Photoaffinity Labeling of the Tetrabenazine Binding Sites of Bovine Chromaffin Granule Membranes†

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Received July 17, 1984

ABSTRACT: An azido derivative of tetrabenazine, a specific inhibitor of the monoamine carrier of chromaffin granule membranes, has been synthesized. In the dark, this compound, ^{3}H-labeled *N*-(3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b*H*-benzo[*a*]quinolizin-2-yl)-4-[(4-azido-2-nitrophenyl)amino]butanamide ([^{3}H]TBA), bound reversibly to purified chromaffin granule membranes. Centrifugation through SP-Sephadex columns was used to separate bound and free [^{3}H]TBA. This technique gave low levels of nonspecific binding and allowed recovery of [^{3}H]TBA–membrane complexes. Scatchard analysis of the data indicated one class of sites with an equilibrium dissociation constant K_D of 50 nM and a density of sites of 40–50 pmol/mg of protein, consistent with reported densities of reserpine and dihydrotetrabenazine binding sites. Competition experiments showed that TBA and tetrabenazine bound to the same site. Irradiation at 435 nm of [^{3}H]TBA–membrane mixtures induced some irreversible binding of the probe to membranes. After irreversible binding of TBA, the number of dihydrotetrabenazine binding sites was decreased, indicating that the probe was covalently bound to the monoamine carrier. [^{3}H]TBA–membrane complexes isolated by centrifugation through SP-Sephadex columns were irradiated, and their radioactivity was analyzed by electrophoresis on sodium dodecyl sulfate/polyacrylamide gels. A polypeptide with a molecular weight of 70 000 was labeled. This polypeptide was different from dopamine β-hydroxylase, and it was not adsorbed on concanavalin A–Sepharose. It is proposed that the monoamine carrier of chromaffin granule membrane has an oligomeric structure, involving a 45K subunit [Gabizon, R., Yetinson, T., & Schuldiner, S. (1982) *J. Biol. Chem. 257*, 15145] and a 70K subunit.

Adrenaline and noradrenaline, the catecholamines of adrenal medulla, are stored in specialized organelles, the chromaffin granules, from which they are released by exocytosis. The concentration of catecholamines in chromaffin granules has been estimated to be 0.7 M (Nordmann, 1984), and this high concentration is the result of an active ATP-dependent transport (Kirschner, 1962; Carlsson et al., 1963). The membranes of chromaffin granules contain a carrier that catalyzes an electrodissipative H^+/neutral amine antiport (Johnson & Scarpa, 1979; Apps et al., 1980; Scherman & Henry, 1980a; Kanner et al., 1980; Knoth et al., 1980). This carrier accumulates catecholamines in response to the H^+ electrochemical gradient (inside positive and acidic) generated by an ATP-dependent electrogenic H^+ pump (Casey et al., 1977; Phillips & Allison, 1978; Johnson & Scarpa, 1979; Scherman & Henry, 1980b). Little is known about the structure of the monoamine carrier. Tetrabenazine (TBZ)[1] and reserpine are specific inhibitors of the carrier (Pletscher, 1976; Scherman & Henry, 1980c). Tritiated derivatives of these drugs have been used in binding studies (Scherman et al., 1983; Scherman & Henry, 1984). [^{3}H]TBZOH binds to chromaffin granule membranes with an equilibrium dissociation constant of 3 nM, and the density of binding sites is 60 pmol/mg of protein, thus indicating that the carrier is not a major component of the membrane (Scherman et al., 1983). [^{3}H]Reserpine binding studies have indicated the presence of a second class of binding sites on the carrier, with a high affinity for reserpine and noradrenaline and no affinity for TBZ (Scherman & Henry, 1984). A purification of the monoamine carrier in a functional state is possible since the conditions of its solubilization have been defined (Maron et al., 1979; Isambert & Henry, 1981; Scherman & Henry, 1983a). Nevertheless, because of the difficulty of the purification, we have attempted the identification of TBZ binding sites by the technique of photoaffinity. Our probe is *N*-(3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b*H*-benzo[*a*]quinolizin-2-yl)-4-[(4-azido-2-nitrophenyl)amino]butanamide (TBA), a derivative of TBZ coupled to an arylazido moiety. In this paper, we present the results of these photolabeling experiments as well as some methodological improvements to the technique. A communication with a similar aim has appeared in the literature (Gabizon et al., 1982). In this work, the probe was a derivative of 5-HT, a substrate of the carrier.

Experimental Procedures

Chemicals. TBZ and reserpine were obtained from Fluka (Buchs, Switzerland). Sodium cyano[^{3}H]borohydride (98.8 mCi/mg), 1-[7,8-^{3}H]noradrenaline (8 Ci/mmol), and ^{14}C-methylated protein mixture were obtained from Amersham International (U.K.). [2,3-^{3}H]-γ-Aminobutyric acid was from

† This work was supported by the CNRS (ER 103), the MIR (Contract 83.C.0915), INSERM (Contract 83 60 14), and the Fondation pour la Recherche Médicale Française.

[1] Abbreviations: TBA, *N*-(3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b*H*-benzo[*a*]quinolizin-2-yl)-4-[(4-azido-2-nitrophenyl)-amino]butanamide; TBZ, tetrabenazine (2-oxo-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b*H*-benzo[*a*]quinolizine); TBZOH, dihydrotetrabenazine (2-hydroxy-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b*H*-benzo[*a*]quinolizine); [^{3}H]TBZOH, [2-^{3}H]dihydrotetrabenazine; $TBZNH_2$, 2-amino-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b*H*-benzo[*a*]quinolizine; NAP–GABA, *N*-(4-azido-2-nitrophenyl)-4-aminobutyric acid; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; DBH, dopamine β-hydroxylase.

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CEA (Saclay, France). Sephadex G-50 (medium) and SP-Sephadex (C-50) were purchased from Pharmacia (Uppsala, Sweden).

Synthesis of $TBZNH_2$. TBZ (0.3 mmol) and ammonium acetate (1.5 mmol) were incubated with sodium borohydride (0.18 mmol) in 10 mL of methanol (dried on 3-Å molecular sieves) for 4 h at room temperature. $TBZNH_2$ was purified by TLC on preparative silica gel plates using $CHCl_3$–methanol (8:2) as the developing solvent and methanol to extract the product from the plates. The two isomers migrated distinctly (R_f 0.3 and 0.4), but generally they were not extracted separately. Primary amine reactivity was measured with trinitrobenzenesulfonic acid (Inman & Dintzis, 1969) and was found to be equivalent to the product concentration estimated from UV spectra by using a molecular absorption coefficient ϵ_{285} = 3600 M^{-1} cm^{-1} in methanol; yield 50%; mass spectroscopy, M^+ = 318; base peak m/z 244 ($M^+ - NH_3$ – isobutyl).

