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Identification of the Melatonin-binding Site MT_3 as the Quinone Reductase 2*

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The regulation of the circadian rhythm is relayed from the central nervous system to the periphery by melatonin, a hormone synthesized at night in the pineal gland. Besides two melatonin G-coupled receptors, mt_1 and MT_2, the existence of a novel putative melatonin receptor, MT_3, was hypothesized from the observation of a binding site in both central and peripheral hamster tissues with an original binding profile and a very rapid kinetics of ligand exchange compared with mt_1 and MT_2. In this report, we present the purification of MT_3 from Syrian hamster kidney and its identification as the hamster homologue of the human quinone reductase 2 (QR_2, EC 1.6.99.2). Our purification strategy included the use of an affinity chromatography step which was crucial in purifying MT_3 to homogeneity. The protein was sequenced by tandem mass spectrometry and shown to align with 95% identity with human QR_2. After transfection of CHO-K1 cells with the human QR_2 gene, not only did the QR_2 enzymatic activity appear, but also the melatonin-binding sites with MT_3 characteristics, both being below the limit of detection in the native cells. We further confronted inhibition data from MT_3 binding and QR_2 enzymatic activity obtained from samples of Syrian hamster kidney or QR_2-overexpressing Chinese hamster ovary cells, and observed an overall good correlation of the data. In summary, our results provide the identification of the melatonin-binding site MT_3 as the quinone reductase QR_2 and open perspectives as to the function of this enzyme, known so far mainly for its detoxifying properties.

Melatonin, a neurohormone produced at night in the pineal gland, is suspected to relay to the peripheral organs the circadian rhythm detected by the central nervous system. Several high affinity melatonin receptors have been identified to date, among which the mt_1 (1) and MT_2 (2) receptors have been cloned from human tissues. The pharmacology of these two receptors is well documented, and several compounds, including melatonin, are ligands with picomolar binding affinity (for review, see Ref. 3). Another putative melatonin receptor was identified on pharmacological grounds, with lower melatonin affinity (nanomolar range), very rapid ligand association/dissociation kinetics, and an original pharmacological profile (4–6). In line with mt_1 and MT_2 receptors, this putative receptor was named MT_3, according to the nomenclature recommendations of the IUPHAR (7). So far, the known inhibitors of MT_3 hardly reach the nanomolar range and encompass an unusually large structural diversity of highly hydrophobic cyclic or polycyclic compounds (Refs. 5 and 6, and for review, see Ref. 3).[1] All pharmacological investigations on mt_1, MT_2, and MT_3 were performed using the radioligand [^{125}I]melatonin, a ligand with high affinity for mt_1 and MT_2 (K_d = 10–200 pM) and with lower affinity for MT_3 (K_d = 3–9 nM). The hamster kidney, liver, and brain have been used as model tissues for MT_3 pharmacological studies, and our recent data confirmed that among a wide range of mammals, this rodent was indeed the best source of MT_3.[1] Hence, the binding specificity for [^{125}I]melatonin competition studies on MT_3 is achieved by preparing material from hamster tissues, and the fast dissociation kinetics is overcome by operating at 4 °C. In addition, iodination of the known very specific MT_3 inhibitor, 5-methoxycarbonylamino-*N*-acetyltryptamine (MCA-NAT),[2] paved the way to more accurate and reliable investigations on MT_3 (5). This ligand, combined with recently improved operating conditions, made possible for us to perform specific MT_3 pharmacological studies at room temperature.[1] Confident in the interest of discovering novel melatoninergic pharmacological targets, we recently set up a biochemical approach to identify and characterize MT_3. The present report describes a specific purification procedure of MT_3 from hamster kidney, which led to a homogeneous single protein of 26 kDa, identified by tandem mass spectrometry as a homologue of the human quinone reductase 2 (QR_2, EC 1.6.99.2). This identification was confirmed by confronting MT_3 pharmacological and QR_2 enzymatic data obtained under different cellular and biochemical conditions corresponding to MT_3 or QR_2 typical conditions. The quinone reductase family comprises two isoforms, QR_1 and QR_2, which have been sequenced (8, 9) and crystallized (10, 11). QR_2 lacks a 47-amino acid C-terminal sequence present in QR_1, resulting in a differ-

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[1] O. Nosjean, J. P. Nicolas, F. Klupsch, P. Delagrange, E. Canet, and J. A. Boutin, submitted for publication.

[2] The abbreviations used are: MCA-NAT, 5-methoxycarbonylamino-*N*-acetyltryptamine; QR_2, quinone reductase 2; OG, octylglucopyranoside; BNAH, dihydrobenzylnicotinamide; DCM, dichoromethane; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; S20098, agomelatine; S26553, *N-methyl*-{1-[2-(acetylamino)ethyl]naphthalen-7-yl}-carbamate; S27145, *N*-[2-(7-amino-1-naphthyl)ethyl]-acetamide.

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TABLE I

Purification of hamster kidney MT_3

Purification of hamster kidney MT_3 was monitored by ^{125}I-MCA-NAT binding, QR_2 enzymatic activity (menadione/BNAH in the presence of dicoumarol), and QR_1 activity (menadione/NADH). Briefly, the tissue homogenate in 0.2 M sucrose (H) was centrifuged at low speed to remove nuclei and unbroken cells (P_1), and the resulting supernatant was incubated in mild detersive conditions (5 mM OG, fraction S1 + OG) in order to separate cytoplasm and loosely membrane-associated material (S_{OG}) from membranes (P_{OG}). The cytoplasm-enriched fraction was dialyzed and fractionated by DEAE ion-exchange chromatography. The MT_3-enriched fractions were pooled and concentrated (DEAE) before final purification by affinity chromatography (affinity). Data are representative of three independent purifications performed with five hamster kidneys as starting material. The affinity eluates were used for activity assays and electrophoresis analysis. The protein content was estimated by densitometry of the electrophoregram in the presence of a bovine serum albumin quantity calibration. Binding data are expressed as fmol/mg (specific binding) or fmol (total binding) of bound ligand. Enzymatic data are expressed as nmol/min/mg (specific activity) or nmol/min (total activity).

