Yeast Sterol C_8-C_7 Isomerase: Identification and Characterization of a High-Affinity Binding Site for Enzyme Inhibitors[†]

Fabian F. Moebius,* Katrin Bermoser, Raphael J. Reiter, Markus Hanner, and Hartmut Glossmann

Institut für Biochemische Pharmakologie, Universität Innsbruck, Peter Mayr Strasse 1, A-6020 Innsbruck, Austria

Received August 12, 1996; Revised Manuscript Received October 31, 1996[⊗]

ABSTRACT: The yeast gene ERG2 encodes a sterol C_8-C_7 isomerase and is essential for ergosterol synthesis and cell proliferation. Its striking homology with the so-called sigma$_1$ receptor of guinea pig brain, a polyvalent steroid and drug binding protein, suggested that the yeast sterol C_8-C_7 isomerase (ERG2) carries a similar high affinity drug binding domain. Indeed the sigma ligands [^{3}H]haloperidol (K_d = 0.3 nM) and [^{3}H]ifenprodil (K_d = 1.4 nM) bound to a single population of sites in ERG2 wild type yeast microsomes (B_{max} values of 77 and 61 pmol/mg of protein, respectively), whereas binding activity was absent in strains carrying ERG2 gene mutations or disruptions. [^{3}H]Ifenprodil binding was inhibited by sterol isomerase inhibitors such as fenpropimorph (K_i = 0.05 nM), tridemorph (K_i = 0.09 nM), MDL28,815 (K_i = 0.44 nM), triparanol (K_i = 1.5 nM), and AY-9944 (K_i = 5.8 nM). [^{3}H]Haloperidol specifically photoaffinity-labeled a protein with an apparent molecular weight of 27 400, in agreement with the molecular mass of the sterol C_8-C_7 isomerase (24 900 Da). 9E10 c-myc antibodies specifically immunoprecipitated the c-myc tagged protein after [^{3}H]haloperidol photolabeling, unequivocally proving that the drug binding site is localized on the ERG2 gene product. Haloperidol, trifluperidol, and ifenprodil inhibited the growth of *Saccharomyces cerevisiae* and reduced the ergosterol content of cells grown in their presence. Our results demonstrate that the yeast sterol C_8-C_7 isomerase has a polyvalent high-affinity drug binding site similar to mammalian sigma receptors and that in yeast sigma ligands inhibit sterol biosynthesis.

In yeast ergosterol is not an obligatory membrane constituent but can be substituted by a variety of precursors. Mutations in ergosterol synthesis confer resistance to polyene antimycotics such as nystatin and amphotericin B. Mutant strains accumulate sterol intermediates, which are normally present only at low level, or even unusual sterols. With the help of these mutants most of the enzymes required for ergosterol biosynthesis from lanosterol were cloned by DNA complementation (Lees et al., 1995). The sterol C_8-C_7 isomerase (ERG2) shifts the double bond in the B ring of fecosterol from the 8(9) to the 7(8) position (Figure 1) without need for a cofactor. The DNA complementing ERG2 deficiency encoded a 24.9 kDa protein that shared no similarities with other enzymes from *Saccharomyces cerevisiae* (Ashman et al., 1991; Arthington et al., 1991). Expression of this DNA normalized the sterol profile of the yeast ERG2 mutant strain WA0, indicating that it was either the enzyme itself or a regulatory subunit. The molecular mechanism of isomerization is not known. Isotopic labeling studies indicate that during the shift of the double bond the sterol molecule takes up a proton at C_9 from the surrounding water or from the enzyme (Lee et al., 1969) (Figure 1). The unstable carbocationic reaction intermediate then releases a proton from C_7 thereby shifting the double bond. The isomerase is one of the targets of agricultural fungicides such as fenpropimorph and tridemorph which were suggested to mimick the unstable reaction intermediate (Rahier et al., 1986; Mercer, 1991).

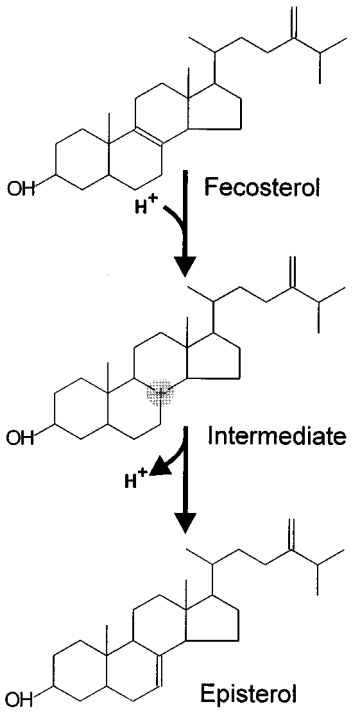

FIGURE 1: Sterol C_8-C_7 isomerization.

[†] This work was supported by a Boehringer-Ingelheim fellowship (F.F.M.) and by grants of the Österreichische Nationalbank (to H.G.) and Fonds zur Förderung der wissenschaftlichen Forschung (Grant P11636 to H.G.). It is part of the doctoral thesis of K.B. presented to the Medical Faculty of the University of Innsbruck.

* To whom correspondence should be adressed. FAX: +43 512-588627.

[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1996.

[1] Abbreviations used: AY-9944, 1,4-bis(2-chlorobenzylaminomethyl)cyclohexane; B_{max}, maximal density of binding sites; BSA, bovine serum albumin; IC_{50}, concentration causing half-maximal inhibition; K_d, dissociation constant; K_i, inhibition constant; k_{+1}, k_{-1}, association and dissociation rate constant, respectively; MDL28,815, *N*-[(1,5,9)-trimethyldecyl]-4,10-dimethyl-8-aza-*trans*-decal-3β-ol; PAGE, polyacrylamide gel electrophoresis; 3-PPP, propyl-3-(3-hydroxyphenyl)-piperidine; SDS, sodium dodecyl sulfate.

S0006-2960(96)01996-4 CCC: $12.00

We recently cloned a protein from guinea pig liver which had 30% identical amino acid residues with sterol C_8-C_7 isomerases from *Magnaporthe grisea*, *Ustilago maydis*, and *S. cerevisiae* (Ashman et al., 1991; Arthington et al., 1991; Keon et al., 1994; Hanner et al., 1996). This protein bound a variety of drugs, so-called sigma ligands, and steroids with high affinity. It had been previously given the provisional name sigma receptor (Walker et al., 1990). Yeast and mammalian proteins shared a similar hydrophobicity plot predicting one putative N-terminal transmembrane segment and two additional stretches of hydrophobic amino acid residues (Keon et al., 1994; Hanner et al., 1996). This prompted us to investigate whether the yeast sterol C_8-C_7 isomerase also carries a high-affinity binding site for sigma ligands and sterol isomerase inhibitors. In the following we provide evidence that the yeast sterol C_8-C_7 isomerase binds the sigma ligands [^{3}H]ifenprodil and [^{3}H]haloperidol, that the morpholine fungicides fenpropimorph and tridemorph bind to this site with picomolar affinity, and, finally, that sigma ligands alter the sterol composition of yeast cells.