Synthesis of [2-^3H]$TBZNH_2$. TBZ (6 μmol) and ammonium acetate (60 μmol) were incubated with 10 mCi (1.6 μmol) of cyano[^{3}H]borohydride in 0.18 mL of dry methanol for 30 min at room temperature. The reaction mixture was purified by HPLC on a C_{18} μBondapak column (Waters, Milford, MA) with methanol–10 mM $(NH_4)CO_3H$ (70:30) as the solvent. The two isomers were eluted at 16 (isomer I, 1.1 mCi) and 21 mL (isomer II, 1.3 mCi); they were collected and lyophilized separately. Both had similar specific activity (2.3 Ci/mmol).

Synthesis of TBA. 4-Fluoro-3-nitrophenyl azide was prepared from 4-fluoro-3-nitroaniline (Fleet et al., 1972). It was coupled to 4-aminobutyric acid essentially as described by Levy (1973) to give NAP–GABA. This compound was recrystallized from ethanol. NAP–GABA was activated by *N*-hydroxysuccinimide. Briefly, dicyclohexylcarbodiimide (0.83 mmol) was slowly added to a mixture of NAP–GABA (0.75 mmol) and *N*-hydroxysuccinimide (0.83 mmol) in 20 mL of dry tetrahydrofuran. The mixture was stirred overnight at room temperature. Acetic acid (0.08 mmol) was added, and solids were filtered off. The solution was dried in vacuo over P_2O_5. The product, *N*-succinimidyl 4-[(4-azido-2-nitrophenyl)amino]butyrate, was about 90% pure as judged by TLC on silica gel plates with $CHCl_3$–methanol (10:1) as the developing solvent. This system separated NAP–GABA (R_f 0.23) from the succinimidyl derivative (R_f 0.68). The succinimidyl derivative was then coupled with $TBZNH_2$. In a typical experiment, the activated NAP–GABA derivative (2.4 μmol) and $TBZNH_2$ (2.0 μmol) were incubated in 0.2 mL of dioxane–H_2O (4:1) for 3 h at room temperature. The reaction mixture was dried under a stream of argon, and the residue was solubilized in methanol and purified by HPLC on a C_{18} μBondapak column with methanol–10 mM $(NH_4)CO_3H$ (70:30) as the solvent and a UV detector operating at 260 nm. The coupled product was detected by monitoring the absorption of the azidophenyl moiety and by the radioactivity of tracer amounts of [^{3}H]$TBZNH_2$. Two products were eluted at 7.8 (compound I) and 9.9 mL (compound II), which contained equivalent amounts of $TBZNH_2$ and arylazido moieties. Experiments with isolated isomers of $TBZNH_2$ indicated that the two products were isomers. Compound II (yield 35%) has been used in all subsequent experiments. Its concentration was estimated by using a molecular absorption coefficient ϵ_{460} = 5900 M^{-1} cm^{-1} in methanol.

Synthesis of [^3H]TBA. (1) [^3H]$TBZNH_2$-Labeled TBA. Isomer II of [^{3}H]$TBZNH_2$ (0.58 μmol, 1.3 mCi) was reacted with the succinimidyl derivative of TBA (0.6 μmol), and the resulting [^{3}H]TBA was purified as described above. [^{3}H]TBA (1.9 Ci/mmol, yield 29%) was stored in methanol at −80 °C. Its purity was periodically checked by HPLC.

(2) [^3H]GABA-Labeled TBA. 4-Amino-[2,3-^{3}H]butyric acid (1 μmol, 5 mCi) was incubated with 4-fluoro-3-nitrophenyl azide (4μmol) and triethylamine (4 μmol) in 30 μL of dimethyl sulfoxide at 70 °C overnight. The mixture was lyophilized, and [^{3}H]NAP–GABA was purified by HPLC with methanol–acetic acid–H_2O (60:1:40) as the solvent. [^{3}H]-NAP–GABA (0.6 μmol) was eluted at 11 mL and was lyophilized. [^{3}H]NAP–GABA was incubated with *N*-hydroxysuccinimide (1.2 μmol) and dicyclohexylcarbodiimide (1.2 μmol) in 50 μL of dry tetrahydrofuran at room temperature overnight. The vial was then dried under a stream of argon. $TBZNH_2$ (2.5 μmol) in 100 μL of tetrahydrofuran–H_2O (3:1) was added, and the mixture was incubated at room temperature. After 3 h, it was dried under argon and purified by HPLC with methanol–30 mM $(NH_4)CO_3H$ (80:20) as the solvent. Isomer II of [^{3}H]TBA (0.12 μmol, 0.57 mCi) was eluted at 10 mL.

In photolabeling experiments, [^{3}H]$TBZNH_2$-labeled and [^{3}H]GABA-labeled TBAs were tried and gave similar results. Most of the experiments described (with the exceptions of those of Figure 5 and Table II) were performed with the latter compound.

Chromaffin Granule Membrane Preparation. Bovine chromaffin granule membranes were prepared by osmotic lysis of granules isolated by centrifugation on a 1.6 M sucrose layer. Unless indicated, membranes were frozen in liquid nitrogen and were stored at −80 °C (Smith & Winkler, 1967; Giraudat et al., 1980).

[^3H]TBA Binding. Membranes (0.01–1.0 mg of protein/mL) were incubated at room temperature with various concentrations of [^{3}H]TBA in 0.3 M sucrose/20 mM Tris–phosphate buffer (pH 7.0). Usually, bound ligand was measured by centrifugation through SP-Sephadex C-50. The gel was equilibrated in the same buffer, poured into 1-mL plastic syringes, and dehydrated by centrifugation at 500 g for 2 min at room temperature (Penefsky, 1977; Pick & Racker, 1979). Samples (0.2 mL) were added to the dehydrated gel and were centrifuged in the same way. Larger samples (1.0 mL) were centrifuged through 3.0 mL of gel with the same results. When nonspecific binding was measured, 7 μM TBZ was added to the incubation mixture, and the gel was equilibrated in the presence of 10 μM TBZ. In some experiments, filtration through C_{18} Sep-pak cartridges (Waters, Milford, MA) were used to adsorb free [^{3}H]TBA. Before being used, the cartridge was washed with 2 mL of methanol, 20 mL of H_2O, and 20 mL of buffer. The sample (0.5 mL) was filtered smoothly, and the resin was washed by 1.5 mL of buffer. The yield of [^{3}H]TBA–membrane complex obtained by this technique was low (about 25%); it was increased by filtering three samples of membrane proteins before the [^{3}H]TBA–membrane complex.

