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Characterization and Properties of Cholesterol Desaturases from the Ciliate *Tetrahymena thermophila*

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ABSTRACT. Live *Tetrahymena thermophila* transforms exogenous cholesterol into 7,22-*bis*, dehydrocholesterol (DHC) by desaturation at positions C7(8) and C22(23) of the cholesterol moiety. In this first report on expression, isolation, characterization, and reconstitution of *Tetrahymena'*s cholesterol desaturases in cell-free extracts, we describe conditions for increasing the expression of both desaturases based on the addition of specific sterols to the culture medium. Reactions performed in vitro, with isolated microsomes, yield only the monounsaturated derivatives, 7-DHC and/or 22-DHC. However, selectivity towards one product can be improved with the addition of specific compounds: β-mercaptoethanol inhibited C22(23) desaturase activity completely, while ethanol selectively increased this activity. Detergent-solubilized microsomes showed no desaturase activity, but partial restoration could be achieved with addition of dilauroylphosphatidylcholine liposomes (25%). Both cholesterol desaturases require molecular oxygen and cytochrome b_5. NADH or NADPH can serve as reduced cofactors, albeit with different efficiency, delivered by reductases present in the microsomal fraction. Azide and cyanide, but not azole compounds, inhibited these desaturases, suggesting a key role for cytochrome b_5 in these reactions.

Key Words. C22(23)-cholesterol desaturase, C7(8)-cholesterol desaturase, induction by sterols, inhibitors, isolation, reconstitution in liposomes, *Tetrahymena*.

Tetrahymena has no sterol requirement and no detectable sterols in its membranes. Instead, it makes and uses tetrahymanol, a compound closely related to hopanoids, the sterol surrogates found in prokaryotes (Raederstorff and Rohmer 1988). However, in response to the addition of sterols to the growth medium, *Tetrahymena* suppresses the formation of tetrahymanol and replaces this compound by the added sterol, either with or without previous modifications. In the case of cholesterol (CHOL), the sterol is converted mainly to the ergosterol analog, 7,22-*bis*, dehydrocholesterol (7,22-DHC), by desaturation at positions C7(8) and C22(23) of the cholesterol moiety (Fig. 1). Formation of minor quantities of the putative precursors, 22-22-DHC and 7-DHC, has also been described (Conner et al. 1966; Mallory and Conner 1970; Mulheirn, Aberhart, and Caspi 1971). These compounds are known to replace tetrahymanol in its structural role (Beedle, Munday, and Wilton 1974; Holz and Conner 1973; Wilton 1983).

Cholesterol desaturation, although very rare in nature, has great potential for biotechnological purposes. The conversion of CHOL into 7-DHC (pro-vitamin D_3) is particularly of practical interest, as it may be used for decreasing CHOL content in foodstuffs of animal origin with simultaneous enrichment in pro-vitamin D compounds (Valcarce et al. 2001, 2002). In addition, the process may be considered an environmentally friendly biological alternative for the production of pro-vitamin D_3 from cholesterol in a single step, instead of the chemical synthesis currently used.

While C22(23) desaturase activity in *T. pyriformis* has been analyzed in relation to substrate specificity and stereochemistry of the reaction (Conner and Landrey 1978; Zander and Caspi 1970), so far C7(8) cholesterol-desaturase activity has not been studied in any living organism. Recently, we isolated and preliminarily characterized this activity in cell-free extracts and microsomal preparations of *Tetrahymena* (Valcarce, Florin-Christensen, and Nudel 2000). However, we still do not know how the expression of these enzymes is regulated.

