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Isolation of the mitochondrial benzodiazepine receptor: Association with the voltage-dependent anion channel and the adenine nucleotide carrier

(isoquinoline carboxamide/flunitrazepam/PK11195/PK14105/porphyrins)

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ABSTRACT **The mitochondrial benzodiazepine receptor (mBzR) has been solubilized with retention of reversible ligand binding, and the associated subunits were characterized. mBzR comprises immunologically distinct protein subunits of 18-, 30-, and 32-kDa. The 18-kDa protein is labeled by the isoquinoline carboxamide mBzR ligand [^{3}H]PK14105, whereas the 30- and 32-kDa subunits are labeled by the benzodiazepine (Bz) ligands [^{3}H]flunitrazepam and [^{3}H]AHN-086. Selective antibodies and reagents identify the 32- and 30-kDa proteins as the voltage-dependent anion channel (VDAC) and the adenine nucleotide carrier (ADC), respectively. While isoquinoline carboxamide and Bz ligands target different subunits, they interact allosterically, as the binding of Bz and isoquinoline carboxamide ligands is mutually competitive at low nanomolar concentrations. Moreover, eosin-5-maleimide and mercuric chloride inhibit [^{3}H]PK11195 binding to the intact receptor via sulfhydryl groups that are present in ADC. VDAC and ADC, outer and inner mitochondrial membrane channel proteins, respectively, together with the 18-kDa subunit, may comprise mBzR at functionally important transport sites at the junction of two mitochondrial membranes.**

Benzodiazepine (Bz) drugs exert their antianxiety, sedative, and anticonvulsant effects through a recognition site on type-A γ-aminobutyric acid receptors (1, 2) in the central nervous system. A distinct "peripheral" Bz receptor in a variety of tissues has the same affinity for diazepam as the central receptor, but differs in its affinity for other drugs (3–5), including the isoquinoline carboxamides PK11195 [1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)isoquinoline-3-carboxamide] and PK14105 [1-(2-fluoro-5-nitrophenyl)-3-isoquinoline carboxylic acid] (6, 7). The peripheral-type receptor is present in kidney (8), adrenal gland, testes (9), and olfactory neurons in the nasal epithelium and olfactory bulb (10). Bz and isoquinolines with high affinity for the peripheral receptors influence numerous physiological processes, including cholesterol transfer in the adrenal gland associated with mitochondrial adrenal steroid synthesis (11–13) and mitochondrial respiration (14, 15). The peripheral receptor, localized to mitochondria by subcellular fractionation (16, 17), is also designated the mitochondrial Bz receptor (mBzR).

The possibility that a complex of proteins comprises mBzR stems from its 34-kDa (18) and 21- to 23-kDa (19) molecular masses observed in radiation analysis as well as the 220-kDa value determined by gel filtration in the presence of digitonin (19). An 18-kDa mitochondrial protein labeled with [^{3}H]PK14105 has been purified and cloned (20, 21). By contrast, the Bz [^{3}H]flunitrazepam ([^{3}H]FNZ) covalently labels a 32-kDa protein which, in preliminary experiments, appeared to represent the voltage-dependent anion channel (VDAC) of mitochondria (22). AHN-086 [1-(2-isothiocyanatoethyl)-7-chloro-1,3-dihydro-5-(4-chlorophenyl)-2*H*-1,4-benzodiazepine-2-one hydrochloride], an isocyanate derivative of Ro 5-4864 [7-chloro-1,3-dihydro-1-methyl-5-(*p*-chlorophenyl)-2*H*-1,4-benzodiazepine-2-one], an mBzR-selective Bz, covalently labels both 32- and 30-kDa proteins in crude mitochondrial membranes (23, 24). We now report purification of the mBzR, which retains the ability to bind both reversible and covalent ligands. We present evidence that the functional receptor requires VDAC (25) as well as the adenine nucleotide carrier (ADC) (26).