SDS/Polyacrylamide Gel Electrophoresis. [^{3}H]TBA–membrane complexes (1 mL) were prepared by centrifugation through SP-Sephadex gels. They were collected in plastic cuvettes and immediately placed in front of a monochromator set at 435 nm (Bausch & Lomb, equipped with an SP-200 mercury light source). Samples were illuminated for 2.5 min at room temperature with stirring. They were diluted to 5 mL with 10 mM Hepes buffer (pH 7.0). The pellets were extracted by 5 mL of ethanol–acetone (1:1), centrifuged at 250000*g* for 20 min, and solubilized in 0.22 mL of a 50 mM phosphate buffer, pH 7.0, containing 2% SDS, 12% sucrose,

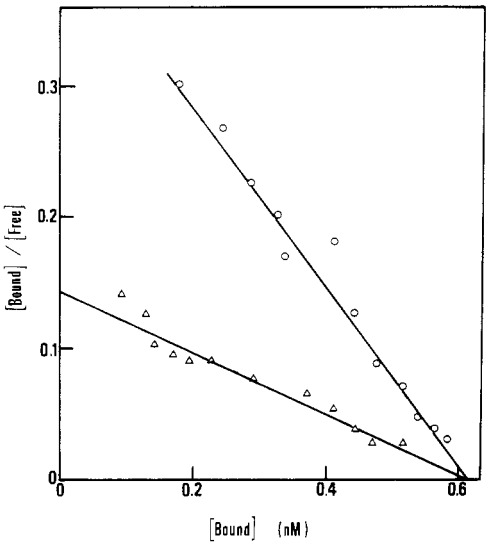

FIGURE 1: Inhibition by TBA of [^{3}H]TBZOH binding. Membranes (0.014 mg of protein/mL) were preincubated in the presence (△) or absence (○) of 85 nM TBA for 1 h at room temperature in 1.0 mL of 20 mM Tris–phosphate buffer (pH 7.2) containing 0.3 M sucrose. [^{3}H]TBZOH (0.78–20 nM) was then added, and the mixture was incubated for 90 min at the same temperature. Bound [^{3}H]TBZOH was determined by filtration on Millipore filters. Nonspecific binding was measured by addition of 2 μM TBZ to the incubation mixture and was subtracted. Points are mean of duplicate determinations.

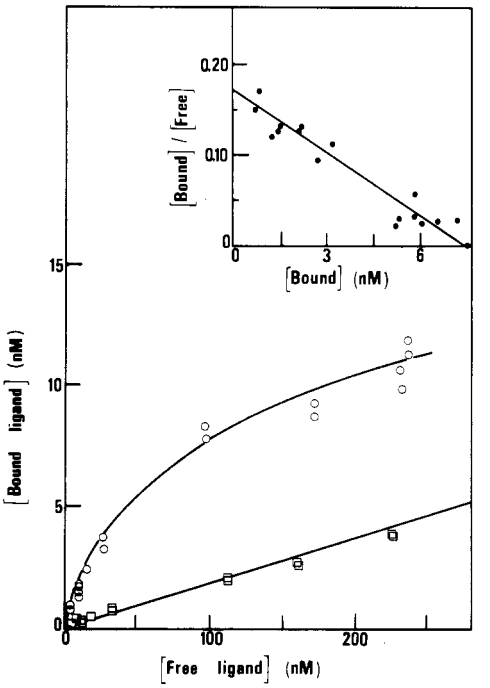

FIGURE 2: Saturation isotherm obtained by filtration of the ligand through SP-Sephadex columns. Membranes (0.15 mg of protein/mL) were incubated with [^{3}H]TBA (5–500 nM) in the absence (○) or presence (□) of 5 μM TBZ for 40 min at room temperature in 0.2 mL of 20 mM Tris–phosphate buffer (pH 7.0) containing 0.3 M sucrose. Inset: Scatchard plot of the data.

30 mM DTT, and 6 mM EDTA. Aliquots (100–200 μL) were subjected to disc electrophoresis according to Weber & Osborn (1969) on 7.5% polyacrylamide gels for 3 h at 15 mA per gel or to Laemmli (1970) on 8% polyacrylamide gels for 2 h at 4 mA per gel. Ethanol–acetone extraction did not change the electrophoresis pattern (Sutton & Apps, 1981). The gels were then sliced in 2-mm fractions, from which the radioactivity was eluted by 0.8 mL of 6 M urea and 1% SDS (Buisson et al., 1976) and measured by scintillation after addition of 7 mL of Aqualuma (Lumac, Schaesberg, Holland).

Other Techniques. ATP-dependent [^{3}H]noradrenaline uptake was measured essentially as described by Phillips (1974), [^{3}H]TBZOH binding as described by Scherman et al. (1983), and DBH activity as described by Wallace et al. (1973). Proteins were estimated by the Lowry procedure with bovine serum albumin as a standard following precipitation in 5% trichloroacetic acid and redissolution in 2% deoxycholate–3% NaOH. Phospholipid estimation was done by assaying inorganic phosphate liberated by perchloric acid hydrolysis.

RESULTS

TBA and TBZOH Binding Occur on the Same Site. [^{3}H]TBZOH binding has been studied in the presence of a constant concentration of TBA, in the dark (Figure 1). A Scatchard plot of the data indicated that the addition of TBA decreased the binding of [^{3}H]TBZOH, without affecting the number of binding sites B_{max}. This result indicates that the two drugs compete for the same site. From these data, the equilibrium dissociation constant for the binding of TBA was also calculated and was found to be 47 nM. The two drugs thus bind to the same site, and the affinity of this site for TBA is an order of magnitude lower than for TBZOH.

Centrifugation through Sephadex Gels as an Assay of [^{3}H]TBA Binding in the Dark. The direct binding of [^{3}H]-TBA to purified chromaffin granule membranes was difficult to investigate by the classical filtration technique, because of the high background due to ligand adsorption on cellulose or glass-fiber filters. Therefore, the bound complex was separated from the free ligand by low-speed centrifugation through Sephadex gels (Penefsky, 1977; Pick & Racker, 1979). In this technique, the gel is dehydrated by a previous centrifugation under the same conditions, thus suppressing dilution of the sample. We found that the negatively charged SP-Sephadex gave better retention of free [^{3}H]TBA than did the neutral Sephadex G-50. In the absence of granule membranes, 2% or less of the layered radioactivity was recovered in the void volume, whereas under the same conditions of centrifugation, the recovery of the membrane protein was higher than 90%. To evaluate the validity of the centrifugation technique for the quantitative estimation of bound ligands, a saturation isotherm of the binding of [^{3}H]TBZOH was performed by this technique. A Scatchard plot of the data indicated a dissociation equilibrium constant K_D of 2.0 nM and a density of sites B_{max} of 27 pmol/mg of protein, which should be compared with values of 1.4 nM and 45 pmol/mg of protein for respectively K_D and B_{max} obtained by filtration on Millipore filters (Figure 1). The technique of filtration through SP-Sephadex gels has thus been utilized to study [^{3}H]TBA binding (Figure 2). The binding observed in the presence of 7 μM TBZ has been considered as nonspecific and has been subtracted to estimate specific binding. A saturation isotherm obtained by this technique is given in Figure 2. Analysis of the data (Figure 2, Inset) showed one class of binding sites characterized by a K_D of 43 nM and a B_{max} of 50 pmol/mg of protein.