EXPERIMENTAL PROCEDURES

Materials. (+)[^{3}H]pentazocine (32 Ci/mmol), [^{3}H]haloperidol (11 Ci/mmol), [^{3}H]ditolylguanidine (40 Ci/mmol), and [^{3}H]ifenprodil (44 Ci/mmol) were obtained from NEN (Vienna, Austria). The enantiomers of emopamil were kindly provided by Knoll AG (Ludwigshafen, Germany); sigma ligands were a gift of Dr. J. Traber (Tropon, Cologne, Germany). Other chemicals were obtained from the following sources: opipramol, Ciba Geigy (Vienna, Austria); triparanol and MDL28,815, Hoechst Marion Roussel Research Institute (Cinicinnati, OH); fenpropimorph and tridemorph, BASF (Limburgerhof, Germany); AY-9944, Dr. P. Benveniste (Strasbourg, France); trifluperidol, RBI (Natick, MA); Bradford Protein Reagent and prestained molecular weight markers, Bio-Rad (Vienna, Austria); 9E10 c-myc antibody, Oncogene (NY, USA). All other chemicals were obtained from Sigma (Vienna, Austria). *S. cerevisiae* strains WA0 and WA10-3-1A and B were kindly provided by Dr. M. Bard (Indianapolis, IN, USA); strain JB811 was from Dr. K. Nasmyth (Vienna, Austria).

Binding Assays. 0.6 nM [^{3}H]pentazocine, 2.0 nM [^{3}H]-ditolylguanidine, 0.8 nM [^{3}H]haloperidol, and 0.7 nM [^{3}H]ifenprodil were incubated in 0.25−1.0 mL 25 mM Tris-HCl, pH 9 (4 °C) for 6 h at 22 °C with yeast microsomes prepared as described previously (Hanner et al., 1995). The protein concentrations were 5−25 μg/mL. Nonspecific binding was measured in the presence of 1 μM of the respective unlabeled drug. Serial dilutions of competing drugs were prepared in dimethylsulfoxide (Boer et al., 1989) and added directly to the assay. The final DMSO concentration was ≤1%, which did not affect specific binding. Separation of bound and free ligand was performed as described previously (Moebius et al., 1993). Binding parameters were calculated by non linear curve fitting to a rectangular hyberbola (K_d, B_{max}) or the general dose response equation (IC_{50}, slope factors (DeLean et al., 1978)). Kinetic constants (k_{+1}, k_{-1}) and K_i values were calculated according to Prinz and Striessnig (1993) and Linden (1982). Protein concentrations were determined according to Bradford (1976), using BSA as a standard. To determine the number of receptors per yeast cell we counted the cells, lysed them with glass beads, and saturated the lysed cells with [^{3}H]-haloperidol.

Growth of S. cerevisiae and Sterol Analysis. S. cerevisiae was grown in 2% (w/v) glucose, 1% (w/v) tryptone, 2% (w/v) yeast extract (YPD) or 2% (w/v) glucose, 0.67% (w/v) yeast nitrogene base, and the amino acids except for leucine. Cells were grown to an OD_{600} of 1.5−2 and harvested by centrifugation. Cell pellets were resuspended in 50 mM Tris-HCl, pH 9 (4 °C), 0.1 mM PMSF, 5 mM EDTA-Tris, and lysed with glass beads. Microsomal membranes were collected by centrifugation (100000*g*, 45 min). After they were washed with 0.5 M KCl, 0.15 M Tris-HCl, pH 9 (4 °C), and centrifuged at 100000*g*, the membranes were resuspended in 5% (w/v) glycerol, 20 mM Tris-HCl, pH 9 (4 °C), shock-frozen in liquid N_2, and stored at −80 °C. From a 200 mL culture approximately 5 mg of microsomal protein was obtained. Solubilization and sucrose density gradient centrifugation were performed as described previously (Moebius et al., 1994). The following strains were used: JB811 (*ade2-1 leu2-3,112 pep4-3 trp1-289 ura3-52*), WAO (*a his7-2 leu2-3,112 ura3-52 erg2-3*), WA10-3-1B (α *ade5 his7-2 leu2-3,112 ura3-52*), and WA10-3-1A (*a ade5 his7-2 leu2-3,112 ura3-52 erg2-4::LEU2*). Growth inhibition experiments were performed by diluting stationary phase cultures of JB811 into fresh YPD medium. Drugs were added in DMSO (final concentration 0.5% (v/v)). Cells were grown to mid-log phase and the OD_{600} was measured. For spectrophotometric scans of non-saponifiable lipids (Molzahn & Woods, 1972), cells were collected by centrifugation and resuspended in distilled water and the OD_{600} was determined. Sterols were extracted from approximately 10^8 cells (10 mg of wet weight) as described (Molzahn & Woods, 1972). The 282 and 271 nm absorptions of sterols extracted from cells grown in the presence of inhibitors were normalized to cells grown in their absence (100%) and to strain WA0, devoid of ergosterol (0%).

Photoaffinity Labeling and SDS−PAGE. 2−3 nM [^{3}H]-haloperidol was incubated with 10−20 μg/mL microsomal protein in 9 mL of 25 mM Tris-HCl, pH 9 (4 °C) for 6 h at 22 °C. 0.25 mL samples were subjected to filtration prior to irradiation. The incubation mix was then transferred to plastic Petri dishes (i.d. 100 mm) and irradiated for the indicated times with a Sylvania G15T8 germicide lamp (6 cm distance). Photolysed membranes were collected by 100000*g* centrifugation and resuspended in SDS sample buffer (Moebius et al., 1993) containing 10 mM *N*-ethylmaleimide or 10 mM dithiothreitol. Proteins were separated on a 15% (w/v) SDS−polyacrylamide gel. The gel was prepared for fluorography with Amplify as described previously (Moebius et al., 1993). For quantification 3 mm gel slices were incubated in 30% (v/v) H_2O_2, 1.25% (v/v) NH_3, for 3 h at 56 °C prior to the addition of scintillation fluid.

Expression of the 9E10 c-myc Tagged ERG2. The open reading frame of the ERG2 DNA obtained from Dr. Martin Bard (pIU411 (Ashman et al., 1991)) was amplified by PCR using pfu polymerase and the primers 5′-AAAAGCTTATGAAGTTTTTCCCACTCC-3′ and 5′-TTTGCGGCCGCTTAGAACTTTTTGTTTTGC-3′ with pIU411 as a template, thereby introducing *Hin*dIII and *Not*I restriction sites. The PCR product was subcloned into YEp351ADC1 or c-myc-YEp351ADC1 (Hanner et al., 1996). Transformation of strain WA0 was performed as described previously (Hanner et al., 1995).