MATERIALS AND METHODS

Unless noted, all reagents were obtained from Sigma. The radiolabeled ligands [^{3}H]Ro 5-4864 (73.8 Ci/mmol; 1 Ci = 37 GBq), [^{3}H]PK11195 (79 Ci/mmol), [^{3}H]FNZ (86.1 Ci/mmol), and [^{3}H]AHN-086 (85 Ci/mmol) were from NEN/DuPont; [^{3}H]PK14105 (87 Ci/mmol) was from Centre d'Etudes Nucleaires, Gif-sur-Yvette, France; unlabeled Bz was a gift of William Scott (Hoffmann–La Roche); and unlabeled PK11195 was from G. Le Fur (Pharmuka Laboratories, Gennevilliers, France). Anti-ADC antiserum was a gift from Hartmut Wolhrab, Boston Biomedical Institute; dodecyl β-D-maltoside (DM) and hydroxylapatite (HA) were from Calbiochem; crude asolectin was from Associated Concentrates (Woodside, NY); Centriprep-30 concentrators were from Amicon; goat anti-rabbit IgG-conjugated alkaline phosphatase was from Boehringer Mannheim; Immobilon type-P filters were from Millipore; low molecular weight prestained standards were from GIBCO/BRL; adult, male Sprague–Dawley rats were from Harlan–Sprague–Dawley.

Preparation of Kidney Mitochondria and Urea-Treated Mitochondria (U-mito). After the rats were decapitated, their kidneys were removed, placed in ice-cold H medium (220 mM D-mannitol/70 mM sucrose/2 mM Hepes, pH 7.4/defatted bovine serum albumin at 0.5 mg/ml) plus 1 mM EGTA, and dissected to remove fatty capsule and fibrous tissue. Kidney mitochondria were prepared by the method of

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Abbreviations: mBzR, mitochondrial benzodiazepine receptor; U-mito, urea-treated mitochondria; Bz, benzodiazepine(s); VDAC, voltage-dependent anion channel; ADC, adenine nucleotide carrier; HA, hydroxylapatite; DM, dodecyl maltoside; Ro 5-4864, 7-chloro-1,3-dihydro-1-methyl-5-(*p*-chlorophenyl)-2*H*-1,4-benzodiazepine-2-one; AHN-086, 1-(2-isothiocyanatoethyl)-7-chloro-1,3-dihydro-5-(4-chlorophenyl)-2*H*-1,4-benzodiazepine-2-one hydrochloride; PK11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)isoquinoline-3-carboxamide; PK14105, 1-(2-fluoro-5-nitrophenyl)-3-isoquinoline carboxylic acid; DCCD, dicyclohexylcarbodiimide; FNZ, flunitrazepam.

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Wehrle *et al.* (27). Mitochondria were either used immediately or stored at a protein concentration of 50 mg/ml at −70°C. To prepare U-mito, mitochondria were diluted to a protein concentration of 2 mg/ml in 20 mM potassium phosphate, pH 6.5/20 mM potassium chloride/4 M urea in a final volume of 3 ml and incubated on ice for 30 min, whereupon 21 ml of 20 mM potassium phosphate, pH 6.5/200 mM potassium chloride was added. Samples were centrifuged at 210,000 × *g* for 75 min. The resulting U-mito membrane pellet was resuspended in half its original volume in buffer B (25 mM Tris, pH 7.7/5 mM EDTA/40 potassium chloride/2.5% ethylene glycol) and used immediately.

Solubilization and Fractionation of mBzR. U-mito were diluted to 2.5 mg of protein per ml in buffer B plus 0.5% DM, solubilized for 15 min on ice, and centrifuged for 60 min at 230,000 × *g*. The soluble fraction was removed, diluted by immediately adding a 4-fold volume of buffer B, and purified by adsorption to HA. Soluble mBzR was diluted with water (1:1, vol/vol) and poured over multiple HA columns (previously washed with 5 ml of 0.5× buffer B) at a ratio of 4 ml per 1.6 g of HA, and the columns were washed in a batch-wise fashion with increasing concentrations (0.5–1.5 M) of potassium phosphate, pH 6.5, in 0.5× buffer B. The fraction with the highest specific activity, corresponding to 1.2 M potassium phosphate, was concentrated 10-fold by Centriprep-30 concentration and stored at −70°C until use. The concentrated mBzR was chromatographed on a FPLC Superose-12B sizing column in the presence of 0.5× buffer B.