Characteristics of [^{3}H]TBA Binding in the Dark. At room temperature, the equilibrium was reached rapidly, about 20 min being required at a concentration of 100 nM. The equilibrium was temperature dependent; the level of [^{3}H]TBA binding observed at 22 °C decreased by a factor of 2 after a 10-min incubation at 0 °C. The kinetics of dissociation were measured by addition of 8 μM TBZ to membranes preincubated with 80 nM [^{3}H]TBA. In this experiment, free and bound ligands were separated by rapid filtration on small

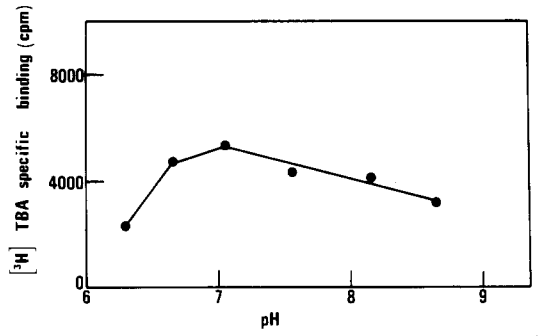

FIGURE 3: pH dependency of [^{3}H]TBA binding. Membranes (0.6 mg of protein/mL) were incubated with [^{3}H]TBA (60 nM) in 0.5 mL of 0.3 M sucrose/20 mM Tris–phosphate buffers at various pH, in the absence or presence of 8 μM TBZ. Duplicate determinations of bound [^{3}H]TBA were performed by centrifugation through SP-Sephadex columns equilibrated at the pH of the incubation mixture. Centrifugation through Sephadex columns and filtration through C_{18} Sep-pak cartridges gave similar pH profiles of the specific radioactivity.

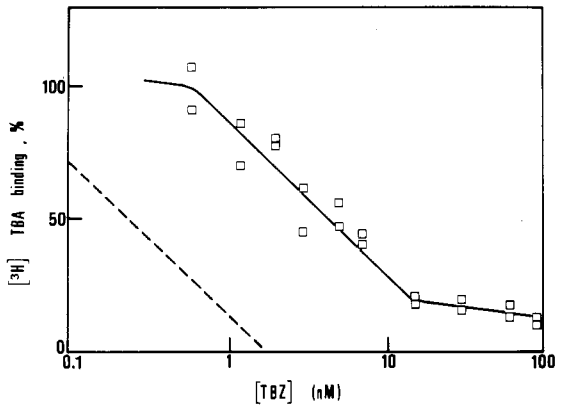

FIGURE 4: Displacement of [^{3}H]TBA by TBZ. Membranes (0.5 mg of protein/mL) were preincubated with TBZ (0–90 nM) for 18 h at room temperature in 0.5 mL of 20 mM Tris–phosphate buffer (pH 7.2) containing 0.3 M sucrose. [^{3}H]TBA (10 nM) was then added, and the mixture was incubated for 2 h at the same temperature. Bound [^{3}H]TBA was determined by centrifugation of 0.2-mL aliquots on SP-Sephadex columns. The dotted line indicates the theoretical slope at midpoint, assuming a Hill number of 1.0.

C_{18}-bonded SiO_2 cartridges, which adsorbed the free ligand. The half-lifetime measured at pH 8.2 and at pH 7.0 is 7 min. [^{3}H]TBZOH which has a pK_A of 7.5 (Scherman & Henry, 1982) has a K_D decreasing with increasing values of the pH, up to pH 8.0 (Scherman & Henry, 1983b). This pH dependency has been attributed to the fact that the monoamine carrier binds only the neutral form of the drug. The effect of the pH on [^{3}H]TBA binding has been studied at a drug concentration of 60 nM (Figure 3). Contrasting with [^{3}H]TBZOH, [^{3}H]TBA binding had a broad pH optimum around pH 7.0. Saturation isotherms had similar characteristics at pH 7.0 and 8.0 (K_D = 43 nM and B_{max} = 50 pmol/mg of protein at pH 7 and K_D = 56 nM and B_{max} = 60 pmol/mg of protein at pH 8).

The specificity of [^{3}H]TBA binding is supported by displacement experiments. [^{3}H]TBA is displaced from its binding site by TBZ (Figure 4). The slope of the curve indicates a competition between the two drugs, thus confirming the results of the experiment of Figure 1. The EC_{50} derived from this experiment is 4 nM, from which a K_D of 3 nM for TBZ can be derived. This figure is consistent with that previously obtained (Scherman et al., 1983). [^{3}H]TBA is also displaced from its binding site by TBA, reserpine, and 5-HT (Table I). The equilibrium dissociation constants for reserpine and TBZ derived from these experiments are similar to those previously reported (Scherman & al., 1983; Scherman & Henry, 1984).

Table I: Displacement of [^{3}H]TBA in the Dark[a]

compounds	EC_{50} (nM)	K_D (nM)[b]
TBZ	3.8	2.9 (1.3)
TBA	40	31
reserpine	55	42 (25)
5-HT	360 × 10^3	277 × 10^3 (240 × 10^3)

[a] Experimental conditions were as described in Figure 4. [b] K_D values were calculated from EC_{50} as described by Jacobs et al. (1975). Figures in parentheses are derived from Scherman et al. (1983) and Scherman & Henry (1984).