Purification step	MT_3 binding assay: ^{125}I-MCA-NAT binding			QR_2 activity assay: menadione/BNAH/dicoumarol activity			QR_1 activity assay: menadione/NADH activity		
	Specific binding	Purification factor	Total binding	Specific activity	Purification factor	Total activity	Specific activity	Purification factor	Total activity
	fmol/mg		*fmol*	*nmol/min/mg*		*nmol/min/mg*	*nmol/min*		*nmol/min*
H	68	1.0	24,870	9.0	1.0	3,278	6.4	1.0	2,330
P_1	73	1.1	7,276	1.6	0.2	157	9.6	1.5	961
S_1 + OG	142	2.1	29,171	14.7	1.6	3,014	15.9	2.5	3,266
P_{OG}	46	0.7	3,164	2.8	0.3	194	12.7	2.0	1,583
S_{OG}	240	3.5	30,006	21.2	2.4	2,646	26.3	4.1	1,808
DEAE	935	13.7	18,512	112	12.5	2,220	0.8	0.1	15
Affinity	702,238	10,312	3,792	90,785	10,115	490	0	0	0

TABLE II

MT_3 pharmacological competitions and QR_2 enzymatic inhibitions

MT_3 pharmacological competitions and QR_2 enzymatic inhibitions on hamster kidney (hamster MT_3/QR_2) and CHO-QR_2 (human QR_2) nuclei-free fractions. The samples were prepared as described under "Materials and Methods," and dialyzed against 20 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$ in order to remove the sucrose. The hamster MT_3/QR_2 sample corresponded to the S_1 fraction of the purification procedure. Data are expressed as K_i (nM) for binding measurements and, since the type of enzymatic competition of each compound was unknown, as IC_{50} (μM) for enzymatic measurements. Menadione and BNAH enzymatic data are represented as K_m (μM). All values are the means of triplicate experiments reproduced three to six times.

	MT_3 binding: ^{125}I-MCA-NAT binding, K_i (nM)		QR_2 activity: Menadione/BNAH/dicoumarol activity, IC_{50} or K_m (μM)	
	Hamster MT_3/QR_2	Human QR_2	Hamster MT_3/QR_2	Human QR_2
Menadione	4,520 ± 853	2,338 ± 861	25.2 ± 2.6 (K_m)	8.9 ± 0.11 (K_m)
BNAH	86 ± 9.9	334 ± 124	60.8 ± 5.1 (K_m)	57.6 ± 4.1 (K_m)
Dicoumarol	2,852 ± 756	1,686 ± 590	566 ± 12	620 ± 26
Estradiol	601 ± 97	3,090 ± 253	>10,000	>10,000
2-Iodomelatonin	8.3 ± 2.3[a]	11.0 ± 2.8	1.2 ± 0.3	29 ± 2.3
S26553	3.0 ± 1.0[a]	62.4 ± 16.4	2.8 ± 1.8	41.4 ± 3.7
MCA-NAT	81 ± 19[a]	642 ± 211	43.5 ± 7.0	376 ± 68
N-Acetylserotonin	146 ± 13.5	207 ± 19	92 ± 34	204 ± 44
Melatonin	277 ± 22[a]	382 ± 99	43 ± 12	246 ± 47
Luzindole	1,156 ± 304[a]	>10,000	>10,000	>10,000
Scrotonin	3,563 ± 1,067	>10,000	>10,000	2,933 ± 384
Tryptamine	>10,000	>10,000	>10,000	>10,000

[a] These data are part of a previous report (Footnote 1).

ent substrate specificity. It is noteworthy that the literature on QR_2 enzymology is rather scarce (12–14). Interestingly, QR_2 was originally discovered in 1962 as a flavoenzyme (12), later re-discovered as the QR_1-related enzyme (14), and was recently found again in porcine kidney as a puromycin aminonucleoside-binding protein (15). We now unveiled a new facet of QR_2 as the melatonin-binding site MT_3, opening new perspectives in melatonin investigations as well as in quinone reduction studies.

EXPERIMENTAL PROCEDURES

Materials

[^{125}I]MCA-NAT (2200 Ci/mmol) was custom synthesized by Amersham Pharmacia Biotech (Orsay, France). 2-Iodomelatonin and MCA-NAT were purchased from Tocris (Bioblock, Illkirch, France), dihydrobenzylnicotinamide was obtained from Maybridge (Interchim, Montluçon, France), and all other reagents were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France).

Partial Purification of MT_3

Frozen hamster kidneys were obtained from Charles River Breeding Laboratories (Saint Aubin les Elbeuf, France). The tissues were thawed, chopped, and added to 5 ml/g of homogenization buffer (50 mM Tris-HCl, pH 7.5, 0.2 M sucrose, 1 mM $CaCl_2$, and Complete™ mixture of protease inhibitors). The cells were gently disrupted using a Dounce homogenizer and unbroken material and nuclei were pelleted at 280 × *g*. The pellet (P_1) was treated identically a second time and the two 280 × *g* supernatants were pooled (S_1) and supplemented with 5 mM final β-octyl glucopyranoside (OG) prior to a 30-min incubation under agitation. Cytoplasm and loose membrane-associated material was recovered in the supernatant of a 100,000 × *g* centrifugation (S_{OG}) and the pellet (P_{OG}) was conserved for analysis. The S_{OG} fraction was dialyzed against 20 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$ and applied to a 6 × 5-cm DEAE Bio-Gel A column (Bio-Rad) pre-equilibrated with the same buffer. The elution of proteins was triggered by a stepwise gradient of 0–1 M NaCl in the application buffer and was monitored by absorbence at 280 nm. The fractions of interest were pooled and dialyzed against the application buffer, concentrated by laying the dialysis tubing onto 20,000 Da polyethylene glycol, and further dialyzed against the application buffer. All procedures were carried out at 4 °C. Most often, the OG solubilization sample S_{OG} was flash frozen in liquid nitrogen and stored until application on the ion-exchange phase. For binding studies, this partially purified MT_3 sample was dialyzed against 20 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$ in order to remove the sucrose which inhibited MCA-NAT binding. The final DEAE sample was frozen identically before further purification or analysis.