Photoaffinity and Covalent Labeling of mBzR. Rat kidney mitochondria and other mBzR fractions were incubated with 20 nM [^{3}H]PK14105 or 100 nM [^{3}H]FNZ (see figure legends for protein concentration) for 30 min in the dark on ice. Samples were then exposed to UV light (254 nm) for 45 min and then quenched with 5× Laemmli sample buffer (15% SDS/0.825 M sucrose/0.325 M Tris·HCl, pH 6.8/5% 2-mercaptoethanol/0.002% bromophenol blue). After covalent labeling with 10 nM [^{3}H]AHN-086 on ice for 60 min, the samples were quenched as above. Nonspecific binding was determined in the presence of 10 μM unlabeled drug. [^{14}C]Dicyclohexylcarbodiimide ([^{14}C]DCCD) labeling (28) and covalent modification with eosin-5-maleimide (29) were as reported.

Bands incorporating the radiolabeled drugs were determined by two procedures. In the first procedure, after resolution on SDS/PAGE (30), gel strips were cut into 2-mm slices, dissolved overnight at 60°C in 0.5 ml of H_2O_2 (37% stock), and assayed directly in 4 ml of formula 963 plus 5% acetic acid. The second procedure involved the fluorography of Immobilon filters previously developed for antigenic identification, permitting accelerated detection of tritiated proteins and establishing the coidentity of antigen and radiolabeled protein. Dried Immobilon filters were soaked in 100% methanol for 10 sec, transferred to a glass tray, soaked for 60 sec in 100% dimethyl sulfoxide, and incubated with a solution of 20% 2,5-diphenyloxazole in 100% dimethyl sulfoxide (31) for 1 min with constant shaking. The opaque filters were then submerged twice in 3 liters of water to precipitate the 2,5-diphenyloxazole, dried, and exposed to film.

Antibodies Against the 32-kDa Protein (VDAC). Highly purified mBzR (1.2 M potassium phosphate fraction from the HA column) was used to immunize rabbits. The immune and preimmune sera were characterized for their reactivity against total rat kidney mitochondria, purified mBzR, and rat kidney VDAC prepared by standard procedures (32). For immunological detection of transferred proteins, antisera were diluted in TTBS (20 mM Tris, pH 7.4/500 mM NaCl/0.05% Tween 20) plus 3% bovine serum albumin and incubated with protein transferred to Immobilon P filters for 2 hr at room temperature. After extensive washing in TTBS and incubation with anti-rabbit alkaline phosphatase secondary antiserum (1:1500) for 1 hr at room temperature, the blot was developed by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate or ^{125}I-labeled protein A (33).

Other Methods. One-dimensional gel electrophoresis was as described (26), and protein was visualized by Coomassie or silver staining (34). Two-dimensional, nonequilibrium gel electrophoresis was as described (35). Membrane (36) and soluble (37) proteins were determined with bovine serum albumin as a standard. Asolectin was prepared in 0.05% DM (38). Radioligand binding was carried out on ice in a total volume of 200 μl, which contained 50 mM Tris and the tritium-labeled drug at 1 nM (with the exception of [^{3}H]FNZ, which was at 100 nM). Nonspecific binding was determined in the presence of 10 μM unlabeled ligand. The final concentration of detergent was <0.05% in all cases, because higher concentrations of DM potently inhibited ligand binding. Samples were filtered over glass-fiber filters (Whatman GF/B) as described (39).