Table II: Irreversible Binding of [^{3}H]TBA after Irradiation

membrane treatment	incubations[a] membranes + [^{3}H]TBA (pmol/mL)	membranes + TBZ + [^{3}H]TBA (pmol/mL)	radioactivity specifically bound[b] (pmol/mL)
incubation in the dark[c]	260	110	150
incubation and extraction in the dark[d]	3	3	0
incubation, irradiation, and extraction[e]	34	14	20

[a] Membranes (0.96 mg of protein/mL) were incubated with [^{3}H]-TBA (1 μM) in the absence or presence of 8 μM TBZ for 45 min. [b] Calculated as the difference between columns 2 and 3. [c] Bound [^{3}H]TBA was determined by centrifugation of 0.2-mL aliquots through SP-Sephadex gels. [d] Samples centrifuged through SP-Sephadex columns were extracted by 1.6 mL of ethanol–acetone (1:1) and centrifuged at 10000g for 50 min; the pellets were solubilized in 0.25 mL of 2% deoxycholate–3% NaOH, and their radioactivity was measured by liquid scintillation in Aqualuma. [e] Samples centrifuged through SP-Sephadex columns were rapidly transferred in front of the monochromator set at 435 nm and irradiated for 2.5 min. The membranes were then extracted by ethanol–acetone as in footnote *d*.

Irreversible Binding of [^{3}H]TBA in the Light. When [^{3}H]TBA–membrane complexes isolated in the dark by filtration on SP-Sephadex gels were extracted by an ethanol–acetone mixture (1:1), the specifically bound radioactivity was completely removed (Table II, line 2). This extraction removed 75% of the membrane phospholipid but did not extract any protein (Sutton & Apps, 1981). If the [^{3}H]TBA–membrane complex was rapidly irradiated at 435 nm after its isolation, then some specific radioactivity was not extractable by acetone–ethanol (Table II, line 3). This formation of an irreversible derivative was very reproducible, with a yield of 23 ± 6% (mean ± SD, n = 16). The same result was observed with [^{3}H]TBZNH$_2$ labeled as well as with [^{3}H]GABA-labeled TBA. The kinetics of this reaction were rapid and under the conditions used, it was terminated in less than 4 min (Figure 5).

It was important to verify that the irreversible binding occurred on the same site as the reversible one. Advantage was taken from the fact that TBA and [^{3}H]TBZOH compete for the same sites (Table III, lines 1 and 2). TBA can be washed out by dilution and centrifugation, thus restoring [^{3}H]TBZOH binding to its initial value (Table III, lines 3 and 4). If the mixture of TBA and membranes was irradiated before the washing step, a 20–30% inhibition of [^{3}H]TBZOH binding was observed (Table III, line 5).

Analysis of the [^{3}H]TBA-Labeled Membranes by Electrophoresis on SDS/Polyacrylamide Gels. As a first attempt to identify [^{3}H]TBA binding sites, mixtures of [^{3}H]TBA and membranes were irradiated at 435 nm, and the proteins were analyzed by gel electrophoresis. By this technique no polypeptide chain was selectively labeled. This negative result was

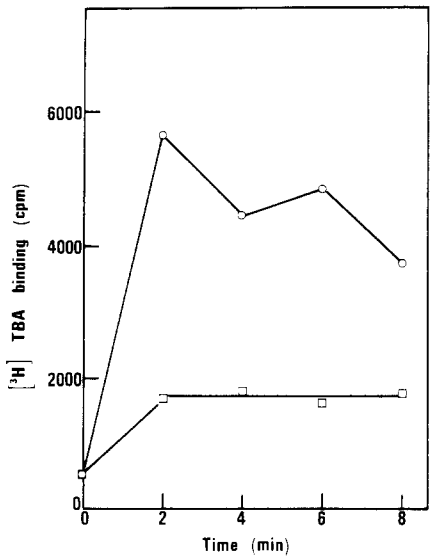

FIGURE 5: Kinetics of the effect of illumination of [^{3}H]TBA–membrane complexes. Membranes (0.76 mg of protein/mL) were incubated with [^{3}H]TBA (340 nM) in the absence (○) or in the presence (□) of 15 μM TBZ in 0.3 M sucrose/20 mM Tris–phosphate buffer (pH 8.15). Aliquots (0.225 mL) were centrifuged through SP-Sephadex columns, rapidly transferred in front of the monochromator set at 435 nm, and irradiated for the indicated period of time. The membranes were then extracted by ethanol–acetone and treated as described in the footnotes to Table II.

Table III: TBA Binds Covalently to [^{3}H]TBZOH Binding Sites

	specific [^{3}H]TBZOH binding[a] (cpm)	inhibition[b] (%)
(1) membranes[c]	3570 ± 260 (n = 3)	control
(2) membranes + TBA[c]	1210 ± 90 (n = 2)	66
(3) washed membranes[d]	2180 ± 100 (n = 4)	control
(4) membranes + TBA, washed[d]	2130 ± 250 (n = 4)	2
(5) membranes + TBA, irradiated, washed[d]	1620 ± 90 (n = 4)	26

[a] [^{3}H]TBZOH (2 nM) binding was measured on membrane samples diluted 10 times in 0.3 M sucrose/Tris–phosphate buffer (pH 7.0) at about 0.01 mg of protein/mL. Nonspecific binding measured in the presence of 2 μM TBZ was subtracted. Measurements are mean ± SD. Similar results have been obtained in two independent experiments. [b] It has been verified that irradiation of the membranes alone did not change [^{3}H]TBZOH binding. [c] Membranes (0.09 mg protein/mL, 1 mL final volume) were incubated without (line 1) or with (line 2) 1.8 μM TBA. [d] Membranes were incubated without (line 3) or with (lines 4 and 5) TBA (1.8 μM) and in line 5, were illuminated at 435 nm for 2.5 min (1 mL final volume). They were then diluted 10 times in sucrose/Tris–phosphate buffer, and after incubation for 10 min at room temperature and 30 min at 0 °C, they were centrifuged for 30 min at 250000g. Pellets were resuspended in 1 mL of the same buffer, and after a 10-fold dilution they were assayed for [^{3}H]TBZOH binding. The protein concentrations of the resuspended pellets were equivalent and were 0.05 mg of protein/mL.

explained by the intensity of [^{3}H]TBA nonspecific binding (Table IV). The level of nonspecific binding was reduced when [^{3}H]TBA–membrane complexes isolated by centrifugation on SP-Sephadex were immediately irradiated (Table IV). Electrophoresis of such complexes revealed a clear peak of radioactivity (Figure 6). No radioactivity peak was observed when 7 μM TBZ or 1 mM 5-HT was added to the incubation mixture before irradiation. Calibration of the gel with ^{14}C-methylated protein markers indicated a molecular weight of 70000 for the labeled polypeptide. In the experiment of Figure 6, the irradiated complex had been extracted with acetone–ethanol (1:1) prior to being electrophoresed. When this step was omitted, a large amount of radioactivity was found in the front of the gel (Figure 7). This radioactivity

Table IV: Decrease of [^{3}H]TBA Nonspecific Binding by Centrifugation on SP-Sephadex Gels

	bound radioactivity[a] (pmol/mL)		
	−TBZ	+TBZ	specific binding
reversible binding,[b] membranes + [^{3}H]TBA	21.2	4.5	16.7
irreversible binding,[c] irradiation of membranes + [^{3}H]TBA[d]	10.2	8.2	2.0
irradiation of the isolated [^{3}H]TBA–membrane complex[e]	3.1	0.6	2.5