Chemical Synthesis of a MT_3 Specific Affinity Phase

The ligand *N*-[2-(7-amino-1-naphthyl)ethyl]-acetamide (S27145, Fig. 1) was prepared in three steps from agomelatine (S20098 (16)) and attached to the polymer resin over a spacer, in two further steps, as

follows. A solution of *N*-[2-(7-methoxy-1-naphthyl)ethyl]acetamide (10 g, 41 mmol) in DCM (50 ml) was treated with BBr_3 (25 ml) at −15 °C under nitrogen. The reaction mixture was kept at −15 °C for 1 h, then poured on, hydrolyzed with 1 N $NaHCO_3$ and extracted with methylethylketone. The organic layer was dried over sodium sulfate and concentrated. The residue was taken up with DCM (100 ml) and the precipitate collected and dried to afford 8.77 g (93%) of *N*-[2-(7-hydroxy-1-naphthyl)ethyl]acetamide (compound *1*). A solution *1* (8.7 g, 38 mmol) in DCM (400 ml) was treated with triethylamine (6.9 ml, 50 mmol) and *N*-phenyl-bistrifluoromethane sulfonimide (20 g, 53 mmol). The reaction mixture was refluxed for 12 h, then cooled, concentrated, taken up with diethyl ether, washed with 1 N $NaHCO_3$, water, and then dried over magnesium sulfate. The crude product was purified on silica gel with DCM/ethyl acetate (95:5) to afford 11.5 g (84%) of *N*-[2-(7-trifluoromethylsulfoxy-1-naphthyl)ethyl]acetamide (compound *2*). A mixture of *2* (4.0 g, 11 mmol) with benzophenonimine (7 g, 38 mmol), bis(diphenylphosphino-1–1′-binaphthalene (310 mg, 0.5 mmol), palladium acetate (70 mg, 0.3 mmol), and cesium carbonate (5 g, 15 mmol) in dimethoxyethane (160 ml) was refluxed under nitrogen for 12 h. The resulting reaction mixture was hydrolyzed with water and extracted with diethyl ether. The organic layer was washed with 10% citric acid and water, then dried over sodium sulfate and concentrated. The resulting crude product was dissolved in tetrahydrofuran (150 ml), treated with 1 N HCl (200 ml), and heated at 60 °C for 1 h. The reaction was cooled, extracted with diethyl ether, and the aqueous phase was brought to pH 11 with NaOH, then extracted with DCM. The organic layer was dried over sodium sulfate, concentrated, and purified on silica gel in DCM/methanol (97:3) to afford 1.44 g of *N*-[2-(7-amino-1-naphthyl)ethyl]acetamide. The corresponding hydrochloride salt (1.61 g, 55%, compound *3*) was obtained by treatment of the base in ethyl acetate with HCl in diethyl ether. Compound *3* (876 mg, 3.31 mmol) and 6-*tert*-butoxycarbonylamino hexanoic acid (1.15 g, 4.97 mmol) were dissolved in DCM (20 ml) and neat HATU (1.89 g, 4.97 mmol) was added in one single portion, followed by a slow addition of diisopropylethylamine (2.57 g, 14.90 mmol). After a 15-min reaction at room temperature, high performance liquid chromatography showed no remaining amine and one major new product. Ethyl acetate (100 ml) was then added and the solution washed 3 times with brine (3 × 25 ml), 1 M HCl (3 × 25 ml), 5% $NaHCO_3$ (3 × 25 ml), and brine (3 × 25 ml), dried over magnesium sulfate and evaporated under vacuum. The yield of {5-[7-(2-acetylamino-ethyl)-naphthalen-2-ylcarbamoyl]-pentyl}-carbamic acid *tert*-butyrate, compound *4* (1.4 g), was 95% and its high performance liquid chromatography purity was 99%. The Boc group was deprotected by treatment of *4* (1.40 g, 3.17 mmol) with trifluoroacetic acid (10 ml) in DCM (20 ml). After 15 min at room temperature, the solution was evaporated under vacuum and lyophilized in water/acetonitrile (9:1). The trifluoroacetic acid salt was then attached to the Novasyn TG carboxy resin (Novabiochem, 0.25 mmol/g) (12 g) on a semiautomatic synthesizer with axial shaking, using HATU (1.21 g, 3.17 mmol) in the presence of diisopropylethylamine (2.05 ml, 11.88 mmol) in a mixture of 200 ml of DCM/DMF (1:1) as solvent. The reaction mixture was shaken for 64 h at room temperature. After filtration, the usual washings were performed: 3 × with DMF and isopropyl alcohol, alternatively and 3 times with DCM. The resin was then capped by treatment with a large excess of glycine methyl ester hydrochloride (3.98 g, 31.7 mmol) using HATU (12.05 g, 31.7 mmol) in the presence of diisopropylethylamine (7.65 ml, 44.4 mmol) in 200 ml of a mixture of DCM/DMF (1:1). The same washings were performed after filtration. Estimation of the substitution level of the resin was carried out by microanalysis of the nitrogen percentage content: 0.11 mmol ligand/g of resin.

