Histidine[77], Glutamic Acid[81], Glutamic Acid[123], Threonine[126], Asparagine[194], and Tryptophan[197] of the Human Emopamil Binding Protein Are Required for in Vivo Sterol Δ8−Δ7 Isomerization[†]

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ABSTRACT: The human emopamil binding protein (hEBP) exhibits sterol Δ8−Δ7 isomerase activity (EC 5.3.3.5) upon heterologous expression in a sterol Δ8−Δ7 isomerization-deficient *erg2-3* yeast strain. Ala scanning mutagenesis was used to identify residues in the four putative transmembrane α-helices of hEBP that are required for catalytic activity. Isomerization was assayed in vivo by spectrophotometric quantification of Δ5,7-sterols. Out of 64 Ala mutants of hEBP only H77A-, E81A-, E123A-, T126A-, N194A-, and W197A-expressing yeast strains contained 10% or less of wild-type (wt) Δ5,7-sterols. All substitutions of these six residues with functionally or structurally similar amino acid residues failed to fully restore catalytic activity. Mutants E81D, T126S, N194Q, and W197F, but not H77N and E123D, still bound the enzyme inhibitor ^{3}H-ifenprodil. Changed equilibrium and kinetic binding properties of the mutant enzymes confirmed our previous suggestion that residues required for catalytic activity are also involved in inhibitor binding [Moebius et al. (1996) *Biochemistry 35*, 16871−16878]. His[77], Glu[81], Glu[123], Thr[126], Asn[194], and Trp[197] are localized in the cytoplasmic halves of the transmembrane segments 2−4 and are proposed to line the catalytic cleft. Ala mutants of Trp[102], Tyr[105], Asp[109], Arg[111], and Tyr[112] in a conserved cytoplasmic domain (WKEYXKGDSRY) between transmembrane segments 2 and 3 contained less than 10% of wt Δ5,7-sterols, implying that this region also could be functionally important. The in vivo complementation of enzyme-deficient yeast strains with mutated cDNAs is a simple and sensitive method to rapidly analyze the functional consequences of mutations in sterol modifying enzymes.

The biosynthesis of cholesterol involves a variety of intermediates and enzymatic systems. After squalene cyclase (EC 5.4.99.7) mediated formation of lanosterol, the ring system is further modified by demethylations at C_{14} and C_4 as well as by removal, shift, and introduction of double bonds in ring B and C and in the side chain. All genes encoding enzymes required for sterol biosynthesis from squalene in *Saccharomyces cerevisiae* have been identified (*1*−*3*). Among the mammalian homologues cloned so far are the lanosterol demethylase (*4*), the C_4-methyloxidase (*5*), the sterol Δ8−Δ7 isomerase [EC 5.3.3.5 (*6*)], the Δ5-dehydrogenase [EC 1.3.3.2 (*7*)], and the Δ7-sterol reductase [EC 1.3.1.21 (*8*)]. The latter enzyme is of outstanding medical significance because a variety of different missense and nonsense mutations in the corresponding human gene (*9*) cause a frequent malformation syndrome [Smith−Lemli−Opitz syndrome (*10*)]. The human sterol Δ8−Δ7 isomerase was purified on the basis of its ability to bind the antiischemic drug (−)-^{3}H-emopamil and was tentatively named emopamil binding protein [EBP[1] (*11*−*13*)]. Complementation of a sterol Δ8−Δ7 isomerization-deficient yeast strain by the EBP cDNA (*6*) suggested that the EBP is a mammalian isoenzyme of the yeast ERG2p. In contrast to the EBP, the ERG2p homologue from vertebrates, that was purified and cloned as a drug binding protein ($sigma_1$ receptor) failed to complement the *erg2-3* mutation (*14*). The human sterol Δ8−Δ7 isomerase (EBP) and the yeast isoenzyme (ERG2p) are structurally unrelated (*15*), most likely because they differ in the substrate specificity (ERG2p, fecosterol; EBP, zymosterol) and the reaction mechanism [ERG2p, cis hydrogen addition−elimination; EBP, trans hydrogen addition−elimination (*16*)].

The molecular features that are indispensable for catalytic activity of enzymes involved in the biosynthesis of sterols from lanosterol are enigmatic. The EBP, like the Δ7-sterolreductase (*8*), is an integral membrane protein with a high proportion of hydrophobic residues (*13*). Its hydrophobicity renders a structural characterization through crystallization and/or NMR challenging. To provide a framework

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[1] Abbreviations: EBP, emopamil binding protein; hEBP, human emopamil binding protein; TMS, transmembrane segment; wt, wild type.

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for future structural studies, we identified amino acid residues that are indispensable for catalytic activity by systematic site-directed mutagenesis of codons for residues in the four putative transmembrane α-helices of human EBP (*13*). We avoided the technical difficulties of the in vitro determination of sterol Δ8−Δ7 isomerase activity (*17*−*22*) by quantifying the ability of mutant enzymes to complement the *erg2-3* mutation (isomerase deficiency) in yeast. In the *erg2-3* mutant strain, Δ8-sterols such as ergostan-5,8,22-trien-3*β*-ol and ergostan-8-en-3*β*-ol accumulate (*23*). Expression of the isomerase results in Δ8−Δ7 isomerization and thereby in the formation of Δ5,7-sterols such as ergosterol (ergostan-5,7,22-trien-3*β*-ol) (*6*, *23*). Such sterols can be quantified spectrophotometrically because the conjugated Δ5,7-ring system of ergosterol has a characteristic UV absorbance profile (*24*).

Residues that, upon substitution with Ala, led to a substantial decrease of catalytic activity of mutant enzymes were substituted with functionally or structurally related residues. We also measured the ability of several substitution mutants with impaired catalytic activity to bind the isomerase inhibitor ^{3}H-ifenprodil because amino acid residues that are indispensable for isomerase function were suggested to be involved in high-affinity inhibitor binding (*25*). To our knowledge this is the first report on the identification of functionally important residues in an enzyme of postsqualene sterol biosynthesis.

EXPERIMENTAL PROCEDURES

Materials. ^{3}H-Ifenprodil (50 Ci/mmol) and ^{125}I-protein A (8 mCi/mg) were from NEN, Vienna, Austria. Other chemicals were obtained from the following sources: Bradford protein reagent and prestained molecular weight markers, Bio-Rad, Vienna, Austria; oligonucleotides, MWG, Penzberg, Germany; all other chemicals, Sigma, Vienna, Austria. *Saccharomyces cerevisiae* strain WA0 was kindly provided by Dr. M. Bard (Indianapolis, IN).