Known sterol desaturases are either soluble or membrane-bound enzymes displaying major differences in relation to substrate specificity, cofactor/s, lipids, cytochromes (CYT), and cytochrome reductases (CRP) requirements, to name a few of the possible variations. A group of sterol C22(23)-desaturases from various yeast genera, all involved in the biosynthesis of ergosterol, has been characterized as belonging to the CYT-P450 superfamily (Hata et al. 1981; Kelly et al. 1995; Lamb et al. 1999). On the other hand, other desaturases, such as the sterol C5(6)-desaturases from liver microsomes (Honjo, Ishibashi, and Ima 1985; Ishibashi and Bloch 1981) and plants (*Zea mays*) (Taton and Rahier 1996), use an electron transport system that includes CYT b_5 and NADH-CYT b_5-CRP. However, this latter requirement could be satisfied to various extents with another reductase, an NADPH-CYT P450-CRP (Grinstead and Gaylor 1982; Kawata, Trzaskos, and Gaylor 1985). These desaturases are similar to 4-methyl sterol oxidases (Kawata, Trzaskos, and Gaylor 1985; Rahier, Smith, and Taton 1997) and fatty acyl coenzyme A desaturases (Oshino and Omura 1973; Strittmatter et al. 1974) and are not considered as members of the P450 superfamily.

In this study, the conditions for increased expression of C7(8) and C22(23) desaturases in live *Tetrahymena*, as well as properties and characterization of both enzymatic activities in cell-free extracts and partially purified microsomal fractions (MFs), were assessed. We describe for the first time microsomal preparations isolated from *Tetrahymena* that function selectively in the C7(8) or C22(23) desaturation of cholesterol to yield the corresponding $\Delta^{5,7}$ and $\Delta^{5,22}$ sterols. A sensitive assay for the desaturation reactions was set up and the properties of both enzymes have been defined with respect to cofactor requirement, inhibitor sensitivity, substrate specificity, and some kinetics parameters. The results obtained so far indicate that in *Tetrahymena thermophila* desaturation at C7(8) and C22(23) involves two inducible enzymes that can be differentially expressed, both requiring exogenous reductant, Cytochrome b_5, and molecular oxygen. The system may be useful for the transformation of natural cholesterol-containing foodstuffs derived from diverse animal tissues.

MATERIALS AND METHODS

Materials. Yeast extract, proteose-peptone, iron citrate, and solvents were purchased from Merck (Darmstadt, FRG). C-18 Ultrasphere (4.6 × 250 mm) high-performance liquid chromatography (HPLC) columns were from Beckman (Palo Alto, CA). [1,2 ^{3}H]-cholesterol (48 Ci.mmole^{-1}) was obtained from New England Nuclear (Boston, MA). CHOL, 22-DHC, 7-DHC and other sterols were from Steraloids, Inc. Newport, RI. Biobeads SM-2 and dye reagents for protein determination were from Bio-Rad (Hercules, CA). Biochemicals were supplied by Sigma Chemical Company (MO) unless otherwise noted.

Microorganism and culture conditions. *Tetrahymena thermophila* strain CU399 were grown in 250-ml Erlenmeyer flasks

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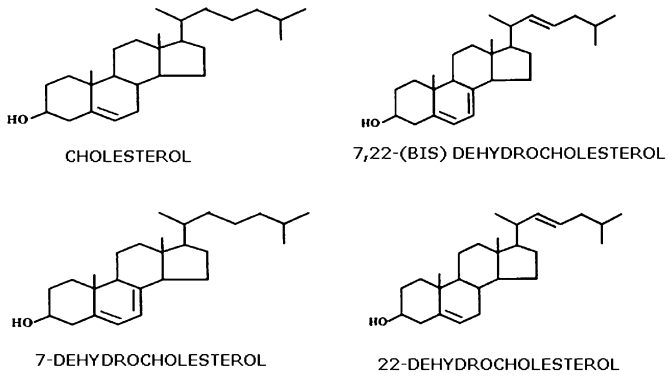

Fig. **1.** Chemical structures of sterol compounds involved in the cholesterol desaturation metabolism of *Tetrahymena thermophila*.

containing 100 ml of a proteose-peptone medium as described previously (Valcarce, Florin-Christensen, and Nudel 2000). It consisted of (w/v) 1% proteose-peptone, 0.1% yeast extract, 0.5% glucose, and 0.003% iron citrate. Cultures were inoculated daily by a 1:10 dilution of a 24-h culture. Cultivation was carried out with rotary shaking (180 rpm) at 29 °C. When indicated, this medium was supplemented with sterols, either CHOL, 7-DHC, or 22-DHC at 2 μg/ml, 5 μg/ml or 20 μg/ml final concentration, prepared from 1 mg/ml stock solutions in ethanol.

