate oxidation whereby ascorbate is consumed both by enzymatic turnover and by the nonenzymatic backreaction. Experiments with chromaffin granule ghosts also resulted in a marked, virtually stoichiometric oxidation of $ASCH_2$ to dehydroascorbate upon incubation of the ghosts with PAESe. Although clearly an extensive neurochemical study will have to be carried out, this observation at least constitutes an initial demonstration of this ascorbate depletion process at the subcellular organelle level. From a pharmacological point of view, the rapid oxygenation of PAESe by DBM with its associated ascorbate oxidation cycle could be of great interest. It is thought that the primary process for the regeneration of reduced ascorbate in adrenergic neurotransmitter vesicles is the semidehydroascorbate reductase/cyt B-561 system (Diliberto & Allen, 1981; Njus et al., 1982; Harnadek et al., 1985), which operates on the semidehydroascorbate produced from ascorbate during normal enzymatic turnover by DBM. Since we have demonstrated both qualitatively and quantitatively that no cyt *c* trappable semidehydroascorbate is produced during the nonenzymatic depletion of fully reduced ascorbate via PAESeO recycling, it is tempting to suggest that, if the electron equivalents for DBM arise solely from the recycling of vesicular semidehydroascorbate, the $ASCH_2$ regenerating system would be incapable of preventing the PAESe-dependent depletion of vesicular ascorbate. If so, selenide substrates may provide a unique approach to the modification of DBM activity for therapeutic purposes.

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Chemical Modification of Bovine Transducin: Effect of Fluorescein 5′-Isothiocyanate Labeling on Activities of the Transducin α Subunit†

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ABSTRACT: Fluorescein 5′-isothiocyanate (FITC) was used to modify the lysine residues of bovine transducin (T), a GTP-binding protein involved in phototransduction of rod photoreceptor cells. The incorporation of FITC showed a stoichiometry of approximately 1 mol of FITC/mol of transducin. The labeling was specific for the T_α subunit. There was no significant incorporation on the $T_{\beta\gamma}$ subunit. The modification had no effect on the transducin–rhodopsin interaction or on the binding of guanosine 5′-(β,γ-imidotriphosphate) [Gpp(NH)p] to transducin in the presence of photolyzed rhodopsin. The dissociation of the FITC–transducin–Gpp(NH)p complex from rhodopsin membrane remained unchanged. However, the intrinsic GTPase activity of T_α and its ability to activate the cGMP phosphodiesterase were diminished by FITC modification. The rate of FITC labeling of the transducin–Gpp(NH)p complex was about 3-fold slower than that of transducin. Limited tryptic digestion and peptide mapping were used to localize the FITC labeling site. The majority of the FITC label was on the 23-kilodalton fragment, and a minor amount was on the 9-kilodalton fragment of the T_α subunit. These results indicate that FITC labeling does not alter the activation of transducin by photolyzed rhodopsin but does affect the GTP hydrolytic activity as well as the GTP-induced conformational change of T_α, which ultimately leads to the activation of cGMP phosphodiesterase.

Central to the phototransduction process in vertebrate retinal rod cells is a light-activated cGMP enzyme cascade which involves the rhodopsin molecule, a GTP-binding protein called transducin (T)[1] and the cGMP phosphodiesterase (PDE) [for a review, see Stryer (1986) and Chabre (1985)]. Subunits of transducin (T_α, M_r 39 000, and $T_{\beta\gamma}$, M_r 36 000 and 8000)

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[1] Abbreviations: ROS, rod outer segment; FITC, fluorescein 5′-isothiocyanate; Gpp(NH)p, guanosine 5′-(β,γ-imidotriphosphate); T, transducin; T_α, α subunit of transducin; $T_{\beta\gamma}$, β and γ subunits of transducin; PDE, cyclic GMP phosphodiesterase; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; MOPS, 4-morpholinepropanesulfonic acid; DEAE, diethylaminoethyl; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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