Site-Directed in Vitro Mutagenesis. Mutations were introduced into the 687 bp hEBP cDNA [HS1 (*13*); here EBP_{high}] by two-step mutagenesis with the proofreading thermostable DNA polymerase from *Pyrococcus furiosus* (Stratagene) as described (*26*). Mutagenic oligonucleotides had a 9−12 bp overlap with the cDNA sequence before and after the mutated codon. PCR amplicons were subcloned with *Xho*I−*Not*I into the 2 *μ* expression vector YEp351ADC1 (*13*) and sequenced with oligonucleotides TCAAGCTATACCAAGCATAC or GATTGGAGACTTGACCAAAC, respectively. DNA sequencing was carried out by Toplab (Martinsried, Germany) with dye terminators and an ABI377 automated sequencer. Presence of the mutation was verified by single 600−650 bp sequence reads. The only second-site mutations that we found were within the mutagenic oligonucleotides, most likely due to technical problems with the synthesis of one batch of oligonucleotides (e.g., I80A: experiment 1, H77P,I80A; experiment 2, L78L,I80A,W83G; experiment 3, H77P,L78L,I80A; the I80A mutation was therefore omitted from further analysis). Minor second-site mutations within the sequence of the mutagenic oligonucleotides were found in the mutants L78A (I76L) and G82A (F84V). In the mutants E81D, E123D, T126S, N194Q, and W197F as well as in W102A, Y105A, D109A, R111A, and Y112A, second-site mutagenesis was ruled out by sequencing of both strands. Numbering of amino acid residues corresponds to the guinea pig EBP sequence.

Growth of S. cerevisiae and Sterol Analysis. Strain WA0 was grown in 2% (w/v) glucose, 1% (w/v) tryptone, and 2% (w/v) yeast extract. Transformed strains were grown in 2% (w/v) glucose, 0.67% (w/v) yeast nitrogen base, and the amino acids except for leucine (*13*). Microsomal membranes were prepared as described (*25*, *27*). For sterol analysis 20 mL liquid cultures were grown for 18 h at 30 °C (OD_{600} 4−7). Cells were collected by centrifugation and resuspended in 0.1 M NaCl and the OD_{600} was determined. Sterols were extracted from approximately 2×10^8 cells (20 mg of wet weight) as described (*25*). Sterols from a strain transformed with the EBP_{high} cDNA and the vector without cDNA insert (mock) were measured in duplicates. The 281 and 271 nm absorptions of the material extracted from strains expressing hEBP mutants were normalized to the mock strain (mock, 0%) and the EBP_{high} strain (wt, 100%). A strain transformed with EBP_{low} was analyzed in parallel as an internal control. For gas chromatographic analysis, sterols were extracted as referenced above (with 4 nmol of exogenous cholesterol as a standard) and the heptane was evaporated. After resuspension in 30 *μ*L of ethyl acetate, the material was separated on a DB5 column (30 m × 0.25 mm) in a Fisons GC8000/MD800 gas chromatograph/mass spectrometer (temperature gradient 200−300 °C at 10 °C/min) with helium as the carrier gas. Experimental mass spectra were compared with reference spectra in the NIST database.

Binding Assay and Immunoblotting. ^{3}H-Ifenprodil (1 nM) was incubated with yeast microsomes (20−400 *μ*g/mL protein) in 0.2 mL of 25 mM Tris-HCl, pH 9 (4 °C) for 12 h at 22 °C. Nonspecific binding was measured in the presence of 0.5 *μ*M (dissociation experiments) and 1 *μ*M (association experiments) ifenprodil (*27*). Bound and free ligand were separated by filtration over Whatman GF/C filters [pretreated with 0.3% (w/v) polyethylenimine] with ice-cold 10 mM Tris-HCl, pH 7.4 (22 °C). Binding parameters (K_d, B_{max}) were determined by nonlinear curve fitting to a rectangular hyberbola. Kinetic constants (k_{+1}, k_{-1}) were calculated as described (*25*). Protein concentrations were determined according to ref *28* with BSA as a standard. Microsomal proteins were separated on SDS−14% (w/v) polyacrylamide gels. After electrophoretic transfer to a PVDF membrane, hEBP was detected with anti-$EBP_{206-223}$ (*13*) and ^{125}I-protein A (1 *μ*Ci/mL) as described (*9*).

RESULTS

Amount of hEBP and Formation of Δ5,7-Sterols Are Not Linear. In our investigation the sterol Δ8−Δ7 isomerase activity of hEBP was measured as the ability to complement the *erg2-3* mutation of yeast. Enzymatic activity was quantified as the formation of Δ5,7-sterols, which we detected by their 271 and 281 nm absorption maxima (Figure 1A). The maximal in vivo enzymatic activity is theoretically limited by the available substrate (Δ8-sterols) within the cells. The amount of hEBP expressed from the open reading frame of the cDNA without 5′- and 3′-noncoding sequence was previously quantified in terms of high-affinity (−)-^{3}H-emopamil binding sites [70 pmol/mg of microsomal protein (*13*)] which is similar to the density of the endogenous