Complete Purification of MT_3

The partially purified MT_3 sample was subjected to affinity chromatography at 4 °C. The phase was synthesized as described above and used in a batch procedure. It was washed three times with 20 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$ and mixed with the sample at a ratio of 50 μg of protein/mg of phase. The incubation was performed for 15 min under gentle agitation after which the sedimentation of the resin was left to occur. The unbound material was removed by pipetting over the supernatant and the phase was washed twice with the equilibration buffer. The bound proteins were eluted by incubating the phase for 15 min in the equilibration buffer supplemented with 50 μM MCA-NAT. The eluate was recovered by pipetting, dialyzed against water, and concentrated on polyethylene glycol as described above. The precipitate obtained in dry ice-cold acetone was dried under a flow of nitrogen and analyzed by Laemmli polyacrylamide gel electrophoresis (17). The proteins were detected in the gel by Coomassie Blue.

Mass Spectrometry Analysis of Purified MT_3

The protein spot in the Laemmli electrophoresis performed after affinity chromatography was excised from the Coomassie Blue-stained gel and washed with 50% acetonitrile. Gel pieces were dried in a vacuum centrifuge and reswollen in 20 μl of 25 mM NH_4 HCO_3 containing 0.5 μg of trypsin (Promega, sequencing grade). After 4 h incubation at 37 °C, the gel pieces were extracted with 5% formic acid and acetonitrile. The extracts were evaporated to dryness. The residues were dissolved in 0.1% formic acid and desalted using a Zip Tip (Millipore). Elution of the peptides was performed with 5–10 μl of 50% acetonitrile, 0.1% formic acid solution. The peptide solution was introduced onto a glass capillary (Protana) for nanoelectrospray ionization. Tandem mass spectrometry experiments were carried out on a Q-TOF hybrid mass spectrometer (Micromass, Altrincham, United Kingdom) in order to obtain sequence information. MS/MS sequence information was used for data base searching using the programs MS-Edman located at the University of California San Francisco and BLAST located at the NCBI.

cDNA-derived Expression of hQR_2 in Hamster Ovary CHO Cells and Preparation of Samples

The QR_2 coding sequence was isolated by reverse transcriptase-polymease chain reaction from human liver mRNA (CLONTECH, Palo Alto, CA) using the 5′ sense primer (5′-GAATTCTCCACCATGGCAGGTAAGAAAGTACTCATGTC-3′, nucleotides 176–202) and the 3′ antisense primer (5′-GCGGCCGCTCATTATTGCCCGAAGTGCCAGTGGGCTGTGC-3′, nucleotides 843–871) generated from the published sequence (Ref. 9; access number JO2888). Liver mRNA (200 ng) was reverse-transcribed with oligo$(dT)_{12–18}$ in accordance with the first-strand cDNA synthesis protocol from Amersham Pharmacia Biotech. Polymerase chain reactions were performed in 100 μl containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2 μl of the single-stranded cDNA preparation, 0.3 μM of each primer, and 2 units of pfu native polymerase (Stratagene) with a 35 cycles program of 94 °C for 1 min, 65 °C for 2 min, and 72 °C for 2 min and a final extension at 72 °C for 8 min. The amplified cDNA was then subcloned in-frame into *Eco*RI and *Not*I site of the pcDNA3.1(+) vector (Invitrogen, San Diego, CA). CHO-K1 cells maintained in Ham's F12 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 500 IU/ml penicillin, and 500 μg/ml streptomycin were transiently transfected by the pcDNA3.1(+)-QR_2 plasmid using LipofectAMINE as described by the manufacturer (Life Technologies). Fourty-eight hours after the beginning of transfection, the adherent cells were washed by phosphate-buffered saline and harvested in 10 ml of homogenization buffer (see "Purification of MT_3," above) and transferred from flask to flask. The cell supension obtained was adjusted to 0.2 M sucrose and spun at 280 × *g*. The resulting nuclei-free supernatant was assayed for protein and MT_3/QR_2 content.

Assays

All assays were performed in triplicate and data presented herein are representative of two to six individual experiments.

Total Protein—The protein concentration was determined by the method of Lowry (18) using bovine serum albumin as a standard.

MT_3 Pharmacological Data—The MT_3 binding was performed according to our original procedure.[1] Briefly, 100 μg (samples from animals) or 20 μg (samples from CHO) of proteins were incubated at 20–22 °C for 10–20 min with 200 pM [^{125}I]MCA-NAT in the presence (nonspecific binding) or absence (total binding) of 10 μM MCA-NAT and, for competition studies, with varying concentrations of test compounds. The final volume was 150 μl in 20 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$. Incubation was stopped by filtration through a 96-well filtration support disposed directly onto a Multiscreen filtering apparatus (Millipore) connected to a vacuum pump, allowing rapid filtration after the samples were loaded using a 96-well pipetting device (Transtar, Costar). The filter-associated radioactivity was measured in a β-scintillation counter (TopCount NXT, Packard). Samples from CHO culture and from the purification of MT_3 up to the ion exchange chromatography step were analyzed on glass fiber filters (GF/B Unifilter, Packard), while elution from the ion-exchange resin was followed using polyvinylidene difluoride filters (Immobilon™ Multiscreen, Millipore) presoaked in methanol and rinsed three times by 200 μl of binding buffer. Alternatively, samples from the final purification steps, affinity chromatography, and ion exchange chromatography as an internal reference, were assayed for MT_3 binding using 96-well format size-exclusion chromatography. Eighty μl of dry Sephadex G-25 fine (APB) were distributed into 96-well format polyvinylidene difluoride filters (Durapore™ Multiscreen, Millipore) using a Multiscreen powder dispensing

apparatus. The exclusion phase was hydrated with 250 μl of 20 mM Tris-HCl, pH 8.5, and spun at 550 $\times$ *g* for 1 min in a 96-well plate basket. The phase was further rinsed three times using 120 μl of the same buffer, left to equilibrate at 4 °C for 30 min, and spun before sample application. The samples were preincubated at 4 °C during 30 min with 200 pM [^{125}I]MCA-NAT as described above, and 120 μl of the mixture were loaded onto the exclusion phase. The plates were immediately spun at 550 $\times$ *g* for 1 min. The free radioligand was excluded from the eluate by diffusion in the chromatographic medium, and 90 μl of the eluate were used for scintillation counting. For competition studies, the data presented are affinity constants (K_i) calculated from specific binding values of logarithmic compound concentrations, according to the method of Cheng and Prusoff (19).