Cell-free extracts, homogenate and, microsomes preparations. *Tetrahymena* cells were harvested by low-speed centrifugation of cultures (1,500 *g* for 10 min at 4 °C) resuspended in 1/10-vol of buffer A (100 mM NaH_2PO_4, pH 7.4; 8.5% sucrose; 0.04% ethylenediamine tetraacetic acid (EDTA), and, when indicated, 0.07% β-mercaptoethanol (β-ME) and frozen overnight. After thawing, the suspension was sonicated 3 × for 30 *s* each with a 5-mm diam. probe in a Vibra Cell sonifier (Sonics & Materials, Inc.) and further centrifuged at 1,500 *g* for 3 min to remove whole cells. This supernatant is referred to as the homogenate fraction. This homogenate was centrifuged at 15,000 *g* for separation of nuclei and mitochondria (M), and the clear post-mitochondrial supernatant (MS) was further centrifuged at 100,000 *g* for 90 min for isolation of microsomes. The MF was separated and resuspended in buffer B (same composition as buffer A supplemented with 20% glycerol and without sucrose) at 20 mg/ml protein concentration. Supernatants of the MFS were also separated and analyzed. All fractions were assayed for cholesterol desaturase activities and protein content. Resuspended microsomes (MF) were used for enzyme-reconstitution assays.

Reconstitution of C7(8) and C22(23) cholesterol desaturase activity in liposomes. Solubilization of C7(8) and C22(23) cholesterol desaturases was performed according to a procedure initially described by Grinstead and Gaylor (1982). For this purpose, 5 ml of the resuspended MF (diluted to 5 mg protein/ml in buffer A without sucrose) was mixed with octylthioglucoside (OTG) to a final detergent concentration of 0.5% (w/v). The mixture was stirred gently at 4 °C for 1 h. After centrifugation at 100,000 *g* for 90 min, the supernatant was recovered and mixed with various liposome preparations.

For these preparations, the following lipid suspensions were assayed: 3% (w/v) egg-phosphatidylcholine, 3% (w/v) asolectin, and 0.05% (w/v) dilauroyl-phosphatidylcholine (DLPC). The milky suspensions were individually sonicated until clear ($OD_{580nm} < 0.1$).

For reconstitution, 0.9 ml of detergent-solubilized microsomes and 0.1 ml of liposome preparations were mixed and incubated at room temperature for 10 min. The detergent content in the mixture was decreased with the addition of 0.5 mg Biobeads SM-2 per ml of suspension, and incubated at 8 °C with gentle stirring for 30 min. Afterwards, the detergent-embedded particles from the upper face were separated.

Assay of other microsomal protein and enzymes. CYT-b_5 was determined spectrophotometrically using difference spectra (reduced–oxidized) as described (Omura and Sato 1964). For this assay, microsomal preparations, usually containing 2–4 mg of protein per ml in 100 mM NaH_2PO_4 and also containing 0.004% (w/v) EDTA (pH 7.4), were scanned at room temperature from 600 to 390 nm at a rate of 1 nm/s, both with and without the addition of a few milligrams of crystalline sodium dithionite, added to the sample 1-ml cuvette.