RESULTS

Purification of mBzR and Assignment of Ligand Binding Sites. The ability of mBzR to reversibly bind ligands is lost following treatment with numerous detergents, but is retained to a limited extent following solubilization with digitonin (39). We have successfully solubilized mBzR with DM, which solubilizes, in active form, membrane proteins containing multiple subunits (40, 41). Whole mitochondria were treated with urea to remove peripheral proteins, providing about a 3-fold enhancement in specific [^{3}H]PK11195 binding (Table 1). Solubilization with DM yielded a further 2-fold purification with retention of 80% of [^{3}H]PK11195 binding of the intact mitochondria. Fractionation of the solubilized extract on HA columns provided a further 10-fold enhanced specific activity in the fraction eluted with 1.2 M salt. This fraction is enriched 60-fold in [^{3}H]PK11195 binding compared to the original mitochondria, with >50% recovery of total binding activity of the original mitochondria, and comprises major proteins of 32 and 30 kDa and a faintly stained 18-kDa protein (Fig. 1*A*). The elution pattern of mitochondrial membrane proteins from HA in the presence of DM differs markedly from methods that employ Triton X-100 (32) or other detergents (42).

To examine the role of the protein subunits of the mBzR complex in ligand recognition, we photolabeled the 1.2 M potassium phosphate HA eluate with specific Bz ligands (Fig. 1*B*). [^{3}H]PK14105 labeled an 18-kDa band. [^{3}H]FNZ was associated with the 32-kDa band, whereas [^{3}H]AHN-086 labeled both the 32- and 30-kDa bands, with more label on the 30-kDa protein. Chromatography of the purified preparation on Superose-12 revealed a single sharp peak of protein (data not shown) coincident with the peak binding of [^{3}H]PK11195, [^{3}H]PK14105, and [^{3}H]Ro 5-4864 (Fig. 1*C*). The

Table 1. Purification of mBzR from rat kidney mitochondria

Fraction	Protein, mg	% total activity	Specific activity, pmol/mg	Fold purification
Mitochondria	330	100	18	—
U-mito	119	100	50	2.8
Soluble Bz	49	91	110	6.1
HA (1.2 M salt)*	3.2	59	1100	60

This procedure was carried out more than 20 times with <5% variation. Crude asolectin (15:1, wt/wt), which markedly stabilized ligand binding to the purified samples (data not shown), was routinely included.

*K_d values of the highly purified mBzR for [^{3}H]PK11195, [^{3}H]Ro 5-4864, and [^{3}H]PK14105 were 15, 25, and 10 nM, respectively. The binding of [^{3}H]PK11195 and [^{3} H]Ro 5-4684 to mBzR was inhibited by protoporphyrin IX with an IC_{50} of 250 nM.