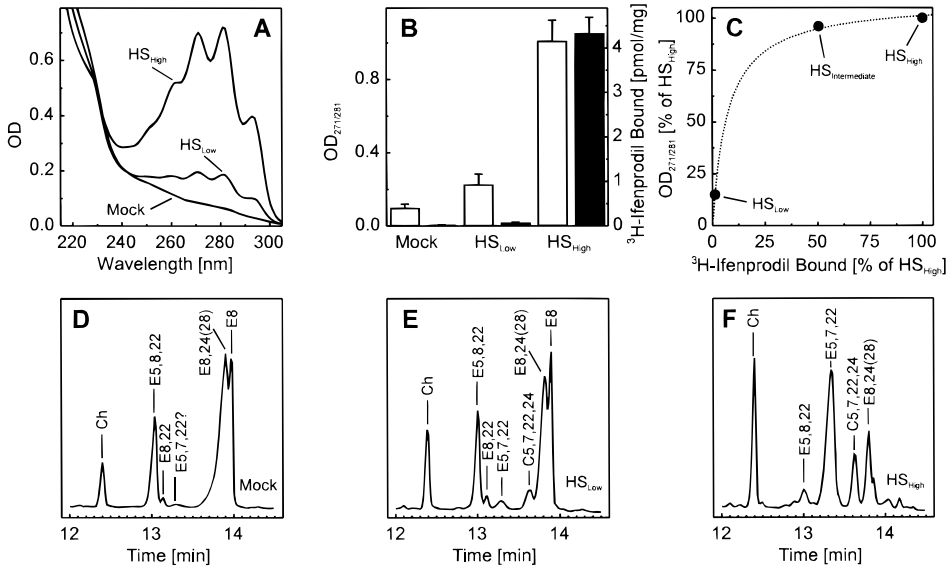

FIGURE 1: Determination of Δ5,7-sterols and ^{3}H-ifenprodil binding sites in strains expressing hEBP at various levels. (A) Spectrophotometric analysis of nonsaponifiable sterols from yeast strain WA0 (*erg2-3*) transformed with the plasmid YEp351ADC1 without insert (mock), the weakly expressed hEBP [HS$_0$ (*13*); EBP$_{low}$, this study], and the overexpressed hEBP [HS$_1$ (*13*); EBP$_{high}$, this study]. Sterols from 20 mg (wet weight) of yeast cell pellets were extracted into 1 mL of heptane. (B) Δ5,7-Sterol content (measured as absolute 271/281 nm absorbance) and hEBP protein (measured as specific ^{3}H-ifenprodil binding activity) for expression constructs EBP$_{low}$ and EBP$_{high}$. The absolute 271/281 nm absorbance (OD$_{271/281}$, open columns, $n = 20$) of sterols extracted from 20 mg of yeast cell pellets into 1 mL of heptan is shown. The specific ^{3}H-ifenprodil binding activity (^{3}H-ifenprodil bound, solid bars, data shown are the mean ± SD from three separate microsomal preparations) was determined at ligand and protein concentrations of 1.0 nM and 40−250 μg/mL, respectively. (C) Relative content in Δ5,7-sterols [measured as 271/281 nm absorbance, normalized to mock (0%) and EBP$_{high}$ (100%), respectively] and hEBP protein [measured as specific ^{3}H-ifenprodil binding sites, normalized to EBP$_{high}$ (100%)] in yeast strains transformed with EBP$_{low}$, EBP$_{intermediate}$, and EBP$_{high}$ cDNAs. Since attempts to make an expression construct with lesser protein expression than EBP$_{intermediate}$ but higher than EBP$_{low}$ failed, the line drawn is suggestive rather than definitive. (D−F) Retention times of sterols extracted from strains transformed with mock (D), EBP$_{low}$ (E), and EBP$_{high}$ (F), respectively. Sterols were identified on the basis of their mass spectra and comparison with the literature (*29*). Ergosterol was identified by comigration with a commercial sample. Ch, cholestan-5-en-3β-ol (exogenous cholesterol, added as a standard); E5,8,22, ergostan-5,8,22-trien-3β-ol; E8,22, ergostan-8,22-dien-3β-ol; E5,7,22, ergostan-5,7,22-trien-3β-ol (ergosterol); E8(24), ergostan-8,24(28)-dien-3β-ol; E8, ergostan-8-en-3β-ol; C5,7,22,24, cholestan-5,7,22,24-tetraen-3β-ol.

isomerase of yeast, the ERG2p (77 pmol/mg of protein, measured with ^{3}H-haloperidol as a ligand (*25*)). For a semiquantitative interpretation of the functional consequences of mutations, it was important to determine whether the amount of hEBP expressed was linear with the formation of Δ5,7-sterols. We previously observed that expression of the full-length cDNA (EBP$_{low}$, open reading frame plus 5′- and 3′-noncoding sequence) resulted in low expression of hEBP (*13*). This construct was therefore employed to investigate whether the reduction of protein expression and the decrease of the Δ5,7-sterol content correlate. This was not the case. Despite >25-fold reduced hEBP expression (*13*), a yeast strain transformed with construct EBP$_{low}$ contained 14% ± 5% ($n = 30$) of EBP$_{high}$ Δ5,7-sterols (Figure 1A), suggesting that the formation of Δ5,7-sterols was not linear with the amount of enzyme protein. To confirm this assumption, we made an expression construct with an expected intermediate expression level (EBP$_{intermediate}$, open reading frame plus 3′-noncoding region). The Δ5,7-sterol content of the strain transformed with EBP$_{intermediate}$ was essentially identical with EBP$_{high}$ [96% ± 7% ($n = 5$)]. The amount of hEBP expressed from EBP$_{low}$, EBP$_{intermediate}$, and EBP$_{high}$ was quantified with ^{3}H-ifenprodil, another high-affinity EBP ligand (*13, 27*), demonstrating that the density of ^{3}H-ifenprodil binding sites and thus hEBP was >50-fold lower in microsomes from the EBP$_{low}$-expressing strain as compared to EBP$_{high}$ (Figure 1B). The expression of the open reading frame with 3′-noncoding sequence (EBP$_{intermediate}$) reduced the protein expression 2-fold [50% ± 14% of EBP$_{high}$ ($n = 3$)]. Our results indicate that the in vivo sterol Δ8−Δ7 isomerase activity of hEBP is not linear with the amount of enzyme protein (Figure 1C), most likely because at a high expression level (which is similar to the endogenous enzyme, the ERG2p) the substrate (Δ8-sterols) becomes limiting for Δ5,7-sterol synthesis. Mutations were introduced into the expression construct with high expression level [EBP$_{high}$ = wild type (wt)] because thereby the in vivo assay became insensitive to minor (2−3-fold) variations of the amount of protein expressed but was still able to detect product formation when enzymatic activity was >50-fold reduced (as in EBP$_{low}$). From our data we conclude that if the amount of mutant and wt protein is identical but the Δ5,7-sterol content of the mutant is <14% of wt, the mutant enzyme is >50-fold less active than wt.

hEBP Expression in the erg2-3 Mutant Produces Δ5,7-Sterols. The spectrophotometric determination of Δ5,7-sterols is simple and allows the analysis of numerous samples. To verify that the assay indeed measured Δ5,7-sterols, we analyzed the sterol composition of strains transformed with mock, EBP$_{low}$, and EBP$_{high}$, respectively, by gas chromatography (Figure 1D−F). Sterols were identified by their mass spectra. The mock-transformed *erg2-3* mutant strain [WA0 (*29*)] contained ergostan-5,8,22-trien-3β-ol, ergostan-8,24-(28)-dien-3β-ol, and ergostan-8-en-3β-ol as major constituents and minor amounts of ergostan-8,22-dien-3β-ol (Figure 1D). Among the sterols from the *erg2-3* mutant a small peak with the mass of ergostan-5,7,22-trien-3β-ol (ergosterol) occurred (Figure 1D), which comigrated with a commercial sample of ergosterol (not shown). This peak was also seen