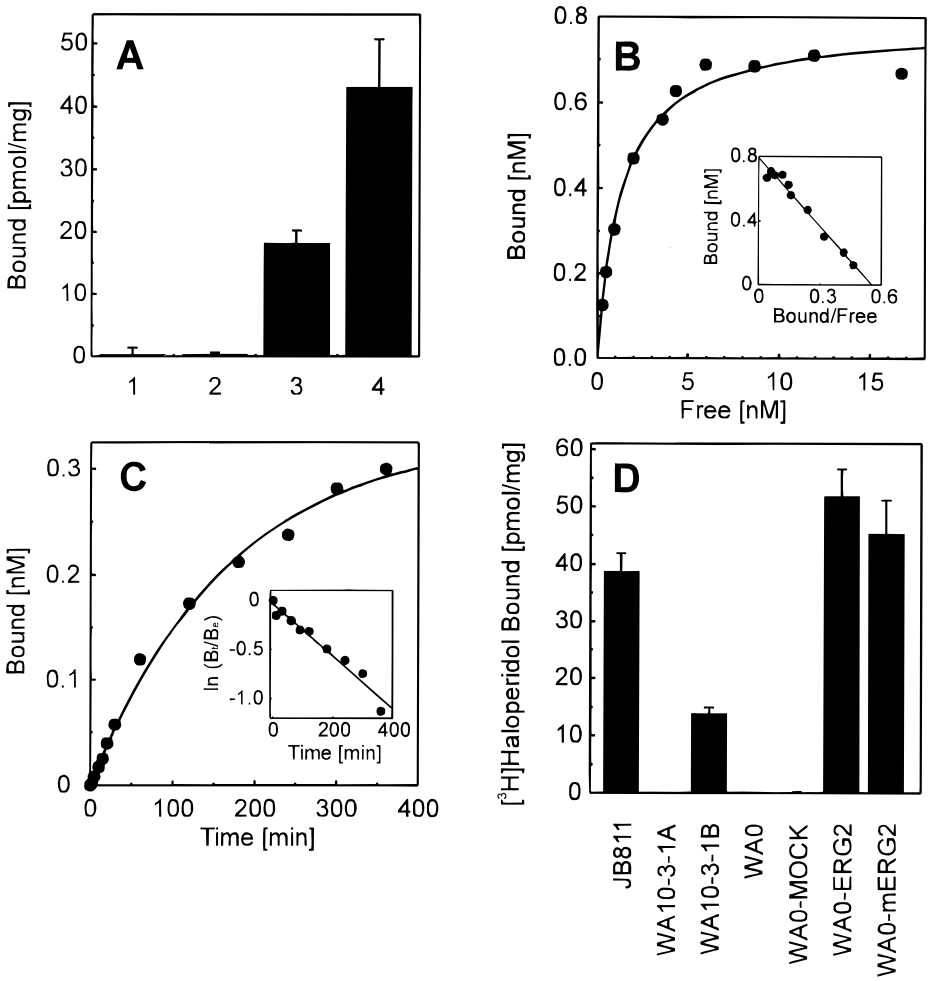

FIGURE 2: Radioligand binding to *S. cerevisiae*. (A) Comparison of (+)-[^{3}H]pentazocine (1), [^{3}H]ditolylguanidine (2), [^{3}H]ifenprodil (3), and [^{3}H]haloperidol (4) binding to membranes from *S. cerevisiae*. Binding was performed at 22 °C at ligand concentrations of 0.6, 2.0, 0.7, and 0.8 nM and protein concentrations of 5, 20, 5, and 2 μg/mL, respectively. Data shown are the mean ±SD ($n = 3$). (B) Saturation analysis with varying concentrations of [^{3}H]ifenprodil at 22 °C at a protein concentration of 15 μg/mL revealing a K_d of 1.3 nM ($n = 3$). The inset shows the linear transformation of the same data. (C) Association of [^{3}H]ifenprodil at 22 °C (ligand concentration 0.4 nM, protein concentration 6 μg/mL protein). The rate constants k_{+1} (52 000 M^{-1} s^{-1}) and k_{-1} (0.000 05 s^{-1}) were determined as described (Prinz & Striessnig, 1993). The inset shows the dissociation of [^{3}H]ifenprodil induced by 0.1 μM unlabeled ifenprodil at 22 °C ($k_{-1} = 0.000\ 05\ s^{-1}$). (D) [^{3}H]Ifenprodil and [^{3}H]haloperidol binding to 5−25 μg/mL of microsomal protein prepared from *S. cerevisiae* strains JB811, WA10-3-1A, WA10-3-1B, and WA0 is shown. Strain WA0 was transformed with the vector YEp351ADC1 carrying no DNA (WA0-MOCK), the ERG2 DNA (WA0-ERG2), or the 9E10 c-myc tagged ERG2 DNA (WA0-mERG2) (see Experimental Procedures).

Immunoblotting and Immunoprecipitation. Immunostaining with a 9E10 c-myc antibody was performed as described previously (Hanner et al., 1996). Immunoprecipitation was performed as described (Hanner et al., 1996) using anti mouse IgG conjugated Sepharose and 1 μg of 9E10 c-myc antibodies (Evan et al., 1985).

RESULTS

Characterization of High-Affinity Drug Acceptor Sites in S. cerevisiae. To clarify whether *S. cerevisiae* contains high-affinity drug binding sites we measured sigma ligand binding to an ERG2 wild type strain of *S. cerevisiae* (JB811). Membranes prepared from this strain were essentially devoid of (+)-[^{3}H]pentazocine and [^{3}H]ditolylguanidine binding activity (Hanner et al., 1996) but contained binding sites for the sigma ligands [^{3}H]ifenprodil and [^{3}H]haloperidol (Figure 2A). [^{3}H]Ifenprodil bound with high affinity ($K_d = 1.4 \pm 0.2$ nM ($n = 3$)) to a single population of binding sites ($B_{max} = 61 \pm 7$ pmol/mg microsomal protein ($n = 3$)) (Figure 2B). Kinetic studies revealed slow association and dissociation of the radioligand (Figure 2C). From the rate constants obtained by fitting the association data to the second order rate equation ($k_{+1} = 45\ 000 \pm 9\ 000$ M^{-1} s^{-1} ($n = 2$), $k_{-1} = 0.000\ 06 \pm 0.000\ 01$ s^{-1} ($n = 2$)) a kinetic dissociation constant of 1.3 ± 0.5 nM ($n = 2$) was calculated. Similar results were obtained with [^{3}H]haloperidol ($K_d = 0.3 \pm 0.1$ nM ($n = 3$), $B_{max} = 77 \pm 8$ pmol/mg of protein ($n = 3$), $k_{+1} = 200\ 000 \pm 4\ 000$ nM^{-1} min^{-1} ($n = 2$), $k_{-1} = 0.000\ 04 \pm 0.000\ 02$ s^{-1} ($n = 2$), kinetically derived $K_d = 0.2 \pm 0.1$ nM ($n = 2$)).

To determine the number of drug receptors per yeast cell, we lysed the cells with glass beads and measured the [^{3}H]-haloperidol binding activity in the lysate. Saturation studies revealed a density of 105 000 ± 3 000 ($n = 4$) [^{3}H]-haloperidol binding sites per cell ($K_d = 0.2 \pm 0.1$ nM ($n = 4$)).