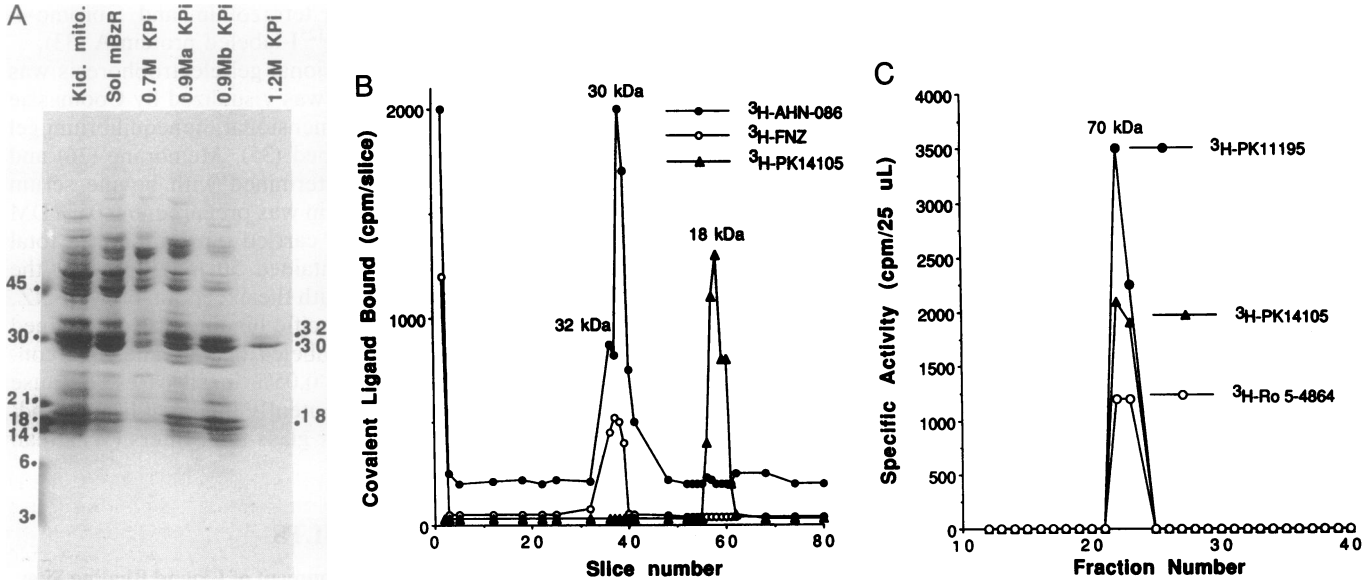


FIG. 1. (*A*) SDS/PAGE profile of purified mBzR. The mBzR was purified from rat kidney according to *Materials and Methods*. Representative fractions were resolved by SDS/PAGE on a 10–20% acrylamide gradient gel. Kid. mito., crude kidney mitochondria (150 μg); Sol mBzR, soluble mBzR (100 μg); 0.7M KPi, 0.7 M potassium phosphate fraction (75 μg); 0.9Ma KPi, 0.9 M potassium phosphate fraction (90 μg); 0.9Mb KPi, 0.9 M potassium phosphate fraction (80 μg); 1.2M KPi, 1.2 M potassium phosphate fraction (15 μg). (*B*) Identification of ligand binding subunits. The concentrated mBzR was labeled with [^{3}H]PK14105, [^{3}H]FNZ, and [^{3}H]AHN-086. Samples were resolved on 12% polyacrylamide gels, sliced, and assayed. Total cpm per slice are plotted to correspond to a Coomassie blue-stained control sample. (*C*) Gel-filtration of purified mBzR. Concentrated mBzR was applied to a 10-cm Superose-12 FPLC column and resolved in the presence of 0.5× buffer B plus 0.05% DM. The resulting fractions were assayed for recovery of active mBzR according to standard filter binding procedures in the presence of crude asolectin (30 μg/ml) prepared according to the method of McEnery *et al.* (38) with the addition of 0.05% DM. The column was calibrated with the following standards: alcohol dehydrogenase (150 kDa), BSA (66 kDa), and cytochrome *c* (12.5 kDa).

calculated Stokes radius is consistent with a native molecular mass for the purified mBzR of 70 kDa.

Identification of 32-kDa Bz Binding Protein as VDAC and the 30-kDa Protein as ADC. [^{3}H]FNZ labeling of intact kidney mitochondria can be recovered almost entirely in purified VDAC (Fig. 2*A*) (22, 32). Antiserum raised against purified mBzR identified a single 32-kDa protein in crude mitochondrial membranes with the same mobility as the [^{3}H]FNZ-labeled protein (Fig. 2*B*). The protein(s) that reacted with this antibody can be resolved by two-dimensional electrophoresis into six isoforms, presumably resulting from post-translational modification, such as phosphorylation (Fig. 2*B*). This constellation of proteins is identical to the 32-kDa [^{14}C] DCCD-labeled VDAC proteins (32, 42, 43) (Fig. 2*B*), indicating that the anti-32-kDa antibody reacts with all possible forms of kidney VDAC. Western blot analysis using antibodies selective for ADC (44) and VDAC (anti-32-kDa antiserum) revealed the copurification by HA chromatography of ADC and VDAC and binding sites for [^{3}H]PK14105 and [^{3}H]FNZ (Fig. 3).

Functional Association of VDAC, ADC, and 18-kDa PK11195 Binding Protein. At short intervals, [^{3}H]PK14105 labels only a single 18-kDa band in crude kidney and liver mitochondria (Fig. 4), whereas after several hours additional labeling is apparent in a 32-kDa band (45). At 2 hr, in the kidney, the 18-kDa band retains the great bulk of radioactivity, whereas in the liver the 32-kDa band is almost as intense as the 18-kDa band. The 32-kDa bands labeled with