QR Enzymatic Activity—The measurements of QR_1 and QR_2 quinone reductase activities were adapted from Jaiswal *et al*. (9) and Zhao *et al.* (14). QR_1 activity was measured using 100 μM menadione as substrate and 100 μM NADH as co-substrate, while QR_2 activity was measured using 100 μM menadione as substrate, 100 μM dihydrobenzylnicotinamide (BNAH) as co-substrate, and 100 μM dicoumarol as QR_1 inhibitor. In both cases, the activities were measured at 25 °C in 200 μl of 20 mM Tris-HCl, pH 7.5, 1 mM OG and the reactions were followed at 440 nm using the intrinsic fluorescence of the two co-substrates with excitation at 340 nm (PolarStar 96-well plate reader, BMG, Offenburg, Germany). Samples were diluted before use in the measurement buffer supplemented with 10% glycerol, in order to apply the desired amount of protein in 20 μl. The instrument was calibrated using a range of co-substrate concentrations. The IC_{50} values were calculated from inhibition curves using semi-logarithmic plots of the compound concentrations (8 points).

RESULTS

Terminology and Assays of MT_3 and QR_2—This work primarily focused on the melatonin-binding site MT_3, which was studied and purified using [^{125}I]MCA-NAT binding (5) as a specific assay, designated herein as the "MT_3 assay." Purification of MT_3 from hamster kidney provided "partially purified MT_3" and "MT_3 purified to homogeneity." The cloning and expression of the human QR_2 gene in CHO provided an identified source of this enzyme, which was assayed using the well described oxidoreduction mechanism involving a quinone (electron acceptor) and a nicotinic derivative (electron donor). For assaying the QR_2 enzymatic activity, menadione was the substrate and a commercially available fluorescent NADH analogue, dihydrobenzylnicotinamide (BNAH, Powell *et al.* (20)), was used for the first time as the co-substrate. Dicoumarol, a potent QR_1 inhibitor and poor QR_2 inhibitor (14) was added to the assay to ensure QR_2 specificity. Control assays of QR_1 activity were performed when necessary, using menadione as the substrate and NADH as the co-substrate, which provided good QR_1 specificity since NADH is a very poor co-substrate for QR_2 (14, 20).[3], Although, in the light of our results presented thereafter, MT_3 and QR_2 seem to designate a unique protein, for convenience in the present report, we alternatively refer to hamster MT_3 or MT_3/QR_2 and human QR_2, depending on the methodological approach involved.

Purification and Identification of MT_3 as QR_2—The purification of the melatonin-binding site MT_3 was performed as described under "Experimental Procedures" and the intermediate fractions were assayed for [^{125}I]MCA-NAT binding and QR_2 enzymatic activity. QR_1 activity was also assayed in all samples as a control. We started the purification of the MT_3 melatonin-binding site by preparing a nuclei-free subcellular fraction, from which MT_3 was recovered with high yield in the supernatant of a 100,000 $\times$ *g* centrifugation after mild detergent treatment (5 mM octylglucoside). Dialysis removed most of the detergent molecules thanks to the high critical micellar concentration of OG (CMC_{OG} = 25 mM (21)). The dialysate was applied to a DEAE anion exchanger, from which MT_3 was eluted by a discontinuous gradient of NaCl. The MT_3 containing fractions were pooled and dialyzed in order to remove the NaCl. Finally, we purified MT_3 to homogeneity using an original affinity phase developed on the basis of the most specific MT_3 ligand known to date, MCA-NAT. The synthetic ligand (S27145, Fig. 1) bears an amine function in position 7 of naphthylethylacetamide, which was substituted by a 6-aminohexanoyl moiety in order to mimic the carbonylamide function of MCA-NAT. The free amino group of the aminohexanoic spacer was used for the attachment to the affinity matrix, a polyethylene glycol-derivatized polystyrene resin. The use of a naphthyl ring eliminated the photosensitivity associated with the indole ring. The affinity chromatographic step was performed at 4 °C in a batch procedure, and the proteins specifically adsorbed on the phase were eluted by 50 μM MCA-NAT. The eluate was analyzed by SDS-polyacrylamide gel electrophoresis, where it appeared as a single band of 26 kDa (Fig. 2). The band was recovered from the electrophoresis acrylamide gel as a trypsin digest and was analyzed by tandem mass spectrometry. The resulting five peptidic sequences were compared with protein data bases for alignment and showed 95% similarity with the human quinone reductase 2, QR_2 (Fig. 3). Table I displays the yields of the successive purification steps as calculated from MT_3 binding and QR_2 enzymatic assay data. The [^{125}I]MCA-NAT binding data are in good agreement with the QR_2 enzymatic data, with a 2.5–3.5-fold enrichment of MT_3/QR_2 in the OG supernatant, and a 12.5–13.5-fold enrichment after DEAE chromatography, while QR_1 was barely detectable in the ion exchange chromatography eluate. After the affinity chromatographic step, the purification of MT_3, as evaluated by [^{125}I]MCA-NAT binding and QR_2 enzymatic assay, reached a 10,000-fold factor of enrichment, confirming the identity of MT_3 with QR_2. Absorption and desorption of the protein preparation from the affinity medium gave a relatively low yield of recovery of MT_3 and QR_2 signals (about 20%), probably due to the rapid kinetics of ligand exchange of MT_3. Indeed, the dissociation constant at room temperature is about 0.3 s^{-1},[1] and performing the affinity chromatographic step at 4 °C could not completely counterbalance the rapid dissociation kinetics.