The detailed characterization of [^{3}H]ifenprodil binding (see Table 1) revealed low affinity for some typical sigma ligands, such as ditolylguanidine, (+)-3-PPP, pentazocine, and nor-allylmetazocine, whereas the high affinity of the [^{3}H]-ifenprodil binding site for haloperidol ($K_i = 0.5 \pm 0.2$ nM ($n = 3$)) was in agreement with the results of the [^{3}H]-

Table 1: Characteristics of the [^{3}H]Ifenprodil Binding Site in Yeast Microsomes (Strain JB811)[a]

compound	[^{3}H]ifenprodil binding	
	K_i (nM)	slope factor
sigma ligands		
ifenprodil	0.9 ± 0.2	1.13 ± 0.15
haloperidol	0.5 ± 0.2	1.24 ± 0.15
opipramol	17 ± 4	0.88 ± 0.03
amiodarone	62 ± 6	1.47 ± 0.24
(+)-emopamil	65 ± 16	1.01 ± 0.17
(−)-emopamil	74 ± 10	1.02 ± 0.10
azidopamil	>10 000	ND
trifluoperazine	500 ± 80	0.96 ± 0.01
(±)-pentazocine	1 020 ± 170	0.98 ± 0.05
ditolylguanidine	1 980 ± 360	0.91 ± 0.06
(+)-3-PPP	1 220 ± 30	0.93 ± 0.05
(+)-norallylmetazocine	4 730 ± 140	0.83 ± 0.09
(−)-norallylmetazocine	14 200 ± 1 800	1.09 ± 0.11
steroids		
testosterone propionate	1 920 ± 150	0.89 ± 0.07
progesterone	4 340 ± 540	0.82 ± 0.08
testosterone	7 760 ± 1 040	0.89 ± 0.05
corticosterone	*b*	ND
isomerase inhibitors		
fenpropimorph	0.05 ± 0.01	1.26 ± 0.09
tridemorph	0.09 ± 0.04	1.56 ± 0.31
trifluperidol	0.15 ± 0.03	1.13 ± 0.07
MDL 28,815	0.44 ± 0.07	1.02 ± 0.10
triparanol	1.5 ± 0.3	0.97 ± 0.07
AY-9944	5.8 ± 2.1	1.12 ± 0.13

[a] 0.7−0.9 nM [^{3}H]ifenprodil was incubated for 6 h at 22 °C at a protein concentration of 4−7 μg/mL in the presence or absence of five to seven concentrations of drugs in 25 mM Tris-HCl, pH 9 (4 °C). Serial drug dilutions were prepared in DMSO. K_i values were calculated according to DeLean et al. (1978). Data shown are the mean ±SD ($n = 3$). For $ZnCl_2$ and $CuCl_2$ IC_{50} values of 4.4 ± 2.3 and 570 ± 100 μM and apparent Hill slopes of 2.26 ± 0.48 and 2.17 ± 0.23 were obtained. [b] No inhibition at 100 μM.

haloperidol binding studies. Steroids such as progesterone and testosterone inhibited [^{3}H]ifenprodil binding with moderate affinity. $ZnCl_2$ inhibited [^{3}H]ifenprodil binding with a steep slope (pseudo Hill slope of 2.3 ± 0.5 ($n = 3$)) and an IC_{50} of 4.4 ± 2.3 μM ($n = 3$). $CuCl_2$ was 100-fold less potent (see legend to Table 1). To ensure that the drug binding sites were membrane associated, we compared [^{3}H]ifenprodil binding in the *S. cerevisiae* homogenate and in membranes obtained by low- (500*g* (P1)) and high-speed (100000*g* (P2)) centrifugation. As expected, the highest densities were found in P2 whereas the 100000*g* supernatant contained essentially no binding activity (not shown). Washing of the membranes with 0.5 M KCl, 0.15 M Tris-HCl, pH 8 (4 °C), increased the density of binding sites (1.6 ± 0.2)-fold ($n = 3$). The binding protein was solubilized efficiently (>90%, $n = 5$) by 1% (w/v) digitonin. The sedimentation coefficient of the digitonin-solubilized [^{3}H]-ifenprodil binding site as determined by ultracentrifuation in 5−30% (w/v) sucrose density gradients (data not shown) was 9.0 ± 0.2 S ($n = 3$).

Strains Devoid of Isomerase Activity Do Not Bind [^{3}H]-Haloperidol and [^{3}H]Ifenprodil. If the high-affinity drug binding sites in *S. cerevisiae* are associated with the sterol C_8-C_7 isomerase they should be absent in *S. cerevisiae* mutant strains which lack the sterol C_8-C_7 isomerase. We therefore measured [^{3}H]haloperidol and [^{3}H]ifenprodil binding to membranes prepared from strain WA10-3-1A (Ashman et al., 1991) in which the ERG2 gene had been disrupted. This strain was devoid of [^{3}H]haloperidol and [^{3}H]ifenprodil (not shown) binding sites whereas these sites were present in the related strain WA10-3-1B (Figure 2D). [^{3}H]Haloperidol and [^{3}H]ifenprodil (not shown) binding sites were also absent in the ERG2 mutant strain WA0 (Ashman et al., 1991) but were restored upon transformation with the ERG2 DNA (Figure 2D). This indicates that these high-affinity drug binding sites were indeed associated with the ERG2 gene product.

Isomerase Inhibitors Potently Inhibit [^{3}H]Ifenprodil Binding. To obtain further evidence that these drug binding domains are associated with the sterol isomerase we measured the affinity of the [^{3}H]ifenprodil binding site for known inhibitors of sterol isomerization. The agricultural fungicides fenpropimorph and tridemorph as well as AY-9944, MDL28,815, and triparanol potently inhibited [^{3}H]ifenprodil binding with K_i values of 0.05−5.8 nM (Table 1). The [^{3}H]-ifenprodil labeled binding site displayed also high affinity for the haloperidol analogue trifluperidol (K_i value 0.15 ± 0.03 nM ($n = 3$), Table 1) which inhibits sterol isomerization in yeast (Sobus et al., 1977).

[^{3}H]Haloperidol Photoaffinity Labels the Sterol C_8-C_7 Isomerase. To prove that the sigma ligand binding sites were localized on the 24.9 kDa protein encoded by ERG2, we determined the apparent molecular mass of the drug binding protein in SDS−PAGE after irreversible labeling. We used the butyrophenone [^{3}H]haloperidol as a photoaffinity label, because the [^{3}H]ifenprodil binding site displayed only low affinity for the arylazide azidopamil (K_i > 10 μM, Table 1). For photoaffinity labeling, microsomal protein was pre-incubated with [^{3}H]haloperidol and irradiated with ultraviolet light. Separation of photolabeled proteins by SDS−PAGE and slicing of SDS-gels revealed labeling of a 27 kDa protein (not shown). Specific photoincorporation depended on the irradiation time and was completely blocked in the presence of 1 μM ifenprodil (not shown). To demonstrate that the pharmacological profile of photoaffinity labeling was identical with the properties of reversible [^{3}H]ifenprodil and [^{3}H]haloperidol binding, we analyzed the reversible [^{3}H]-haloperidol binding activity by filtration of a sample (Figure 3A) prior to photolysis and SDS−PAGE. The fluorogram obtained after 35 days of exposure showed that photo-affinitylabeling of a 27.4 ± 0.7 kDa ($n = 4$) polypeptide (Figure 3B) reflected the pharmacological profile of reversible [^{3}H]ifenprodil and [^{3}H]haloperidol binding: ifenprodil and haloperidol (0.1 μM) as well as $ZnCl_2$ (100 μM) blocked photoaffinitylabeling as well as reversible binding, whereas pentazocine (1 μM) and ditolylguanidine (1 μM) had essentially no effect. $CuCl_2$ (100 μM) did not affect binding but inhibited photoincorporation. Reduction of disulfide bonds with dithiothreitol prior to SDS−PAGE neither affected migration nor photoincorporation (Figure 3B, lanes 1 and 8, respectively). To unequivocally demonstrate that the photolabeled 27 kDa protein was the ERG2 gene product we expressed the ERG2 DNA with an N-terminal 9E10 c-myc epitope in the ERG2 mutant strain WA0. A monoclonal antibody against this epitope (Evan et al., 1985) recognized a 28 kDa protein in Western blots (Figure 4A) and immunoprecipitated the [^{3}H]haloperidol photolabeled protein under denaturating conditions (Figure 4B), whereas no immunostaining or immunoprecipitation of the non-tagged ERG2 were observed.