[a] The columns give total binding (column 1), nonspecific binding determined in the presence of 8 μM TBZ (column 2) and specific binding obtained as the difference between columns 1 and 2. [b] Membranes (1.0 mg of protein/mL) were incubated with [^{3}H]TBA (65 nM) in 0.3 M sucrose/20 mM Tris–phosphate buffer (pH 7.0). Reversible binding was measured by centrifugation through SP-Sephadex columns. [c] The irradiated samples were washed and extracted by ethanol–acetone, as described for gel electrophoresis. [d] Irradiation of [^{3}H]TBA membrane mixtures for 2.5 min at 435 nm. Gel electrophoresis of these samples indicated multiple radioactivity peaks with no clear displacement by TBZ. [e] Irradiation of [^{3}H]TBA membrane complexes isolated by centrifugation on SP-Sephadex gels as described under Experimental Procedures. Gel electrophoresis of these samples indicated a major peak of radioactivity with a molecular weight of about 65000.

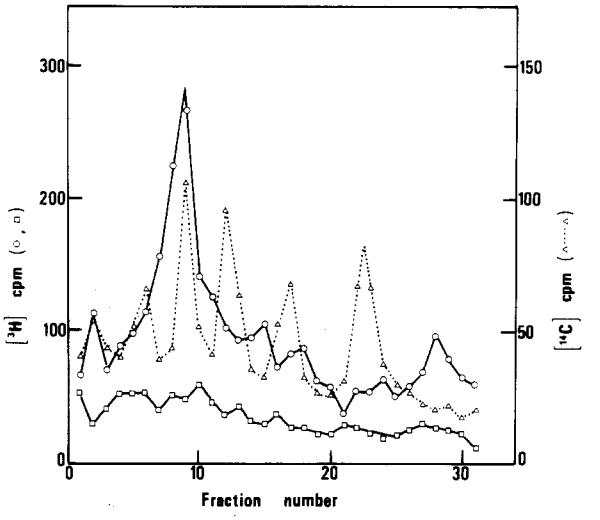

FIGURE 6: SDS/polyacrylamide gel electrophoresis of irradiated [^{3}H]TBA–membrane complexes. Membranes (0.8 mg of protein/mL) were incubated with [^{3}H]TBA (100 nM) in the absence (○) or presence (□) of 7 μM TBZ in 0.3 M sucrose/20 mM Tris–phosphate buffer (pH 7.0). Samples (1.3 mL) were processed as described under Experimental Procedures. ^{14}C-Labeled protein markers (△) were mixed with the [^{3}H]TBA sample before electrophoresis. Electrophoresis was performed according to Weber & Osborn (1969). Marker molecular weights were the following: myosin, 200K; phosphorylase b, 92.5K; bovine serum albumin, 69K; ovalbumin, 46K; carbonic anhydrase, 30K; lysozyme, 14.3K.

is likely to be due to free or lipid-bound [^{3}H]TBA derivatives. However, the possibility of the labeling of a minor peptide of low molecular weight has not been investigated. Use of chromaffin granule membranes prepared in the presence of protease inhibitors (5 μg/mL soybean trypsin inhibitor, 100 μg/mL bacitracin, 1 mM benzamidine, 0.1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride) did not change the electrophoretic pattern. In addition, the same 70K polypeptide was labeled when the experiment was performed immediately after preparation of the membranes, on nonfrozen material.

We have attempted to decrease the duration of the time required for the isolation of [^{3}H]TBA–membrane complex.

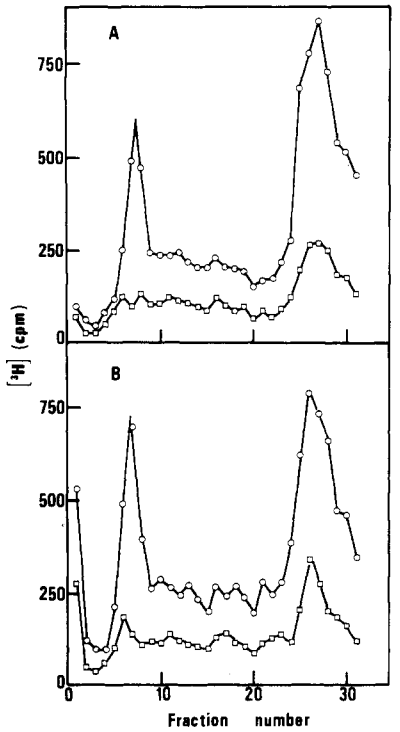

FIGURE 7: Gel electrophoresis of reduced (A) or nonreduced (B) [^{3}H]TBA–membrane complexes. Experimental conditions were similar to those of figure 6, but irradiated membranes were not extracted by acetone–ethanol. The washed membranes were solubilized by the described SDS buffer with (A) or without (B) 30 mM DTT. The molecular weight of the [^{3}H]TBA-labeled peaks was about 70 000.

The best results have been obtained by rapid filtration of [^{3}H]TBA–membrane mixtures through small SiO_2 cartridges, directly in a cuvette in front of the monochromator. The electrophoretic patterns of samples treated in this way indicated a radioactivity peak with a molecular weight of 70 000 and a low background. We have also noted that [^{3}H]TBA probes tritiated on the TBZ or the GABA moiety labeled the same 70K polypeptide.

[^{3}H]TBA-Labeled Polypeptide Is Not Dopamine β-Hydroxylase (DBH). DBH is the major component of chromaffin granule membrane (Hortnagl et al., 1972). In the presence of reducing agents, it migrates on SDS/polyacrylamide gels as a monomer with a molecular weight of 75 000 (Wallace et al., 1973). It was thus of importance to verify that our results did not originate in some low-affinity interaction between [^{3}H]TBA and this protein. TBA (up to 0.7 μM) and TBZ (2.4 μM) were not inhibitors of DBH activity (data not shown). Moreover, in the absence of reducing agents, DBH migrates as a dimer with a molecular weight of 150 000. This shift from M_r 75 000 to 150 000 is considered to be characteristic of DBH (Cahill & Morris, 1979). Omission of the reducing agent did not change the mobility of the [^{3}H]TBA-labeled component (Figure 7).