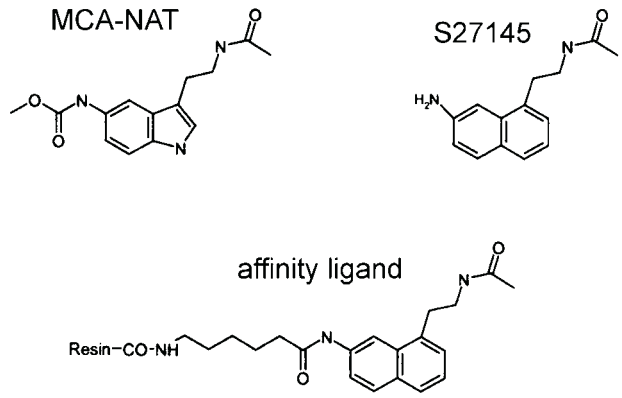

FIG. 1. **Structure of MCA-NAT, S27145, and the affinity ligand [(7-[6-aminohexanoylamido]-1-naphthyl)ethyl]acetamide branched on polyethylene glycol-derived polystyrene beads through a C_6 spacer.**

Cloning, Overexpression, and Tissue Distribution of QR_2—The human QR_2 gene was amplified from total RNA of human liver (9) and was used for the preparation of CHO cells transiently expressing QR_2. Four clones were prepared and transfected in CHO cells. MT_3 binding as well as QR_2 enzymatic activity were assayed on a nuclei-free fraction obtained as described under "Experimental Procedures." Data in Fig. 4 show an average of 33-fold increase in [^{125}I]MCA-NAT binding in CHO-QR_2, and an average of 259-fold increase in QR_2 activity as compared with native CHO cells. Hence, the amplifica-

[3] O. Nosjean and J. A. Boutin, unpublished observations.

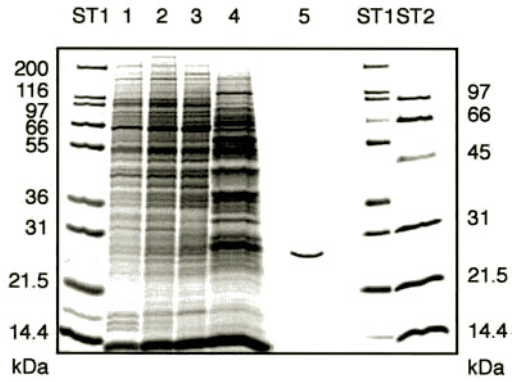

FIG. 2. **Purification of MT_3 from Syrian golden hamster kidney followed by SDS electrophoresis.** 20 μg of proteins was applied to each lane, except for *lane 5* where the amount applied was evaluated at 400 ng by densitometry. *Lane 1*, Dounce homogenate; *lane 2*, nuclei-free fraction (S_1); *lane 3*, cytosol-enriched fraction (S_{OG}); *lane 4*, 30–40 mM NaCl DEAE eluate containing MT_3; *lane 5*, affinity chromatography eluate, ST1 Novex molecular weight standards, ST2 Bio-Rad molecular weight standards. The affinity chromatography eluate was dialyzed against deionized water, concentrated in its dialysis tubing on high molecular weight polyethylene glycol, precipitated by dry ice-cold acetone, dried under a flow of nitrogen, and redisolved in 10 μl of deionized water. Values in the margins indicate molecular weights in kDa; *left scale*, Novex molecular weight markers, *right scale*, Bio-Rad molecular weight markers. The gel was stained by Coomassie Blue.

MAGKKVLIVYAHQEPKSFNGSLKNVAVDELSRQGCTVTVSDLYAMNFE
VLLLYAHQEPFSFNGSLK

PRATDKDITGTLSNPEVFNYGVETHEAYKQRSLASDITDEQKKVREAD
YGLEAYEAYK TSDLLEEQR

LVIFQFPLYWFSVPAILKGWMDRVLCQGFAFDIPGFYDSGLLQCKLAL
LAL

LSVTTGGTAEMYTKTGVNGDSRYFLWPLQHGTLHFCGFKVLAPQISFA
LSLTTGGTAEMYTK VLAPQLSFG

PEIASEEERKGMVAAWSQRLQTIWKEEPIPCTAHWHFGQ
LDVSSEEER

FIG. 3. **Sequence alignment between human QR_2 and the peptidic fragments obtained after purification of MT_3.** *First line*, sequence of quinone reductase 2 from *Homo sapiens* (Swiss Prot P16083). *Second line*, sequence information obtained by tandem mass spectrometry on affinity purified MT_3. For these sequences, *L* represents either leucine (L) or isoleucine (I) since these two amino acids have the same nominal mass. For the same reason, *Q* represents either glutamine (Q) or lysine (K). *Light shade*, amino acid homolog to its counterpart in the QR_2 sequence. *Heavy shade*, amino acid identical to its counterpart in the QR_2 sequence.

tion of QR_2 in CHO cells led to a strong increase of both MT_3 and QR_2 specific signals. The lower amplification rate observed with [^{125}I]MCA-NAT binding was probably due to an overestimation of MT_3 in native CHO cells (5 fmol/mg), because this value fell within the high background level obtained with the binding technique developed for MT_3 (20–30% specific signal of total).

Ligand Specificity for MT_3 and QR_2 Assays—Several series of compounds were tested for their potency to inhibit either [^{125}I]MCA-NAT binding (MT_3 assay) or menadione/BNAH oxidoreduction (QR_2 assay) on hamster kidney-purified MT_3/QR_2 and on transfected human QR_2 (Table II). The MT_3 ligands showed an affinity for hamster MT_3/QR_2 in the nanomolar range. Iodomelatonin was the most potent ligand, but also bound very tightly to mt_1 and MT_2. As expected, melatonin and other melatoninergic compounds displayed no or low affinity for MT_3/QR_2. The QR_2 co-substrate BNAH was a ligand as