Sigma Ligands Modulate the Growth and Sterol Composition of S. cerevisiae. To assess the biological significance

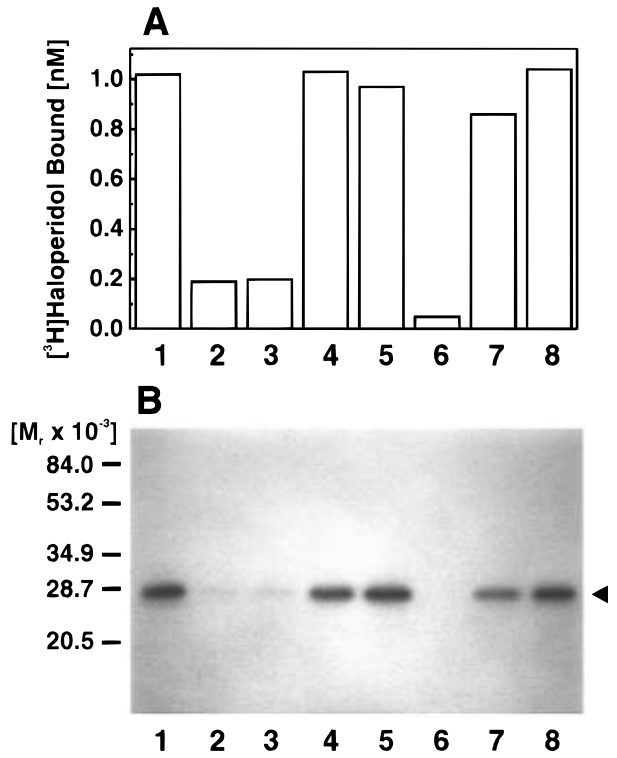

FIGURE 3: Photoaffinity labeling of the sterol C_8-C_7 isomerase with [^{3}H]haloperidol. 25 μg/mL of microsomal protein were incubated in the dark with 2 nM [^{3}H]haloperidol in 8 mL 25 mM Tris-HCl, pH 9 (4 °C). After 6 h at 22 °C 0.25 mL samples were filtrated to quantify reversible binding. (A) Pharmacological profile of reversible [^{3}H]haloperidol binding in the absence (lanes 1 and 8) or presence of 0.1 μM haloperidol (lane 2), 0.1 μM ifenprodil (lane 3), 1 μM pentazocine (lane 4), 1 μM ditolylguanidine (lane 5), 100 μM $ZnCl_2$ (lane 6), 100 μM $CuCl_2$ (lane 7). (B) After photolysis for 6 min the membranes were collected by centrifugation, resuspended in SDS sample buffer containing 10 mM *N*-ethylmaleimide (lanes 1−7) or 10 mM dithiothreitol (lane 8), and separated on a 15% (w/v) SDS−polyacrylamide gel. The gel was fixed, treated with Amplify, and subjected to fluorography for 35 days.

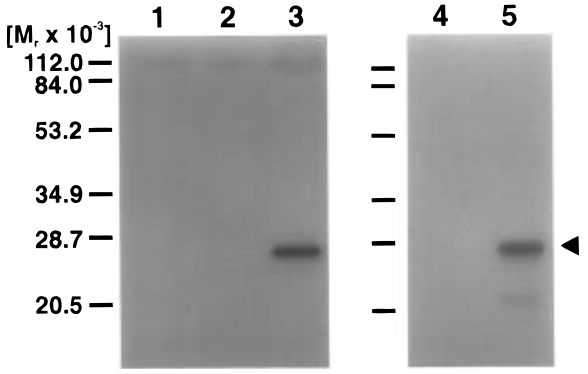

FIGURE 4: 9E10 c-myc immunoblotting and immunoprecipitation of the [^{3}H]haloperidol photolabeled 9E10 c-myc tagged sterol C_8-C_7 isomerase. For immunoblotting (lanes 1−3) 5 μg of microsomal protein prepared from strain WA0 transformed with the vector alone (lane 1), with the vector carrying the ERG2 DNA (lane 2), or with the 9E10 c-myc tagged ERG2 DNA (lane 3) were separated on a 15% (w/v) SDS−polyacrylamide gel, blotted, and immunostained with 10 ng/mL of 9E10 c-myc IgG as described (Moebius et al., 1994). 10 μg/mL of microsomal yeast protein from WA0 cells transformed with the vector carrying the ERG2 DNA (lane 4) or the 9E10 c-myc tagged ERG2 DNA (lane 5) were incubated with 4 nM [^{3}H]haloperidol. After photolysis samples were subjected to immunoprecipitation with 1 μg of c-myc antibody coupled to anti mouse IgG conjugated sepharose as described in Experimental Procedures.

of high-affinity haloperidol and ifenprodil binding to the ERG2 gene product, we tested whether sigma ligands modulate the aerobic growth of *S. cerevisiae* (Figure 5A). Haloperidol and ifenprodil at 50 μM slowed cell growth (initial $OD_{600} \approx 0.15$). 50 μM haloperidol and ifenprodil reduced 12 h growth by 63 ± 12% ($n = 5$) and 67 ± 5% ($n = 5$) as compared to 50 μM of the antifungal agents trifluoperazine and miconazole, whereas the hydrophobic β-adrenoceptor blocker (±)-propranolol as well as the sigma ligands pentazocine, (−)-norallylmetazocine, ditolylguanidine, and (+)-3-PPP (50 μM, data not shown) had no effect. A more pronounced growth inhibition was observed when inhibition experiments were started with lower initial cell numbers (Figure 5B). With an initial $OD_{600} \approx 0.04$, concentrations of 4 ± 2 ($n = 4$), 6 ± 2 ($n = 3$), and 8 ± 1 ($n = 4$) μM haloperidol, trifluperidol, and ifenprodil, respectively, inhibited growth measured after 16 h by 50% (slope factors 1.6 ± 0.5, 1.6 ± 0.2, and 3.6 ± 1.0, respectively).