Finally, in another set of experiments, advantage was taken from the fact that DBH is a glycoprotein with a high affinity for concanavalin A. [^{3}H]TBA-labeled membranes were solubilized by low SDS concentrations, and the soluble components were filtered through a concanavalin A–Sepharose column, according to Fischer-Colbrie et al. (1982). A large fraction (75%) of the radioactivity was not retained on the column, and analysis by electrophoresis on polyacrylamide gels indicated that this fraction was associated with the 70K polypeptide (Figure 8). In the same experiment, the densitometric scanning of the protein stained with Coomassie blue is shown. From the comparison of the two profiles, it is obvious

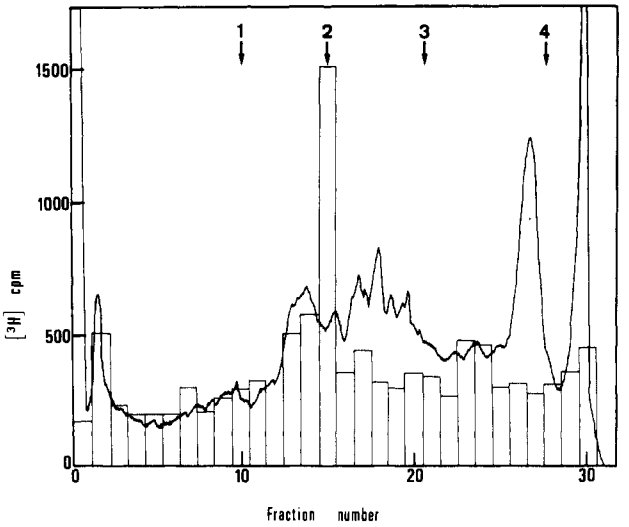

FIGURE 8: Gel electrophoresis of membranes labeled with [^{3}H]TBA and filtered through concanavalin A–Sepharose. Membranes (0.5 mg of protein/mL) were incubated with 70 nM [^{3}H]TBA in 6.0 mL of 0.3 M sucrose/20 mM Tris–phosphate buffer (pH 7.2). The sample was centrifuged through SP-Sephadex gels, irradiated, and washed as described under Experimental Procedures. The pellet was solubilized by 2.0 mL of 0.07% SDS in 100 mM NaCl/100 mM phosphate buffer (pH 7.4). The mixture was centrifuged at 160000*g* for 45 min. The supernatant was filtered through concanavalin A–Sepharose gel as described by Fischer-Colbrie et al. (1982). The filtrate was delipidated and concentrated according to Wessel et al. (1984). The samples were then analyzed by electrophoresis according to Laemmli (1970). The electrophoretogram shows the radioactivity of gel slices and a densitometric scan of Coomassie stained protein. Arrows indicate molecular weight markers: 1, phosphorylase *b*; 2, bovine serum albumin; 3, ovalbumin; 4, carbonic anhydrase.

that [^{3}H]TBA specifically labels a minor component of the membrane.

Discussion

We describe the synthesis of a probe, TBA, intended to label the TBZ binding site. The binding in the dark of [^{3}H]TBA to chromaffin granule membranes has been followed by the technique of centrifugation on Sephadex gels, originally proposed by Pick (Penefsky, 1977). This technique has several advantages over the classical filtration assay. The membrane–ligand complex is obtained in solution and can thus be easily manipulated. Moreover, the level of nonspecific binding is low. In several experiments, we have verified that it was not very different from the radioactivity recovered in the absence of granule membranes, thus suggesting that nonspecific binding controls contained principally free ligand. Nevertheless, the reliability of the technique from the quantitative point of view has to be assessed. From binding data obtained at different [^{3}H]TBA concentrations and assuming a K_D of 50 nM, we can calculate a density of sites B_{max} of 38 ± 19 pmol/mg of protein (SD, n = 26). This figure can be considered as being in good agreement with that obtained by filtration for [^{3}H]TBZOH binding sites (Scherman et al., 1983).

[^{3}H]TBA binds in the dark to the same site than TBZ since (i) both drugs have similar densities of sites (Figures 1 and 2), (ii) TBA inhibits competitively the binding of [^{3}H]TBZOH (Figure 1), (iii) TBZ displaces [^{3}H]TBA from its sites (Figure 4), and (iiii) the dissociation constants of TBZ, reserpine, and 5-HT derived from [^{3}H]TBA displacement experiments are similar to those derived from [^{3}H]TBZOH displacement ones (Table I). The derivatization of TBZ to TBA has thus only decreased the affinity of this new compound for chromaffin granule membrane. This change of affinity may be the result

of the introduction of an extra amino group in the TBZ moiety since $TBZNH_2$ has an affinity of the same order as TBA, as judged from [^{3}H]TBZOH displacement and noradrenaline uptake inhibition experiments (data not shown). This amino group is likely to have a pK_A higher than 7.5, the pK_A of the ring tertiary amine of TBZOH (Scherman & Henry, 1982). The observed affinity decrease might thus result from the presence of a positive charge in the TBZ moiety, a hypothesis consistent with the fact that the carrier binds only the neutral form of substrate and inhibitor monoamines (Scherman & Henry, 1983b). Though we have not systematically investigated the acido–basic properties of TBA, we have noted a difference in the pH dependency of [^{3}H]TBZOH and [^{3}H]-TBA binding. Increasing the pH from 7.0 to 8.0 decreases the K_D of the former drug from 7.0 to 4.7 nM (Scherman & Henry, 1983b) and did not change that of the latter one.

[^{3}H]TBA binding in the dark is reversible. This is shown by (i) the displacement of [^{3}H]TBA by TBZ (Figure 4), (ii) direct measurement of the rate of dissociation, and (iii) the fact that the ligand can be extracted by organic solvents (Table II) or washed away (Table III). Nevertheless, bound [^{3}H]-TBA can be separated from free ligand by rapid centrifugation through SP-Sephadex columns since the half-lifetime of the complex (7 min) is larger than the time required to isolate it (2 min). Under the conditions used (50–100 nM [^{3}H]TBA), it can be calculated that 80% of the particulate ligand is specifically bound and that 80% of that fraction is present as a membrane–ligand complex after centrifugation through SP-Sephadex. These conditions are thus suitable to get high levels of specific labeling. Irradiation of isolated [^{3}H]TBA complexes gives rise to some irreversible binding of the probe. Irreversibly bound [^{3}H]TBA is washed neither by dilution and centrifugation (Table III) nor by organic solvents (Table II). Irreversible binding labels a polypeptide with a molecular weight of 70K (Figure 6). The labeling is considered to be specific for the following reasons: (i) Irradiation of TBA–membrane complexes decreases the number of [^{3}H]TBZOH binding sites and the number of [^{3}H]TBZOH binding sites photoinactivated is similar to the yield of [^{3}H]TBA irreversible binding (Table III). This result argues against a transfer of the probe to a protein of the vicinity during isolation of [^{3}H]TBA–membrane complexes. Such an artifact is also ruled out by the fact that the isolation time can be decreased to less than 15 s by filtration through SiO_2 cartridges, without affecting the labeling of the 70 k polypeptide. (ii) The peak of radioactivity at 70K is not observed when TBZ of 5-HT was added to the incubation mixture before irradiation. In the experiment of Figure 7, the concentration of TBZ is 7 μM, but similar results were obtained with a 500 nM drug concentration. (iii) A comparison of the densitometric scanning of the protein stained with Coomassie blue and of the [^{3}H]-TBA radioactivity profile (Figure 8) indicates that the probe labels a minor component of the membrane and is not associated in a nonspecific manner with the peptidic material. Since the major protein of chromaffin granule membrane, DBH, has a molecular weight of 75K, care has been taken to demonstrate that [^{3}H]TBA does not label DBH as a result of nonspecific or low-affinity binding. Therefore, we propose that the TBZ binding site is borne by a 70K polypeptide.