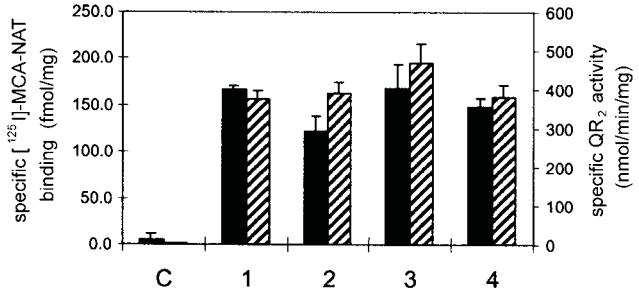

FIG. 4. **^{125}I-MCA-NAT binding (*plain bars*) and QR_2 enzymatic activity (*striped bars*) of control (*C*) and QR_2 transfected (*1–4*) CHO cells.** The four clones were prepared as described under "Experimental Procedures," transfected in CHO-K1 cells and the cells were cultured for 2 days before harvesting. The cells were gently disrupted and the nuclei fraction was removed by a 280 $\times$ *g* centrifugation. Specific assays were performed in triplicates on the resulting fraction.

potent as MCA-NAT, while, surprisingly, menadione displayed only poor affinity for hamster MT_3/QR_2. Dicoumarol, known to poorly inhibit QR_2 (14) exhibited poor affinity for MT_3/QR_2, while estradiol, which diminishes quinone reductase activity in Syrian hamster kidney (22), had an intermediate affinity (about 600 nM).

The pattern of inhibition of [^{125}I]MCA-NAT binding described above for hamster MT_3/QR_2 was conserved when the same assay was applied to a human QR_2 preparation. Nonetheless, the compounds exhibited a 2–20-fold lower affinity for human QR_2 compared with hamster MT_3/QR_2. This hamster/human difference was also observed with the enzymatic data, where IC_{50} were 1.5–20-fold higher with human QR_2 compared with hamster MT_3/QR_2. Besides, hamster MT_3/QR_2 and human QR_2 had a similar Michaelis affinity for menadione and BNAH, with a K_m of about 60 μM for BNAH, comparable with the K_m of NAD(P)H for QR_1 (20, 23). Dicoumarol was confirmed as a poor inhibitor of QR_2, with an IC_{50} of about 600 μM. Interestingly, the MT_3 compounds proved to be relatively good inhibitors of the enzyme, especially of the hamster preparation where the IC_{50} of iodomelatonin and S26553 were in the micromolar range, well below the K_m of the substrates. On the contrary, and similarly to the binding data, the mt_1/MT_2 ligands did not inhibit the QR_2 enzymatic activity.

DISCUSSION

The first in-depth biochemical investigation of MT_3, which was previously known solely on the basis of its peculiar melatoninergic pharmacological profile, and was often compared with the two well described mt_1 and MT_2 melatonin membrane receptors (4–7, 24) is reported here. The MT_3 melatonin-binding site was purified to homogeneity using classical biochemical tools, and was identified by partial peptide sequencing as the homologue of the human quinone reductase 2, with 57 out of 71 amino acid identity. Furthermore, several clues support the identification of MT_3 as the hamster homologue of QR_2. First, MT_3 has always exhibited enzyme-like kinetics of association/dissociation of its ligands (5, 6), which has long been a hint to its pharmacological characterization with classical techniques. The identification of MT_3 as an enzyme therefore explains this point. Second, both MT_3 and QR_2 showed hydrophobic properties, while not behaving as genuine membrane proteins. Indeed, when hamster kidney membranes were prepared in the absence of detergent, MT_3 loosely associated with membrane components and fractionated almost equally in the pellet and in the supernatant of an ultracentrifugation.[3] This is consistent with the elution from the DEAE column at low ionic strength (30–40 mM NaCl), and with the previously described hydrophobic properties of QR_2 (9, 14, 20, 25). Third, the present

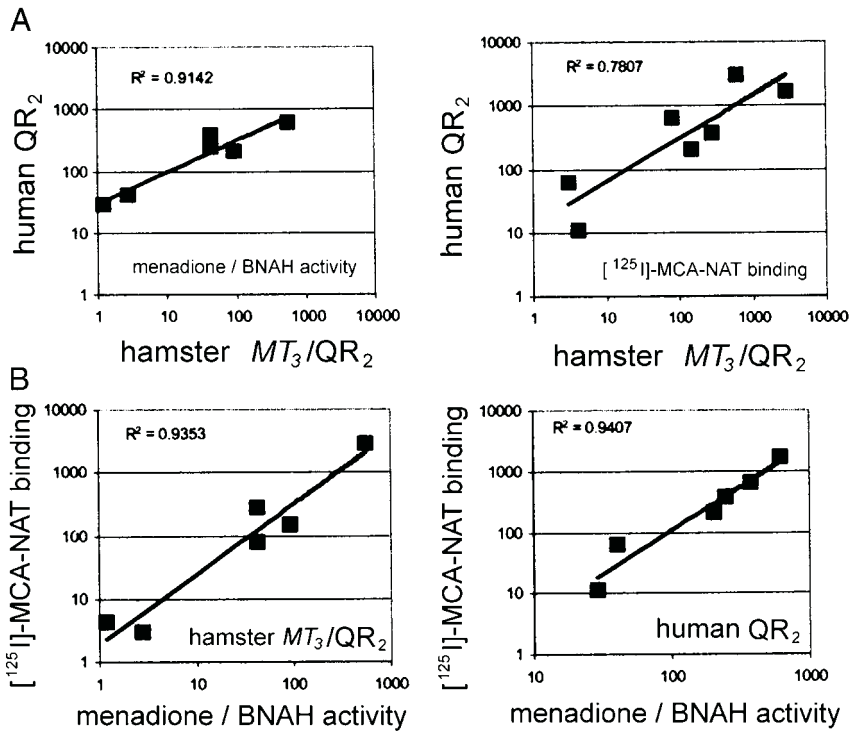

FIG. 5. **Comparative data obtained from pharmacological competition and enzymatic inhibition measurements performed on DEAE-purified hamster MT_3/QR_2 and overexpressed human QR_2.** Data are represented as follows. *A, left panel*, correlation of enzymatic data from hamster MT_3/QR_2 (*horizontal*) *versus* human QR_2 (*vertical*), *right panel*, correlation of pharmacological data from hamster MT_3/QR_2 (*horizontal*) *versus* human QR_2 (*vertical*). *B, left panel*, correlation of enzymatic (horizontal) *versus* pharmacological (vertical) data from hamster MT_3/QR_2, and *right panel*, correlation of enzymatic (horizontal) *versus* pharmacological (*vertical*) data from human QR_2. Data are extracted from Table II, except for substrates (menadione and BNAH) and very low affinity compounds (K_i or $IC_{50} > 10{,}000$).