If sigma ligands do not only bind to the sterol C_8-C_7 isomerase but also inhibit the enzymatic activity, haloperidol and ifenprodil should prevent the conversion of fecosterol to episterol and hence inhibit ergosterol synthesis. We therefore analyzed the ergosterol content of cells grown in the absence or presence of haloperidol, trifluperidol and ifenprodil by spectrophotometric scanning of the non-saponifiable lipids extracted from equal amounts of cell mass. All three drugs reduced the amount of 5,7-sterols (UV absorption peaks at 293, 282, and 271 nm, respectively, (Nes, 1985)) in a concentration dependent manner (Figure 5C−E), whereas 50 μM (±)-propranolol, ditolylguanidine, and (+)-3-PPP had no effect (not shown). 20 μM ifenprodil induced an additional absorption peak at 250 nm (Figure 5E). The spectrophotometric profile obtained from cells grown in the presence of 10 μM haloperidol or ifenprodil was similar to the profile of the ERG2 mutant strain WA0 (Figure 5C,E) as well as to the profile obtained from cells grown in the presence of 0.02 μM fenpropimorph (Figure 5F) which inhibits sterol C_8-C_7 isomerase and Δ^{14}-reductase (Marcireau et al., 1990) or of 1 μM of miconazole (not shown) which is an inhibitor of lanosterol demethylase (Mercer, 1991). Concentrations of 0.016 ± 0.004 ($n = 4$), 1.9 ± 0.2 ($n = 4$), 2.6 ± 0.1 ($n = 3$), and 5.2 ± 0.3 ($n = 4$) μM fenpropimorph, haloperidol, trifluperidol, and ifenprodil, respectively, inhibited ergosterol biosynthesis by 50% (slope factors 2.1 ± 1.0, 1.3 ± 0.4, 1.2 ± 0.1, and 1.0 ± 0.1, respectively).

DISCUSSION

Yeast Sterol C_8-C_7 Isomerase Carries a High-Affinity Drug Binding Site. Our evidence that not only the mammalian $sigma_1$ receptor but also the homologous yeast sterol C_8-C_7 isomerase forms a high-affinity drug acceptor site is as follows: The ERG2 wild type yeast strain JB811 contained high densities (B_{max} value of 60−80 pmol/mg of protein) of high-affinity binding sites for the sigma ligands [^{3}H]haloperidol and [^{3}H]ifenprodil, whereas this activity was absent in strains WA10-3-1A and WA0 in which the ERG2 gene had been disrupted or mutagenized (Ashman et al., 1991). Binding activity was restored upon expression of the ERG DNA. This confirmed the association of these binding sites with the ERG2 gene product. The yeast binding site

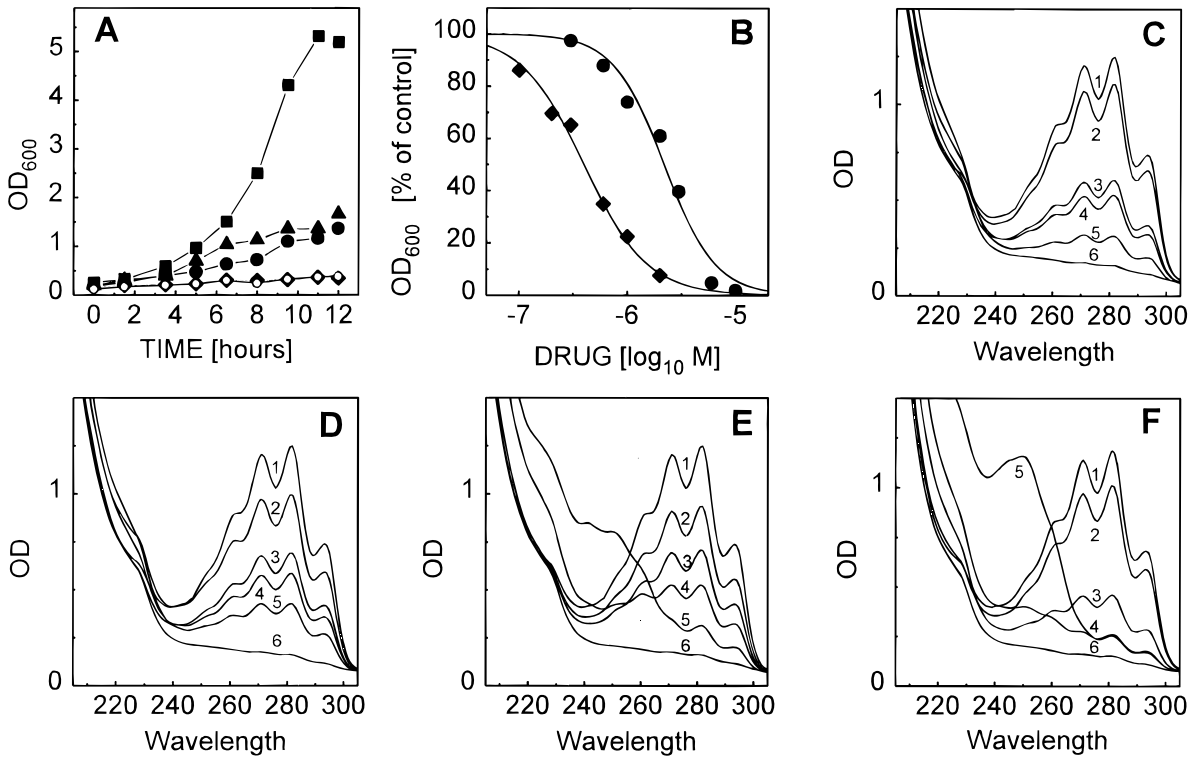

FIGURE 5: Modulation of growth and sterol profile of *S. cerevisiae* by sigma ligands. (A) *S. cerevisiae* strain JB811 was grown in YPD at 30 °C in suspension in the absence (■) or presence of 50 μM haloperidol (●), 50 μM ifenprodil (▲), 50 μM trifluoperazine (◆), or 50 μM miconazole (○). The OD_{600} was measured at the indicated times. (B) Concentration-dependent inhibition of growth by haloperidol (◆) and ifenprodil (●). Cells were grown 25 h to stationary phase and diluted 1:1500 into fresh YPD medium containing haloperidol or ifenprodil. Data were normalized to the growth in the absence of drug. One of two experiments is shown (haloperidol IC_{50} = 0.4 μM, ifenprodil IC_{50} = 1.9 μM). C−F Sterol analysis from strain WA0 (6) or strain JB811 grown in the absence (1) or presence of 1 (2), 2 (3), 5 (4), and 10 (5) μM haloperidol (C); 1 (2), 2 (3), 5 (4), and 10 (5) μM trifluperidol (D); 2 (2), 5 (3), 10 (4), and 20 (5) μM ifenprodil (E); and 0.01 (2), 0.02 (3), 0.05 (4), and 0.1 (5) μM fenpropimorph (F).