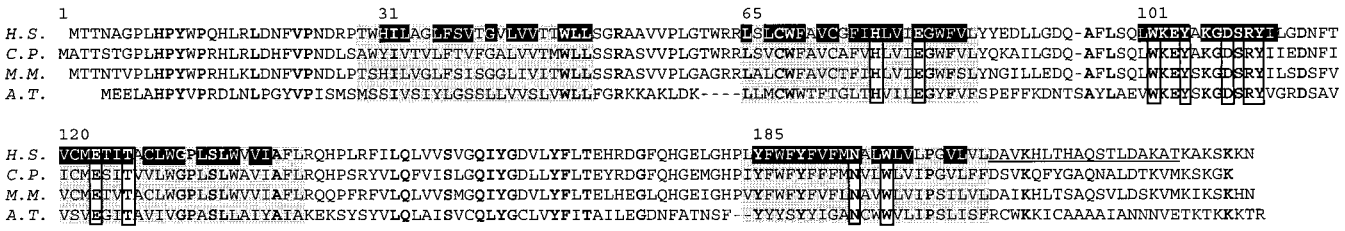

FIGURE 2: Amino acid sequence alignment of sterol Δ8−Δ7 isomerases from *Homo sapiens* (*H.S.*, GenBank accession no. Z37986), *Cavia porcellus* (*C.P.*, GenBank accession no. Z37985), *Mus musculus* (*M.M.*, GenBank accession no. X97775), and *Arabidopsis thaliana* (*A.T.*, GenBank accession no. AF030357). Putative TMS (*13*, gray boxes), residues conserved throughout all four sequences (boldface type), residues substituted with Ala (black boxes), and residues essential for catalytic activity in vivo (white boxes) are indicated. Numbers refer to the position of the residues in the amino acid sequence of the guinea pig EBP. The residues corresponding to the synthetic peptide $EBP_{206-223}$ (*13*) are underlined.

Table 1: In Vivo Activity of Mutant Enzymes in the *erg2-3* Strain WA0[a]

TMS1			TMS2			TMS3			TMS4		
amino acid	codon	activity (% wt)	amino acid	codon	activity (% wt)	amino acid	codon	activity (% wt)	amino acid	codon	activity (% wt)
H31A	GCT	69 ± 7	L65A	GCG	82 ± 9	V120A	GCG	62 ± 5	Y185A	GCC	64 ± 6
I32A	GCA	73 ± 9	L67A	GCT	63 ± 9	C121A	GCC	95 ± 14	F186A	GCC	56 ± 3
L33A	GCT	47 ± 3	C68A	GCC	94 ± 7	M122A	GCG	28 ± 11	W187A	GCG	56 ± 10
L36A	GCC	62 ± 7	W69A	GCG	17 ± 6	E123A	GCA	10 ± 5	F188A	GCT	94 ± 6
F37A	GCC	74 ± 6	F70A	GCT	95 ± 3	T124A	GCC	91 ± 10	Y189A	GCC	32 ± 7
S38A	GCT	74 ± 9	V72A	GCG	93 ± 10	I125A	GCC	78 ± 9	F190A	GCT	21 ± 6
V39A	GCC	80 ± 7	C73A	GCT	83 ± 8	T126A	GCA	4 ± 4	V191A	GCC	91 ± 8
G41A	GCG	94 ± 7	F75A	GCC	88 ± 9	C128A	GCC	81 ± 8	F192A	GCC	92 ± 8
L43A	GCA	103 ± 10	I76A	GCT	33 ± 7	L129A	GCG	81 ± 8	M193A	GCT	53 ± 6
V44A	GCC	76 ± 10	H77A	GCC	9 ± 4	W130A	GCG	70 ± 4	N194	AGCT	5 ± 3
V45A	GCG	79 ± 9	L78A[b]	GCG	74 ± 8	G131A	GCA	69 ± 7	L196A	GCG	62 ±5
W48A	GCG	87 ± 10	V79A	GCG	75 ± 7	L133A	GCC	77 ± 6	W197A	GCG	5 ± 2
L49A	GCG	108 ± 13	E81A	GCG	5 ± 6	S134A	GCC	101 ± 12	L198A	GCG	50 ± 5
L50A	GCG	96 ± 8	G82A[c]	GCC	56 ± 9	L135A	GCG	92 ± 8	V199A	GCG	76 ± 6
			W83A	GCG	97 ± 11	W136A	GCG	104 ± 12	V203A	GCC	71 ± 1
			F84A	GCC	72 ± 8	V138A	GCG	94 ± 11	L204A	GCT	77 ± 5
			V85A	GCT	68 ± 6	I139A	GCC	90 ± 12			

[a] The codon introduced by site-directed mutagenesis in transmembrane segments (TMS) is given. Data shown are mean ± SD ($n = 4-7$). Catalytic activity was quantified by spectrophotometric determination of Δ5,7-sterols in cell pellets from strains expressing mutant hEBPs and normalized to wt (100%) and mock (0%) transformed strains, respectively. [b] Additional I76L mutation. [c] Additional F84V mutation.

in an erg2 disruptant (*30*), suggesting that it is not produced by leakiness of the *erg2-3* mutation. In the strain transformed with EBP_{low}, the amount of ergostan-5,7,22-en-3β-ol was increased and an additional peak tentatively identified as cholestan-5,7,22,24-tetraen-3β-ol appeared (Figure 1E). The sterol profiles from a strain transformed with EBP_{high} (Figure 1F) and ERG2 (not shown), respectively, were similar but not identical. The most prominent difference was the accumulation of zymosterol (identified by comigration with a standard) in ERG2p- but not in hEBP-expressing strains. The sterol analysis confirmed that expression of hEBP in the *erg2-3* mutant strain WA0 indeed resulted in the formation of Δ5,7-sterols depending on the amount of isomerase protein expressed.

Ala Scanning Mutagenesis in TMS1−4. Enzymatic cholesterol modification is supposed to take place within the membrane of the endoplasmic reticulum. Therefore site-directed mutagenesis was focused on the four putative TMS (*13*). The amino acid sequence alignment of the EBP from man, mouse, and guinea pig identified amino acid residues that are identical or similar throughout mammals (Figure 2). Such residues were substituted with Ala by changing the nucleotide sequence of the codon (Table 1). Ala was chosen because its substitution in α-helices in the core of proteins has minimal effects on secondary structure (*31*). Replacement by Ala is therefore expected to reduce or increase the size of the amino acid residues without causing global conformational changes in the α-helix. Due to the hydrophobic nature of the TMS (*13*), most substituted residues were either hydrophobic (Leu, Ile) or aromatic (Trp, Phe, Tyr). Immunoblotting with a sequence-specific antiserum [anti-$EBP_{206-223}$, (*13*)] verified that reduction of UV-absorbing sterols was not due to impaired protein expression (data not shown).