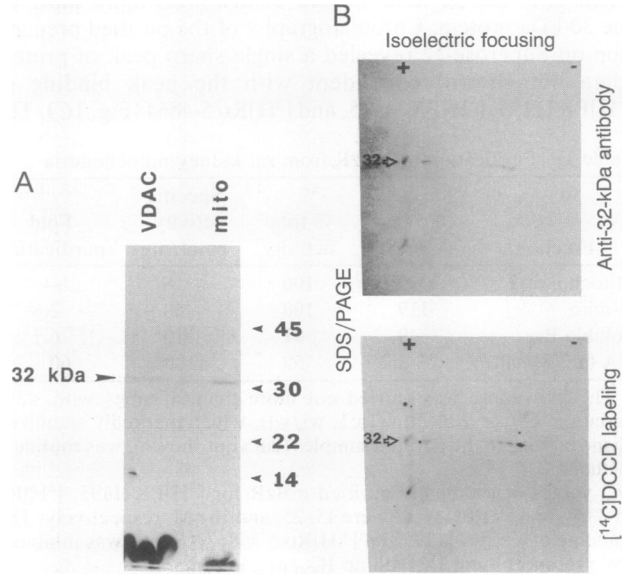


FIG. 2. Identification of the 32-kDa component of mBzR. (*A*) The [^{3}H]FNZ-labeled band is VDAC. Purified rat kidney mitochondria (2 mg/ml) were photolabeled with 100 nM [^{3}H]FNZ, the reaction was immediately quenched with 10 μM cold Ro 5-4864, and mitochondria (mito) were centrifuged at 210,000 × *g* for 30 min. The resulting pellet was resuspended in 20 mM KCl/20 mM potassium phosphate, pH 6.5, and extracted with 3% Triton X-100, and VDAC was purified over HA according to published procedures (32). Samples were resolved by electrophoresis on 12% PAGE gels and transferred overnight to Immobilon filters. Lane 1, autoradiogram of [^{3}H]FNZ-labeled VDAC (40 μg); lane 2, autoradiogram of [^{3}H]FNZ-labeled kidney mitochondria (100 μg). (*B*) Crossreactivity of [^{14}C] DCCD-labeled proteins with anti-32-kDa antibody. Rat kidney mitochondria were labeled overnight at 4°C with 10 nM [^{14}C]DCCD and centrifuged at 210,000 × *g*; the resulting pellet was resuspended in 50 mM Tris (pH 7.5) at a protein concentration of 5 mg/ml. [^{14}C]DCCD-labeled mitochondria (25 μl) were resolved on nonequilibrium isolectric focusing tube gels in the presence of pH 3–10 Ampholytes for 3 hr. The tube gels were then carefully affixed to 12% PAGE gels and resolved overnight. The gels were transferred to Immobilon, incubated with anti-32-kDa antiserum (*Upper*), followed by treatment with 2,5-diphenyloxazole/dimethyl sulfoxide and then fluorographed (*Lower*).

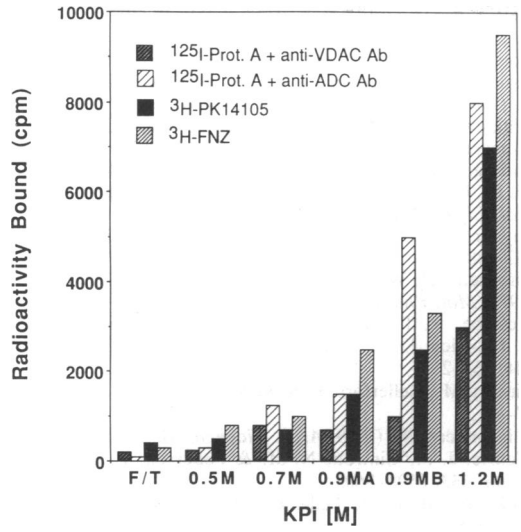


FIG. 3. Copurification of VDAC, ADC, and ligand binding sites by HA chromatography. Soluble mBzR were photolabeled with [^{3}H]PK14105 and [^{3}H]FNZ and then resolved by HA chromatography. Aliquots (20 μl) were assayed after filtration to remove unbound ligand. Also, resulting HA fractions were resolved by SDS/PAGE, transferred to Immobilon filters, and probed with anti-VDAC and anti-ADC antibodies (Ab), followed by reaction with ^{125}I-labeled protein A (^{125}I-Prot. A) secondary antibody. The filters were sliced and assayed directly in a GammaRIA counter, and the quantity of ^{125}I-labeled protein A bound per lane was plotted per HA fraction.