displayed the expected high affinity (K_i = 0.05−6 nM) for drugs known to inhibit sterol isomerization in eukaryonts such as trifluperidol (Sobus et al., 1977), triparanol (Campagnoni et al., 1977), AY-9944 (Schmitt & Benveniste, 1979), MDL28,815 (Gerst et al., 1988), tridemorph (Raederstorff & Rohmer, 1987), and fenpropimorph (Rahier et al., 1986; Raederstorff & Rohmer, 1987). The slopes of the inhibition curves were close to unity. Therefore the interaction of the isomerase inhibitors with the [^{3}H]ifenprodil binding site is most likely competitive. Irreversible labeling with the light sensitive (Panaggio & Greene, 1983) butyrophenone [^{3}H]haloperidol revealed photoincorporation into a protein with an apparent molecular weight of 27 400 in SDS−PAGE close to the calculated mass of the ERG2 gene product (24.9 kDa) (Arthington et al., 1991). This photolabeled protein carries the high-affinity binding site, because the pharmacological profile of reversible binding and irreversible labeling were essentially identical. When the ERG2 DNA was tagged with a c-myc epitope and the tagged isomerase was expressed in an ERG2 mutant strain, 9E10 c-myc antibodies specifically immunoprecipitated the [^{3}H]-haloperidol photolabeled protein. This unequivocally demonstrates that the high affinity [^{3}H]haloperidol binding domain is localized on the protein encoded by ERG2. Our data prove that the yeast sterol C_8−C_7 isomerase carries a polyvalent drug binding site which probably overlaps with the binding domain for sterol isomerase inhibitors.

Binding Site of S. cerevisiae Is Related with Mammalian Sigma Binding Sites. The detailed characterization of the drug binding sites for [^{3}H]haloperidol and [^{3}H]ifenprodil in *S. cerevisiae* revealed that they both share the slow association and dissociation of the radioligand (Figure 2C) with the (+)-[^{3}H]pentazocine binding site on the mammalian $sigma_1$ receptor (Hanner et al., 1996). The affinity of haloperidol and ifenprodil for the yeast sterol C_8−C_7 isomerase (K_d of 0.3 and 1.4 nM, respectively) was essentially identical with their affinity for the [^{3}H]pentazocine labeled $sigma_1$ receptor of guinea pig liver (K_i of 0.2 and 2 nM, respectively (Hanner et al., 1996)). Whereas the mammalian (+)-[^{3}H]pentazocine binding site was sensitive to divalent cations only at high concentrations (Basile et al., 1992), $ZnCl_2$ inhibited [^{3}H]-ifenprodil binding to ERG2 with an IC_{50} of 4.4 μM. $CuCl_2$ was 100-fold less potent, indicating that ERG2 carries a selective divalent cation binding site. The pharmacological profile of drug binding to ERG2 differed from that of the mammalian $sigma_1$ binding site showing low affinity for the tricyclic benzomorphans pentazocine and norallylmetazocine, the phenylpiperidine (+)-3-PPP, and the guanidine derivative ditolylguanidine. However, it had in common with the guinea pig $sigma_1$ receptor the high affinity for haloperidol, ifenprodil, and opipramol (Hanner et al., 1996) as well as the moderate affinity for progesterone and testosterone. The rank order of potency (progesterone > testosterone ≫ corticosterone) was the same as for the mammalian (+)-[^{3}H]pentazocine-labeled $sigma_1$ receptor (Hanner et al., 1996). We propose to classify the high-affinity drug binding domains on the yeast sterol C_8−C_7 isomerase as sigma binding sites because this protein is structurally related to the mammalian $sigma_1$ binding protein (Hanner et al., 1996),

Table 2: Comparison of the K_i Values of the [^{3}H]Ifenprodil Binding Site and the EC_{50} Values for Inhibition of Ergosterol Biosynthesis[a]

compound	K_i (nM)	EC_{50} (μM)	EC_{50}/K_i
fenpropimorph	0.05	0.016	320
trifluperidol	0.15	1.9	12 700
haloperidol	0.5	2.6	5 200
ifenprodil	0.9	5.2	5 800

[a] K_i and EC_{50} values were determined as described in Experimental Procedures.

binds sigma ligands with high affinity and has affinity for steroids.

Sigma Ligands Inhibit the Sterol C_8-C_7 Isomerase of S. cerevisiae. Haloperidol and ifenprodil, but not pentazocine and ditolylguanidine, inhibited the growth of *S. cerevisiae*. It has been suggested that the fungistatic activity of fenpropimorph is due primarily to the inhibition of ergosterol biosynthesis rather than the accumulation of toxic sterols (Marcireau et al., 1990). Disruption of the sterol C_8-C_7 isomerase-encoding gene is indeed lethal in the absence of exogenous ergosterol (Silve et al., 1996). The sterol profile of cells grown in the presence of haloperidol, trifluperidol, and ifenprodil showed a concentration-dependent reduction of 5,7-sterols. Ifenprodil induced the formation of an additional 250 nm peak which was also seen after growth in the presence of 0.1 μM fenpropimorph. It probably originates from ignosterol formed upon inhibition of the Δ^{14}-reductase (ERG24 gene product (Lees et al., 1995)) which is an additional target of fenpropimorph and other sterol isomerase inhibitors (Mercer, 1991).

Trifluperidol ($K_i = 0.15$ nM) as well as triparanol ($K_i = 1.5$ nM) inhibit sterol isomerization in yeast and lead to the accumulation of Δ^8-sterols (Sobus et al., 1977; Campagnoni et al., 1977). Trifluperidol differs from haloperidol only by the presence of a trifluoromethyl group instead of a chlorine substituent. We therefore propose that the observed inhibition of ergosterol biosynthesis by haloperidol and ifenprodil is due to reduced activity of the sterol C_8-C_7 isomerase. Our findings are in line with the block of cell proliferation by the sigma ligand SR31747 which is mediated by the yeast sterol C_8-C_7 isomerase (Silve et al., 1996). The discrepancy between the EC_{50} values determined *in vivo* and the K_i values measured *in vitro* (Table 2) might be due to adaptive changes upon inhibition, such as the compensatory induction of enzymatic activity, competition of the accumulating substrate fecosterol, and intracellular drug metabolism. Acidification of the medium, drug extrusion mechanisms, and trapping of drugs in hydrophobic membrane compartments could also reduce the number of free drug molecules in the cell.

Consequences for Mammalian Sigma Receptors? Our finding that the yeast sterol C_8-C_7 isomerase binds sigma ligands with high affinity supports our hypothesis that the so-called $sigma_1$ receptor is related to the yeast sterol C_8-C_7 isomerase. Previous studies on the effects of sigma ligands in mammals suffered from the lack of information on the function of the ligand binding protein, and it was therefore not possible to connect the observed effect to a biochemical pathway. In yeast the pharmacological effect of sigma ligands, i.e., the inhibition of ergosterol biosynthesis and growth can be rationally explained considering the function of the sigma ligand binding protein which is the sterol C_8-C_7 isomerase. If we take into account the altered membrane permeability in yeast ergosterol biosynthesis mutants (Welihinda et al., 1994; Gaber et al., 1989; Prendergast et al., 1995) and the known effects of cholesterol on the activity of membrane proteins (Renaud et al., 1986; Chang et al., 1995; Bodovitz & Klein, 1996; Bennett & Simmonds, 1996; Schroeder et al., 1996), it is conceivable that some of the effects of sigma ligands in mammalian neurons are mediated by changes in the composition of membrane lipids.