Our results were arbitrarily stratified by the extent of activity reduction. Ala substitution of most aromatic residues (Phe^{37}, Phe^{70}, Phe^{84}, Phe^{186}, Phe^{188}, Phe^{192}, Trp^{48}, Trp^{83}, Trp^{130}, Trp^{136}, and Trp^{187}) had no major effect on Δ5,7-sterol content (56−104% of wt, Table 1). Neither were the Δ5,7-sterols substantially reduced in most strains expressing enzymes with Ala mutations of hydrophilic residues (His^{31}, Cys^{68}, Cys^{73}, Cys^{121}, Ser^{38}, Thr^{124}, and Ser^{134}) (69−101% of wt, Table 1). Out of 64 Ala mutants only 11 resulted in a Δ5,7-sterol content <35% of wt (Table 2). In six mutants the Δ5,7-sterol content was ≤10% of wt, suggesting that these six residues are major determinants of catalytic activity.

Substitution of His^{77}, Glu^{81}, Glu^{123}, Thr^{126}, Asn^{194}, and Trp^{197} with Similar Residues. Although Ala substitution in transmembrane α-helices has minimal effects on secondary structure (*31*), it is impossible to verify that mutations do not impair structural integrity of membrane proteins, e.g., by eliminating charge pairs. His^{77}, Glu^{81}, Glu^{123}, Thr^{126}, Asn^{194}, and Trp^{197} were substituted with structurally or

Table 2: Alanine Mutations That Resulted in a Major Loss of Catalytic Activity[a]

mutation	TMS	catalytic activity (% wt)
	≤10% wt	
H77A	2	9 ± 4
E81A	2	5 ± 6
E123A	3	10 ± 5
T126A	3	4 ± 4
N194A	4	5 ± 3
W197A	4	5 ± 2
	11−35% wt	
W69A	2	17 ± 6
I76A	2	33 ± 7
M122A	3	28 ± 11
Y189A	4	32 ± 7
F190A	4	21 ± 6

[a] Allocation of residues to putative transmembrane α-helices (TMS) is indicated (see Figure 2). Data shown were taken from Table 1.

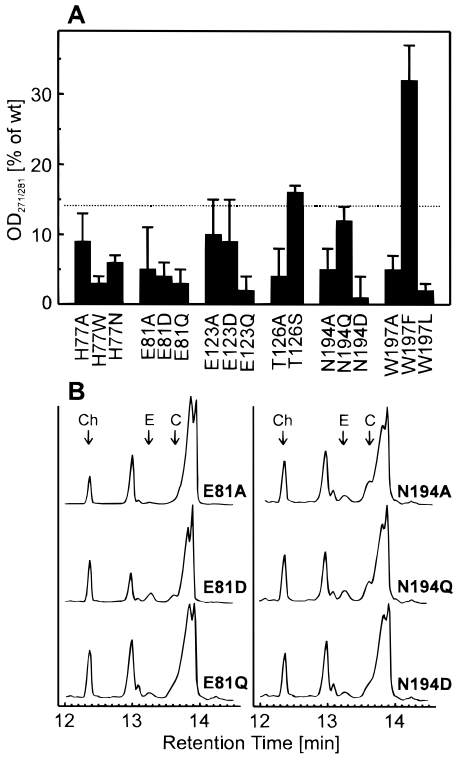

FIGURE 3: (A) Relative Δ5,7-sterol content of mutants with substitution of His[77], Glu[81], Glu[123], Thr[126], Asn[194], and Trp[197]. The 271/281 nm absorbance from strains transformed with mutants of hEBP was measured [$OD_{271/281}$, normalized to mock (0%) and wt (100%)] in nonsaponifiable lipid extracts from 20 mg (wet weight) of yeast cell pellets. Data shown are the mean ± SD ($n = 4-7$). The dotted line represents the relative content in Δ5,7-sterols in a strain transfected with EBP_{low}. The following codon changes were introduced: H77N (AAC), H77W (TGG), E81D (GAT), E81Q (CAG), E123D (GAC), E123Q (CAA), T126S (TCA), N194Q (CAG), N194D (GAT), W197F (TTC), and W197L (TTG). (B) Gas chromatographic analysis of sterols extracted from substitution mutants of Glu[81] and Asn[194]. Ch, cholestan-5-en-3β-ol (exogenous sterol added as a standard); E, ergostan-5,7,22-trien-3β-ol (ergosterol); C, cholestan-5,7,22,24-tetraen-3β-ol.

functionally similar amino acid residues to confirm that they are indeed major determinants of catalytic activity (Figure 3A). Most residues could be replaced by other proteinogenic amino acids that differ either in the length of the carbon backbone or in the functional group [but have a similar

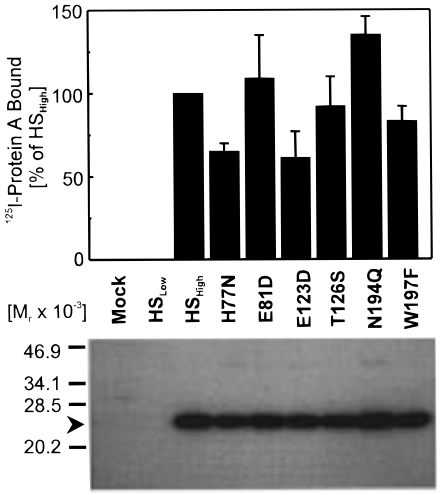

FIGURE 4: Protein expression of mutant proteins. Microsomal protein (50 μg) from yeast strains transformed with the indicated mutant cDNAs were separated on SDS−14% (w/v) polyacrylamide gels and electrophoretically transferred to a PVDF membrane. Immunostaining with rabbit anti-$EBP_{206-223}$ was carried out as described (*13*). Immunoreactivity was quantified by incubation with ^{125}I-labeled protein A (NEN, Vienna, Austria; 1 μCi/mL), membrane slicing, and γ-counting (mock, 0−12 cpm; EBP_{high} (=wt), 819−3318 cpm). Data shown (upper panel) are mean ± SD ($n = 3$) normalized to EBP_{high} (=wt). Prior to slicing, the PVDF membranes were exposed to Kodak Xomat AR film (lower panel, 36 h exposure). The arrow designates the migration of the 26.4 kDa (*13*) hEBP.