purification shows a good correlation between the purification factors calculated from "MT_3 data," *i.e.* [^{125}I]MCA-NAT binding, and from "QR_2 data," *i.e.* menadione enzymatic reduction. This observation is of particular interest regarding the last step of purification which led to a 10,000-fold enrichment of both [^{125}I]MCA-NAT binding and QR_2 activity. Fourth, the affinity step which purified QR_2 to homogeneity was designed on the basis of a typical MT_3 ligand, MCA-NAT, which had poor structural similarity with either QR_2 substrates or inhibitors known to date. Nevertheless, these observations clearly called for additional data showing the correlation between the presence of MT_3 and QR_2 signals. For this purpose, the human QR_2 gene was inserted in a vector and transfected in CHO cells, leading to the apparition of both MT_3 and QR_2 phenotypes, which were otherwise absent in native cells. In itself, this result definitively associated MT_3 and QR_2 signals, and led to the unambiguous identification of MT_3 as the hamster homologue of the human QR_2.

Thereafter, it was of particular interest to compare the properties of MT_3 ligands (iodomelatonin, MCA-NAT, S26553, *N*-acetylserotonin, and melatonin), mt_1- and MT_2-specific melatoninergic ligands (luzindole, serotonin, and tryptamine), and QR-active compounds (menadione, BNAH, dicoumarol, and estradiol) toward hamster MT_3/QR_2 and human QR_2 in order to build up relevant cross-comparisons. The [^{125}I]MCA-NAT binding and the QR_2 enzymatic data were in good agreement altogether, and the compounds evaluated showed similar relative affinities for hamster MT_3/QR_2 and human QR_2. Nevertheless, it is noteworthy that there was a 2–20-fold higher affinity of the compounds for hamster MT_3/QR_2 relative to human QR_2, most probably due to interspecies difference in the protein sequence and properties, as was already reported for a rat to human QR_1 comparison (26). Besides, the two QR_2 substrates, menadione and BNAH, displayed very contrasted affinities to hamster MT_3/QR_2 and human QR_2, the former being a poor competitive inhibitor of [^{125}I]MCA-NAT binding. These differences in binding affinities may reflect the difference of behavior of these two compounds toward the catalytic site of MT_3/QR_2. Indeed, we suggest that menadione has a low affinity for a reduced FAD-containing enzyme which seems to be present in binding experiments, while in contrast BNAH has a higher affinity for the protein bearing this intermediate catalytic site.

Furthermore, all the values for the inhibition of [^{125}I]MCA-NAT binding were in the nanomolar range, while those of menadione/BNAH oxidoreduction were in the micromolar range. As these parameters are not often compared, this difference of 3 orders of magnitude is surprising at first glance. However, when making such a comparison, one must bear in mind the dynamic ping-pong mechanism occurring at the active site, as opposed to the simple ligand exchange occurring in the binding experiments. For this reason, comparison of absolute affinity constants is not relevant, in contrast to correlation representations. Indeed, Fig. 5 shows a good correlation between the data discussed above, whether the comparison applies to the enzymatic (Fig. 5*A, left panel*) or binding (Fig. 5*A, right panel*) data from hamster MT_3/QR_2 and human QR_2, or conversely, when it applied to the binding and enzymatic data obtained on hamster MT_3/QR_2 (Fig. 5*B, left panel*) or human QR_2 (Fig. 5*B, right panel*) preparations.

In conclusion, we have purified to homogeneity the hamster kidney melatonin-binding site MT_3, which, after sequencing by mass spectrometry, was identified as the hamster homologue of the human quinone reductase 2 (EC 1.6.99.2). It was further demonstrated an overall good correlation between data collected from [^{125}I]MCA-NAT binding (MT_3 specific assay) and from menadione/BNAH + dicoumarol oxidoreduction (QR_2 specific assay) experiments. The purification scheme led to a 10,000-fold enrichment in both the MT_3 binding and the QR_2 activity, and we showed that QR_2 activity and MT_3 binding both appeared after QR_2 transfection in CHO cells. Furthermore, we found that inhibition data obtained with various compounds classically involved in MT_3 or QR_2 inhibition were correlated. Taken together, these results show that the former putative melatonin-binding site MT_3 is now identified as the quinone reductase QR_2. The relative abundance of the MT_3 signal ([^{125}I]melatonin and, more specifically, [^{125}I]MCA-NAT binding) in hamster organs compared with other mammals has tempered the pharmacological interest of this melatonin-bind-

ing site to date. It is now suggested that hamster QR_2 has an inhibition specificity different enough from human QR_2 to have appeared until now as a distinct protein called MT_3, being in fact only an inter-species homologue. Furthermore, hamster MT_3/QR_2 and, to a lower extent, human QR_2, show interesting binding affinities for melatonin and MT_3-specific ligands. The oxidoreductive properties of the QR_2 open the way for a novel enzymatic investigation of the highly debated antioxidant properties of melatonin (for review, see Ref 27). Hence, in addition to the G protein-coupled receptors of melatonin (mt_1 and MT_2) and the transferase arylalkylamine *N*-acetyltransferase which controls the limiting step of melatonin biosynthesis (28), MT_3/QR_2 appears as a fourth molecular target to explore the multiple facets of melatonin action.

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