[^{3}H]PK14105 in kidney and liver correspond closely to the 32-kDa band labeled with [^{3}H]FNZ and that stains with the anti-VDAC antibody. The 65-kDa band is believed to be a dimer of VDAC. The transfer of radiolabel from the 18-kDa to the 32-kDa band suggests that these two protein subunits are closely associated in mBzR, permitting migration of the label between the two subunits.

Evidence that the 18-kDa protein cannot of itself provide ligand binding comes from an examination of the role of sulfhydryl groups. The cloned 18-kDa protein contains no cysteine (20). Eosin-5-maleimide and mercury are protein-modifying reagents that react with reduced sulfhydryl groups of cysteine. [^{3}H]Ro 5-4864 binding is abolished by both reagents, whereas eosin-5-maleimide and mercury maximally reduce [^{3}H]PK11195 binding 80% and 40%, respectively (Fig. 5*A*). Eosin itself has no influence on ligand binding (data not shown). In crude mitochondria, eosin-5-maleimide covalently labels the 30-kDa ADC, the 31-kDa phosphate carrier, and larger molecular mass proteins (Fig. 5*B*, lane 1; ref. 29). In the purified mBzR preparation, only the ADC

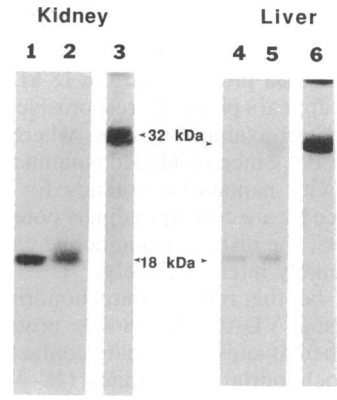
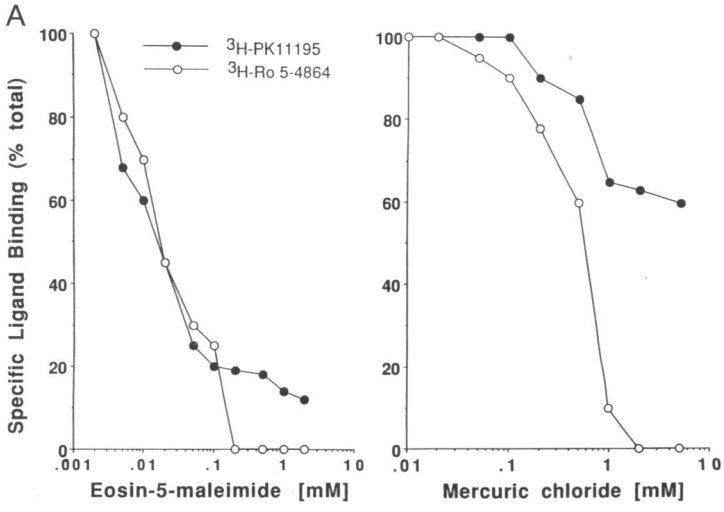


FIG. 4. [^{3}H]PK14105 photolabels both the 18-kDa isoquinoline carboxamide binding protein and VDAC. Purified rat kidney and liver mitochondria were photolabeled with 20 nM [^{3}H]PK14105 and 100 nM [^{3}H]FNZ at a protein concentration of 1 mg/ml for stated lengths of time and then quenched with 5× Laemmli sample buffer. The samples (100 μl) were resolved on 12% PAGE gels, transferred to Immobilon filters, fluorographed, and exposed to film. Kidney mitochondria (lanes 1–3) and liver mitochondria (lanes 4–6) were labeled with [^{3}H]PK14105 for 1 hr (lanes 1 and 4) or for 2 hr (lanes 2 and 5) or were labeled with [^{3}H]FNZ for 1 hr (lanes 3 and 6). The bands at the top of lanes 3 and 6 are aggregates or dimers of VDAC.

protein is modified (Fig. 5*B*, lane 2). The pronounced effects of mercury and eosin-5-maleimide at concentrations that interact with cysteine indicate that the 18-kDa protein cannot of itself account for ligand binding to mBzR in kidney or adrenal mitochondria (data not shown) and that ADC is required for ligand binding.