volume *V* (*32*)]. The following amino acids were used for substitution: Gln and Asp replacing Glu[81/123]; Ser replacing Thr[126]; Asp and Gln replacing Asn[194]; Phe (aromatic) and Leu (hydrophobic) replacing Trp[197]. The chemical properties of His are unique. His[77] was therefore replaced by Trp, which carries a nitrogen atom in the ring system in a position similar to His, and by Asn, which is isosteric with His (*33*). Out of all substituting mutations only T126S (16% ± 1%) and W197F (32% ± 5%) partially restored catalytic activity as quantified by Δ5,7-sterol content (Figure 3A). Sterol analysis by gas chromatography confirmed that most substitutions abolished catalytic activity almost completely (Figure 3B and data not shown). The presence of a peak comigrating with ergosterol in the mock-transformed *erg2-3* strain (Figure 1D) prevented a reliable determination whether a mutant hEBP had minute residual catalytic activity or was enzymatically completely inactive. Protein expression from mutated cDNAs was verified by immunoblotting with a sequence-specific antiserum [(*13*), Figure 4 and data not shown]. Quantification of immunoreactivity with ^{125}I-labeled protein A revealed no major differences in the expression level of hEBP with mutations H77N, E81D, E123D, T126S, N194Q, and W197F (Figure 4).

^{3}H-Ifenprodil Binding to Substitution Mutants H77N, E81D, E123D, T126S, N194Q, and W197F. Residues that are essential for isomerase function were suggested to be also involved in high-affinity inhibitor binding (*25*). Therefore the binding properties of several of the substitution mutants were characterized with ^{3}H-ifenprodil as a ligand. The binding activity in microsomes from hEBPs with H77N and E123D mutations was not different from the endogenous background in microsomes from a mock-transformed yeast strain (data not shown) devoid of an ERG2p-associated high-affinity ^{3}H-ifenprodil binding site (*25*). Saturation analysis

Table 3: ^{3}H-Ifenprodil Binding Properties of hEBP Mutants[a]

	k_{+1}	k_{-1}	kinetic K_d
wt	1.0[b]	1[c]	1[d]
H77N	NBA	NBA	
E81D	0.9 ± 0.5	16 ± 3	21
E123D	NBA	NBA	
T126S	1.2 ± 0.1	5 ± 1	4
N194Q	3.8 ± 1.3	55 ± 21	17
W197F	0.8 ± 0.1	5 ± 1	6

[a] Data from individual experiments were normalized to wt (=1). Data shown are mean ± SD (n = 3). NBA, no detectable specific ^{3}H-ifenprodil binding activity. [b] $k_{+1} = (28 \pm 6) \times 10^3$ s^{-1}. [c] $k_{-1} = (140 \pm 10) \times 10^{-6}$ s^{-1}. [d] Kinetic K_d = 5.3 nM.

with ^{3}H-ifenprodil revealed no major changes in B_{max} but an increase of the K_d in microsomes from mutants E81D, T126S, N194Q, and W197F (data not shown). To confirm that the reduced affinity was due to destabilization of the enzyme−inhibitor complex, we measured the dissociation of ^{3}H-ifenprodil from the hEBP (Table 3). The increase of the dissociation rate constants as compared to wt ($k_{-1(wt)} = 140 \times 10^{-6}$ s^{-1}) was even larger than expected from the equilibrium binding experiments (not shown) for mutants E81D (16- ± 3-fold, n = 3), T126S (5- ± 1-fold, n = 3), N194Q (55- ± 21-fold, n = 3), and W197F (5- ± 1-fold, n = 3). Except for mutant N194Q (4- ± 1-fold increase), the association rate constants were identical with wt (Table 3). Our results suggest that residues which are required for sterol Δ8−Δ7 isomerization also stabilize the enzyme−inhibitor complex.

Ala Substitutions in the Linker between TMS2 and TMS3. In the course of this study the amino acid sequence of the EBP-related sterol Δ8−Δ7 isomerase of *Arabidopsis thaliana* became available (GenBank accession no. AF030357). The sequence alignment with the amino acid sequences of the mammalian EBPs revealed a high degree of evolutionary conservation in an 11 amino acid residue domain in the linker between TMS2 and TMS3 (Figure 2) proposed to be oriented toward the cytosol (*13*). This prompted us to investigate the functional consequences of Ala substitutions in this conserved sequence and in the two adjacent hydrophobic residues (Leu^{101}−Ile^{113}). Strains expressing hEBPs with Ala substitution of Trp^{102}, Tyr^{105}, Asp^{109}, Arg^{111}, and Tyr^{112} contained almost no Δ5,7-sterols as determined either by Δ5,7-sterol content (Figure 5A) or by sterol analysis by gas chromatography (Figure 5B), despite sufficient hEBP expression as verified by immunoblotting (Figure 5A).

DISCUSSION

The mutant proteins cannot be kinetically characterized with present methodology. To distinguish whether His^{77}, Glu^{81}, Glu^{123}, Thr^{126}, Asn^{194}, and Trp^{197} are involved in catalysis or substrate binding, it is necessary to determine whether these mutations modify K_m or V_{max}. For several reasons it is technically extremely demanding to characterize the in vitro properties of mutant enzymes. First, the substrate, zymosterol, is commercially not available. Second, large amounts of microsomal protein or an increased density of enzyme by pharmacological treatment (*20*, *22*) is required because, similar to other enzymes of postsqualene cholesterol biosynthesis, the V_{max} is low. Third, the substrate concentration cannot be increased arbitrarily because the solubility of zymosterol is limited. Although the density of the yeast-expressed wt hEBP (EBP_{high}) is sufficient for kinetic analysis [V_{max} = 0.325 nmol min^{-1} (mg of protein)$^{-1}$ $K_{m(zymosterol)}$ = 25 μM (*27*)], it is impossible with present methodology to characterize the kinetics of sterol Δ8−Δ7 isomerization by mutants in which the catalytic activity is 25-fold or more reduced because not enough product for reliable quantification is formed (Y.-K. Paik, personal communication).