Previous work from this laboratory (Scherman et al., 1983) has indicated that the TBZ binding site is associated with the monoamine transporter, and it can thus be proposed that the TBA-labeled 70K polypeptide is the transporter or one of its subunits. Nevertheless, Gabizon et al. (1982), using a different probe, ANPA–5-HT, derived from 5-HT, have labeled a 45K polypeptide by the same technique. Experiments are now in progress to demonstrate that the TBA-labeled polypeptide is directly associated with amine transport. This hypothesis is interesting since it suggests an oligomeric structure for the monoamine carrier of chromaffin granules. Since the two probes are different, it can be proposed that the 70K subunit carries TBZ binding sites, whereas the 45K one has a site of high affinity for the substrate. In a previous communication (Scherman & Henry, 1984), it has been proposed that the carrier has two monoamine binding sites: sites R_1 of high affinity for substrates and reserpine and with no affinity for TBZ and sites R_2 with a high affinity for TBZ and low affinities for substrates and reserpine. We can now speculate that R_1 sites are borne by the 45K subunit whereas R_2 sites belong to that of 70K.

ACKNOWLEDGMENTS

We are indebted to P. Fellman, E. Favre (Institut de Biologie Physico-Chimique), H. Gozlan (Collège de France), P. Jaudon, and Y. Hoppiliard (Ecole Polytechnique) for help in chemical synthesis. We thank the Service Vétérinaire des Abattoirs de Mantes for collecting bovine adrenals.

Registry No. TBA, 96557-41-4; [2-^{3}H]$TBZNH_2$-labeled TBA, 96557-37-8; [2,3-^{3}H]GABA-labeled TBA, 96557-38-9; $TBZNH_2$, 96614-27-6; NAP-GABA, 58775-36-3; [2-^{3}H]$TBZNH_2$, 96557-39-0; [2,3-^{3}H]NAP-GABA, 96557-40-3; tetrabenazine, 58-46-8; *N*-succinimidyl 4-[(4-azido-2-nitrophenyl)amino]butyrate, 62618-07-9; 4-amino[2,3-^{3}H]butyric acid, 13048-68-5; 4-fluoro-3-nitrophenyl azide, 28166-06-5.

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Photoaffinity Labeling of the Pactamycin Binding Site on Eubacterial Ribosomes†

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Received November 30, 1984

ABSTRACT: Pactamycin, an inhibitor of the initial steps of protein synthesis, has an acetophenone group in its chemical structure that makes the drug a potentially photoreactive molecule. In addition, the presence of a phenolic residue makes it easily susceptible to radioactive labeling. Through iodination, one radioactive derivative of pactamycin has been obtained with biological activities similar to the unmodified drug when tested on in vivo and cell-free systems. With the use of [^{125}I]iodopactamycin, ribosomes of *Escherichia coli* have been photolabeled under conditions that preserve the activity of the particles and guarantee the specificity of the binding sites. Under these conditions, RNA is preferentially labeled when free, small ribosomal subunits are photolabeled, but proteins are the main target in the whole ribosome. This indicates that an important conformational change takes place in the binding site on association of the two subunits. The major labeled proteins are S2, *S4*, S18, *S21*, and L13. These proteins in the pactamycin binding site are probably related to the initiation step of protein synthesis.

The study of the mode of action of antibiotics has the 2-fold interest of opening up possibilities to potentiate their therapeutic activity and of providing important data that can help us to understand the structure and activity of their biological targets.

Pactamycin, a drug initially developed as a potential antitumor agent, is especially interesting as an inhibitor of protein synthesis in eukaryotic and prokaryotic systems (Goldberg, 1974). It blocks the initial steps of the protein synthesis process [for a review, see Vázquez (1979)] either by interfering with the association of the large ribosomal subunit with the 40S initiation complex or by blocking the ribosome after the formation of the first peptide bond (Kappen & Goldberg, 1976). In any case, the drug binds preferentially to the small subunit and has lower affinity for the whole riosome and practically none for the large subunit when tested at low inhibitory concentrations (McDonald & Goldberg, 1970).

The identification of the ribosomal components involved in the antibiotic binding site is an important step in understanding its mode of action. Affinity labeling techniques are probably the most direct method to approach this problem and, in fact, have been used fruitfully in the case of many ribosomal effectors (Cooperman, 1980). The applicability of this technique is usually restricted to cases where radioactively labeled and chemically reactive derivatives are available. Pactamycin, having in its molecule a potentially photoactive (Cowan & Drisko, 1978) acetophenone group, as well as a phenolic group susceptible to radioactive iodination, has the appropriate conditions for the identification of the components of its ribosomal binding site using photoaffinity procedures.

The identification of these components in *Escherchia coli* ribosomes would allow us, considering the activity of the drug, to establish correlations between ribosomal components involved, directly or indirectly, in the initiation step of protein synthesis.

Materials and Methods

Iodination of Pactamycin. Pactamycin (25–50 μg) in 50 mM phosphate, pH 7.5, and 50% ethanol was treated with 0.5–0.6 mCi of Na^{125}I by a modification of the chloramine T method that avoids direct contact between the oxidant compound and the drug, as reported previously (Tejedor & Ballesta, 1982).

After treatment, the reaction mixture was separated by an LH20 Sephadex column (0.9 × 20 cm), equilibrated, and eluted with 96% ethanol. Fractions (0.5 mL) were collected and checked in a γ counter. The fractions containing the drug were pooled, evaporated, dissolved in 200 μL of Cl_2CH_2, and

† This work has been supported by institutional grants from the Fondo de Investigaciones Sanitarias (FIS) and by personal grants from the Comision Asesora de Investigación Científica y Técnica (Spain).

0006-2960/85/0424-3667$01.50/0