DISCUSSION

Here, we provide evidence for a functional requirement of VDAC, ADC, and the 18-kDa PK11195 binding subunit in the intact mBzR: (*i*) The purified mBzR contains VDAC and ADC along with the 18-kDa component; and all three proteins, despite differing molecular masses, copurify and migrate as a single sharp peak upon gel chromatography. (*ii*) The 32-kDa FNZ binding site is identical to VDAC; furthermore, binding of the Bz AHN-086 involves both VDAC and ADC, which either contains Bz binding sites or is in close proximity to a Bz binding protein. (*iii*) Sulfhydryl reagents potently inhibit ligand binding to mBzR; the Bz binding site is more sensitive than the PK11195 binding site. Eosin-5-maleimide, a potent inhibitor of ligand binding, interacts selectively with ADC in purified mBzR. (*iv*) [^{3}H]PK14105,



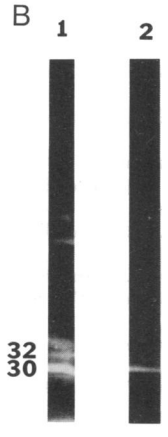


FIG. 5. Sulfhydryls in mBzR. (*A*) Effect of sulfhydryl reagents on ligand binding to mBzR. Purified rat kidney mitochondria were incubated for 30 min on ice with stated concentrations of eosin-5-maleimide and mercuric chloride and assayed for ligand binding. (*B*) Eosin-5-maleimide labels ADC in mBzR. Rat kidney mitochondria (100 μg; lane 1) and purified mBzR (20 μg; lane 2) were incubated, respectively, with 10 μM eosin-5-maleimide in the dark on ice according to published procedures (29). The reaction was terminated by the addition of 5× Laemmli sample buffer, and the samples were resolved on 12% PAGE gels. The gels were then visualized with a 260/280 nm transilluminator and photographed. The labeling was completely prevented by 1 mM dithiothreitol (data not shown).

which initially targets the 18-kDa subunit, migrates to a 32-kDa component, indicating a close association of VDAC, the presumed 32-kDa protein, and the 18-kDa subunit. The 18-kDa protein appears primarily responsible for recognition of isoquinoline carboxamide ligands, whereas VDAC and ADC recognize Bz. Since unlabeled isoquinoline carboxamides compete with nanomolar potency for [^{3}H]Ro 5-4864 binding and since Bz are correspondingly potent in competing for [^{3}H]PK11195, the distinct isoquinoline carboxamide and Bz sites presumably interact closely.

What might be the role in mitochondrial function for mBzR, comprising VDAC, the 18-kDa protein, and ADC? VDAC is enriched at sites of intimate contact between inner and outer mitochondrial membranes (46–48), which may provide a channel comprising ADC and VDAC for substrates such as ATP (49, 50). Since hexokinase binds to VDAC at the outer mitochondrial membrane (51), ATP likely passes through ADC and VDAC at these junctions. Similarly, mBzR is associated with the transport of cholesterol into mitochondria for adrenal steroid biosynthesis (52) and with porphyrin binding. The 18-kDa PK11195 binding protein shares homology with the CrtK protein of *Rhodobacter capsulatus* (53), which is implicated in carotenoid synthesis and transport (54) and is proposed to recognize porphyrins. Porphyrins are the most potent known endogenous ligands for mBzR (55, 56). Porphyrin transport in and out of mitochondria provides substrates for cytochrome biosynthesis and other porphyrin-containing proteins essential for mitochondrial integrity. We propose that a ternary complex of the 18-kDa protein, VDAC, and ADC forms a transport assembly, which interacts with both endogenous and Bz ligands mediating multiple cellular functions (57–60).

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