Mutations modify the catalytic center rather than the global protein conformation. An important caveat of site-directed mutagenesis is that the substituting amino acid residue could impair protein folding nonspecifically. We chose Ala for scanning the transmembrane segments of hEBP because the substitution of residues in α-helices with Ala in the core of proteins has minimal effects on secondary structure (*31*). To

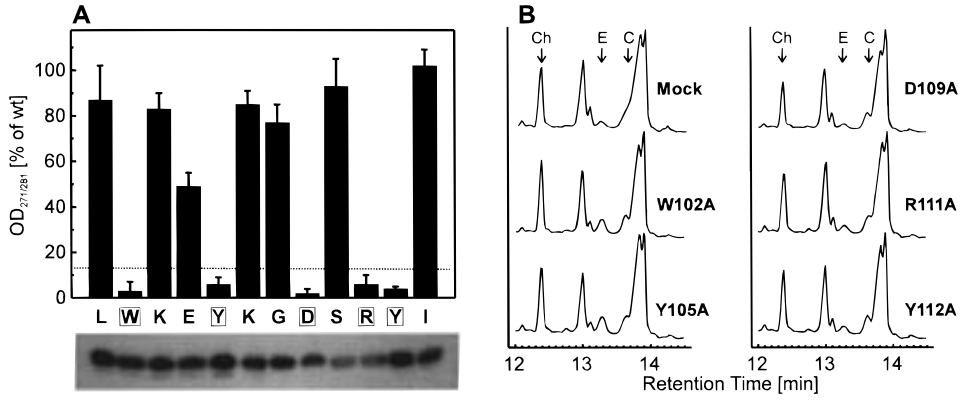

FIGURE 5: Functional consequences of Ala substitution of residues in the conserved cytoplasmic linker [Leu^{101} (L)−Ile^{113} (I)] between TMS2 and TMS3 (Figure 2). (A) Relative content in Δ5,7-sterols (upper panel, quantified as 271/281 nm absorbance and normalized to mock (0%) and wt (100%)] in yeast strains transformed with cDNAs with Ala mutations. Data shown are the mean ± SD (n = 4−7). The dotted line represents the relative content in Δ5,7-sterols in a strain transformed with EBP_{low}. The following codon changes were introduced: L101A (GCT), W102A (GCT), K103A (GCA), E104A (GCG), Y105A (GCT), K107A (GCG), G108A (GCA), D109A (GCC), S110A (GCT), R111A (GCA), Y112A (GCT), and I113A (GCT). To verify expression of substituted mutant proteins (lower panel), 50 μg of microsomal protein from yeast strains transformed with the indicated cDNAs were separated on an SDS−14% (w/v) polyacrylamide gel and transferred electrophoretically to a PVDF membrane. Immunostaining with rabbit anti-$EBP_{206-223}$ was carried out as described (*13*). Immunoreactivity was detected by incubation with ^{125}I-labeled protein A (NEN, Vienna, Austria; 1 μCi/mL) and autoradiography (72 h exposure). (B) Gas chromatographic analysis of sterols extracted from mutant and mock transformed strains. Ch, cholestan-5-en-3β-ol (exogenous sterol added as an internal standard); E, ergostan-5,7,22-trien-3β-ol (ergosterol); C, cholestan-5,7,22,24-tetraen-3β-ol.

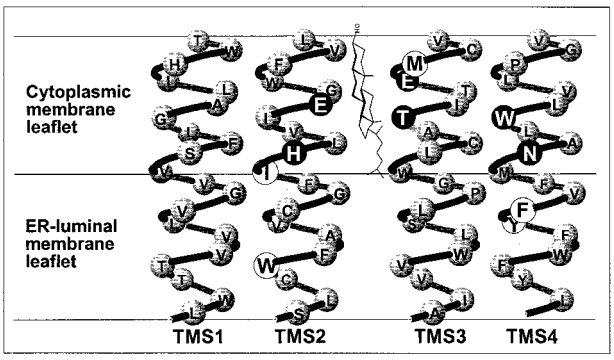

FIGURE 6: Model of the four putative transmembrane segments (TMS1−4) of hEBP. Amino acid residues that could not be substituted by Ala without a reduction in Δ5,7-sterol content to ≤10% (●) (TMS2, H = His[77] and E = Glu[81]; TMS3, E = Glu[123] and T = Thr[126]; TMS4, N = Asn[194] and W = Trp[197]) and <35% (○) (TMS2, W = Trp[69] and I = Ile[76]; TMS3, M = Met[122]; TMS4, Y = Tyr[189] and F = Phe[190]), respectively, of wt are indicated. The sterol substrate symbolizing the catalytic cleft is drawn approximately to scale.

rule out that the mutations disrupted charge pairs we also replaced—if possible—the residues that we identified as major determinants of catalytic activity with functionally similar amino acid residues, e.g., E81D, E123D, and N194Q. Numerous missense mutations in the Δ7-sterol reductase gene from patients with the Smith−Lemli−Opitz syndrome reduced protein expression >95%, most likely by rendering the protein unstable due to misfolding (*9*). We therefore verified expression of mutant hEBP by immunoblotting. The expression of the mutants H77N, E81D, E123D, T126S, N194Q, and W197F was essentially identical with that of wt hEBP. Four of the mutant enzymes (E81D, T126S, N194Q, and W197F) with reduced catalytic activity still bound the enzyme inhibitor ^{3}H-ifenprodil, suggesting that the global confirmation was not severely impaired.

We therefore assume that these mutations do not reduce catalytic activity by disrupting protein folding. Instead, the enzymatic conversion of Δ8-sterols by substitution mutants is most likely reduced because functional groups required for catalysis are either missing (H77N, E81Q, E123Q, N194D, and W197L), modified (T126S and W197F), or shifted to a localization in which they cannot function properly because they fail to fulfill critical distance requirements (E81D, E123D, and N194Q).

The Residues Identified Are Conserved and Localized in TMS2−4. All the residues that could not be substituted by Ala without a >50-fold loss of catalytic activity are localized in TMS2−4 (Table 2). We therefore suggest that TMS1 does not form part of the substrate binding site or catalytic cleft. His[77], Glu[81], Glu[123], Thr[126], Asn[194], and Trp[197], which are major determinants of catalytic activity, are conserved among the sterol Δ8−Δ7 isomerases from cress (*Arabidopsis thaliana*) and mammals (*Homo sapiens*, *Mus musculus*, and *Cavia porcellus*). The tentative model of the four TMS of hEBP (Figure 6) is based upon the hydrophobicity analysis and a C-terminal endoplasmic reticulum retention sequence (*13*). The residues required for sterol Δ8−Δ7 isomerization are adjacent within the α-helices (TMS2, His[77] and Glu[81]; TMS3, Glu[123] and Thr[126]; TMS4, Asn[194] and Trp[197]) and all lie in the cytoplasmic halves of the TMS. Although it remains to be determined experimentally which residues in the α-helices face each other, proximity of His[77], Glu[81], Glu[123], Thr[126], Asn[194], and Trp[197] is consistent with the topology model (Figure 6). Thus these six residues may line the catalytic cleft, which is symbolized by the sterol structure in Figure 6. We therefore suggest that sterol Δ8−Δ7 isomerization takes place in the cytoplasmic leaflet of the membrane of the endoplasmic reticulum, in line with the NADPH requirement of the sterol reductases (*34*).