Our work demonstrates that the ERG2 gene product carries a high-affinity binding site for sterol isomerase inhibitors. This lends support to the assumption that the ERG2 gene encodes the sterol C_8-C_7 isomerase enzyme itself and not a regulatory subunit. The evolutionarily conserved affinity of the yeast and mammalian binding sites for haloperidol and ifenprodil suggests that the binding domains for these drugs are identical with the sterol acceptor site or with the catalytic center. The isomerization reaction involves a carbocationic reaction intermediate generated by proton transfer but the required donor and acceptor amino acid residues for the proton and the molecular structures of the sterol and inhibitor binding sites are not yet known. [^{3}H]Ifenprodil binding and [^{3}H]haloperidol photolabeling can be combined with site-directed mutagenesis to elucidate the molecular basis of high-affinity drug binding by mapping the amino acid residues which are part of the inhibitor binding site. These mutants can be also used to identify amino acid residues required for sterol C_8-C_7 isomerization, thereby providing insight into the molecular mechanism of the isomerization reaction. This will enable us to investigate whether the same amino acid residues which are essential for isomerase function are also important for high-affinity inhibitor binding.

ACKNOWLEDGMENT

We are grateful to Dr. M. Bard (Indianapolis, IN) for providing the yeast strains WA0 and WA10-3-1A and B and the ERG2 DNA, B. Fiechtner for expert technical assistance, and Dr. J. Striessnig for invaluable discussion and enthusiastic support.

REFERENCES

Arthington, B. A., Hoskins, J. A., Skatrud, P. L., & Bard, M. (1991) *Gene 107*, 173.

Ashman, W. H., Barbuch, R. J., Ulbright, C. E., Jarrett, H. W., & Bard, M. (1991) *Lipids 26*, 628.

Basile, A. S., Paul, I. A., Mirchevich, A., Kuijpers, G., & De Costa, B. (1992) *Mol. Pharmacol. 42*, 882.

Bennett, P. J., & Simmonds, M. A. (1996) *Br. J. Pharmacol. 17*, 87.

Bodovitz, S., & Klein, W. L. (1996) *J. Biol. Chem. 271*, 4436.

Boer, R., Grassegger, A., Schudt, C., & Glossmann, H. (1989) *Eur. J. Pharmacol. 172*, 131.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.

Campagnoni, C., Holmlund, C. E., & Whittaker, N. (1977) *Arch. Biochem. Biophys. 184*, 555.

Chang, H. M., Reitstetter, R., Mason, R. P., & Gruener, R. (1995) *J. Membr. Biol. 143*, 51.

DeLean, A., Munson, P. J., & Rodbard, D. (1978) *Am. J. Physiol. 4*, E97.

Evan, G. I., Lweis, G. K., Ramsay, G., & Bishop, J. M. (1985) *Mol. Cell. Biol. 5*, 3610.

Gaber, R. F., Copple, D. M., Kennedy, B. K., Vidal, M., & Bard, M. (1989) *Mol. Cell. Biol. 9*, 3447.

Gerst, N., Duriatti, A., Schuber, F., Taton, M., Benveniste, P., & Rahier, A. (1988) *Biochem. Pharmacol. 37*, 1955.
Hanner, M., Moebius, F. F., Weber, F., Grabner, M., Striessnig, J., & Glossmann, H. (1995) *J. Biol. Chem. 270*, 7551.
Hanner, M., Moebius, F. F., Flandorfer, A., Knaus, H.-G., Striessnig, J., Kempner, E., & Glossmann, H. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 8072.
Keon, J. P. R., James, C. S., Court, S., Baden-Daintree, C., Bailey, A. M., Burden, R. S., Bard, M., & Hargreaves, J. A. (1994) *Curr. Genet. 25*, 531.
Lee, W.-H., Kammereck, R., Lutsky, B. N., McCloskey, J. A., & Schroepfer, G. J. (1969) *J. Biol. Chem. 244*, 2033.
Lees, N. D., Skaggs, B., Kirsch, D. R., & Bard, M. (1995) *Lipids 30*, 221.
Linden, J. (1982) *J. Cyclic Nucleotide Res. 8*, 163.
Marcireau, C., Guilloton, M., & Karst, F. (1990) *Antimicrob. Agents Chemother. 34*, 989.
Mercer, E. I. (1991) *Lipids 26*, 584.
Moebius, F. F., Burrows, G. G., Striessnig, J., & Glossmann, H. (1993) *Mol. Pharmacol. 43*, 139.
Moebius, F. F., Hanner, M., Knaus, H.-G., Weber, F., Striessnig, J., & Glossmann, H. (1994) *J. Biol. Chem. 269*, 29314.
Molzahn, S. W., & Woods, R. A. (1972) *J. Gen. Microbiol. 72*, 339.
Nes, W. R. (1985) *Methods Enzymol. 111*, 3.
Panaggio, A., & Greene, D. S. (1983) *Drug Dev. Ind. Pharm. 9*, 485.
Prendergast, J. A., Singer, R. A., Rowley, N., Rowley, A., Johnston, G. C., Danos, M., Kennedy, B., & Gaber, R. F. (1995) *Yeast 11*, 537.
Prinz, H., & Striessnig, J. (1993) *J. Biol. Chem. 268*, 18580.
Raederstorff, D., & Rohmer, M. (1987) *Eur. J. Biochem. 164*, 421.
Rahier, A., Schmitt, P., Huss, B., Benveniste, P., & Pommer, E. H. (1986) *Pestic. Biochem. Physiol. 25*, 112.
Ramsey, R. B., Fredericks, M., & Fischer, V. W. (1977) *J. Neurochem. 28*, 1317.
Renaud, J. F., Schmid, A., Romey, G., Nano, J. L., & Lazdunski, M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8007.
Schmitt, P., & Benveniste, P. (1979) *Phytochemistry 18*, 445.
Schroeder, F., Woodford, J. K., Kavecansky, J., Wood, W. G., & Joiner, C. (1996) *Mol. Membr. Biol. 12*, 113.
Silve, S., Leplatois, P., Josse, A., Dupuy, P. H., Lanau, C., Kaghad, M., Dhers, C., Picard, C., Rahier, A., Taton, M., Le-Fur, G., Caput, D., Ferrara, P., & Loison, G. (1996) *Mol. Cell. Biol.* 16**,** 2719.
Sobus, M. T., Holmlund, C. E., & Whittaker, N. F. (1977) *J. Bacteriol. 130*, 1310.
Walker, J. M., Bowen, W. D., Walker, F. O., Matsumoto, R. R., De Costa, B., & Rice, K. C. (1990) *Pharmacol. Rev. 42*, 355.
Welihinda, A. A., Beavis, A. D., & Trumbly, R. J. (1994) *Biochim. Biophys. Acta 1193*, 107.

BI961996M