Trp[102], Tyr[105], Asp[109], Arg[111], and Tyr[112] in the TMS2−3 Linker Are Also Required for in Vivo Catalysis. The amino acid sequence alignment of sterol Δ8−Δ7 isomerases from mammals and plants identified a conserved cytoplasmic domain in the TMS2−3 linker sequence (WKEYXKGDSRY, Figure 2), suggesting that this region is important for catalysis. In mutants with Ala substitution of Trp[102], Tyr[105], Asp[109], Arg[111], or Tyr[112] the catalytic activity was indeed >50-fold reduced (Figure 5). This cytoplasmic sequence lies between Glu[81] and Glu[123] in TMS2 and TMS3, respectively, which were suggested to be part of the catalytic cleft. Conversion of Δ8-cholestenol to Δ7-cholestenol occurs through the uptake of solvent hydrogen from the medium (*18, 19*), implying that a proton or a water molecule can enter the catalytic cleft. Thus one candidate function of residues in the conserved WKEYXKGDSRY domain is proton or water delivery to the catalytic cleft. Alternatively, some of these residues could be involved in the binding of the OH group at C_3 of the sterol molecule which in the estrogen receptor donates a hydrogen bond to Glu[353], which interacts with Arg[394] (*35*). A similar function of Asp[109] or Arg[111] of hEBP is conceivable.

Catalytic Cleft and Inhibitor Binding Site Overlap. In all six mutants with impaired catalytic activity (H77N, E81D, E123D, T126S, N194Q, and W197F) the ^{3}H-ifenprodil binding properties were different from those of wt. His[77] and Glu[123] are important determinants of inhibitor binding because no specific binding activity was found in H77N and E123D mutants. In mutants E81D, T126S, N194Q, and W197F the enzyme−inhibitor complex was less stable, indicating that the wt residues provide additional interaction sites for the drug. The modified binding properties of all six mutants confirmed our suggestion, based on the pharmacological similarities between the structurally unrelated isomerase from yeast (ERG2p) and mammals (EBP) (*25, 27*), that the inhibitor binding site and the catalytic cleft overlap.

Putative Function of Residues in the Reaction Mechanism. The general acid-based reaction mechanism was suggested by labeling studies with tritiated water, substrate, and product (ref *18* and references therein). The donor residue protonates zymosterol at $C_{9\alpha}$. The resulting carbocationic reaction intermediate is deprotonated by the acceptor residue at $C_{7\beta}$ (*16*). A major difference between isomerization in yeast and mammals is that it occurs through cis ($C_{9\alpha-7\alpha}$) and trans ($C_{9\alpha-7\beta}$) hydrogen addition−elimination, respectively (*16*). In the cis reaction the proton donor and acceptor could be identical, whereas in the trans reaction two separate functional groups are required (*18*). On the basis of the reversibility of sterol Δ8−Δ7 isomerization, both the proton donor and the proton acceptor were suggested to act primarily as acids (*18*). Among the residues required for catalysis, Glu[81] and Glu[123] in TMS2 and TMS3 but also His[77] in TMS2 are candidates for the postulated acid function. Among the few enzymes of isoprenoid biosynthesis with known three-dimensional structure is the squalene−hopene cyclase (*36*).

It is similar to EBP because it forms a functional homodimer (*13*, *36*) and cyclizes squalene by enzyme-mediated protonization, formation of a carbocationic intermediate, and deprotonization. In the cyclase the reaction is presumably started by a positive charge on the pair $Asp^{376}-His^{451}$ that is stabilized by the negative pair $Asp^{374}-Asp^{377}$ and protonates C_3 (*36*). Pentalene synthesis also occurs through a carbocationic intermediate and deprotonization: $His^{309}-Ser^{305}$ of the enzyme is the likely catalytic base, whereas Phe^{77} and Asn^{219} are suggested to stabilize the carbocation (*37*). It is therefore conceivable that in hEBP His^{77} is coupled to Glu^{81}, Glu^{123}, or Thr^{126}. Glu^{81} or Glu^{123} could be the residues that deliver or receive the proton, respectively, possibly through a hydrogen-bonding network (e.g., with His^{77}), whereas Asn^{194} and Trp^{197} could stabilize the carbocationic reaction intermediate. In both the squalene cyclase and the pentalene synthase the central cavity is lined by numerous aromatic residues, resulting in further stabilization of the short-lived carbocation (*36*, *37*). The EBP contains a strikingly high percentage of aromatic residues in its TMS (*13*), suggesting that π-electrons of Trp^{69}, Tyr^{189}, and Phe^{190} of hEBP might provide minor interaction sites for the carbocationic reaction intermediate. Additional structural information is required to confirm or dismiss this interpretation of our findings.

Our work identified six residues in the cytoplasmic halves TMS2−4 of the human emopamil binding protein that cannot be substituted with functionally or structurally related residues without a major loss of catalytic activity. We suggest that these residues, His^{77}, Glu^{81}, Glu^{123}, Thr^{126}, Asn^{194}, and Trp^{197}, line the catalytic cleft and are part of the binding site for the enzyme inhibitor ifenprodil. Five additional residues, Trp^{102}, Tyr^{105}, Asp^{109}, Arg^{111}, and Tyr^{112}, in a conserved cytoplasmic linker sequence between TMS2 and TMS3 could be also required for catalysis or substrate binding. The pharmacological properties of the isomerases (ERG2p and EBP) and the sterol reductases (Δ14-, Δ7-, and Δ24-sterol reductase) overlap, most likely because their catalytic clefts recognize similar pharmacophores (*27*). Identification of amino acid residues indispensable for catalytic activity of the yeast ERG2p and the mammalian Δ7-sterol reductase will clarify whether they have structural features in common with EBP. A challenging problem is the identification of the residues that form the proton delivering group [to $C_{9\alpha}$ in the isomerases (*16*) and to $C_{8\beta}$ in the Δ7-sterol reductase (*34*)]. In vivo measurement of enzymatic activity is sensitive and avoids the technical difficulties of the in vitro characterization of sterol biosynthetic enzymes. In vivo complementation of yeast mutants combined with spectrophotometric Δ5,7-sterol quantification can be easily modified (e.g., in *erg5*/*erg6* double mutants) to identify major molecular determinants of 7-dehydrocholesterol reduction through the Δ7-sterol reductase defective in the Smith−Lemli−Opitz syndrome (*9*).

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