CYT-b_5-CRP and CYT-P450-CRP were measured by the change in absorbance of cytochrome *c* at 550 nm after addition of NADH or NADPH, respectively. The molar extinction coefficient for cytochrome *c* was considered as 19.6/mM/cm for both CRP's (Kawata, Trzaskos, and Gaylor 1985). One unit of activity is equivalent to the reduction of 1 μmol of the acceptor per min at 25 °C.

Analysis of sterols. Total lipids were extracted by a modification of the Bligh and Dyer (1959) method. Aliquots of 1 ml from cultures were saponified by addition of 1 vol of 2 M NaOH prepared in methanol/water (1:1 v/v) and heated at 60 °C for 1 h. After cooling and mixing, 5.6 ml of chloroform/methanol (3/2) was added. The sterols were extracted into the lower phase, concentrated under nitrogen, and separated by HPLC on a C-18 Ultrasphere column using methanol/water (98:2, v/v) as mobile phase at 41 °C. The absorbancy of the eluates was monitored at 205 and 285 nm. An increase in the latter is indicative of the CHOL desaturation at position 7, with formation of conjugated $\Delta^{5,7}$-dienes. Formation of $\Delta^{5,22}$-dienes was monitored at 205 nm, either directly or using radiolabeled CHOL (see below). To aid quantitation, stigmasterol (100 μg/ml) was added as an internal standard to the samples prior to saponification. This sterol separates cleanly from CHOL and its derivatives under the conditions used. The identity of each compound was established by GC–MS (Casabuono and Pomilio 1997; Valcarce, Florin-Christensen, and Nudel 2000).

Bioconversion of radiolabeled cholesterol in cell cultures. Culture media were supplemented with [1,2 ^{3}H] CHOL (0.75 μCi/ml, final concentration) prior to inoculation with *Tetrahymena* cells. Radiolabeled CHOL was added from a 1 mCi/ml stock solution in ethanol, by dilution in 5 ml of the culture medium, followed by 1-h incubation with shaking at 30 °C to help dispersion of CHOL into the medium. To this suspension, the ciliate cultures were added (3×10^5 cells/ml). Samples were drawn and analyzed both for mass sterol composition and radioactivity recovered in each of the cholesterol derivatives, by HPLC coupled to a Flo-one Beta Radio 105 chromatography detector, following the manufacturer's instructions (Radiomatic, Canberra Company). For this purpose, samples were previously saponified, extracted with solvent, and concentrated as indicated above. The amounts of cholesterol derivatives are expressed as a percentage of the initial radioactive CHOL added to the suspension (%). The values correspond to the means (M) ± standard deviation (SD) of three separate experiments.

Standard assay for C7(8) and C22(23) desaturase activity. The incubation mixtures routinely contained 1 ml of sample, either homogenate fraction (10 mg/ml protein) or microsomes (1 mg/ml protein) or solubilized microsomal preparations (0.2–0.7 mg protein/ml) suspended in buer A or B also containing [1,2 ^{3}H] CHOL (3 μCi/ml, 60 nM, final concentration). The reaction was started by adding 1 mM NADH under gentle stirring at 30 °C for 3 h under an oxygen atmosphere. When non-radiolabeled CHOL was added to the reaction mixture, the final concentration was up to 1 mM. Additions were made from 20 mM stock solution in ethanol, followed by 1-h incubation with shaking at 30 °C, to help dispersion. The reaction was stopped by adding 1 ml of 2 M

NaOH prepared in methanol/water (1:1 v/v) and heated at 60 °C during 1 h. After cooling the sterols were extracted twice with a total vol. of 10-ml *n*-hexane. The extracts were evaporated under nitrogen. The resulting residue was suspended in 100 µl of absolute ethanol and an aliquot (50 µl) was injected and analyzed by HPLC coupled to a radioactive detector as described above. Desaturase activity is expressed either as a percentage (%) of the cholesterol derivatives formed from the initial radioactive cholesterol added to the suspension or as pmol of product formed per min and per mg of protein (pmol/mg/min). Results shown are the (M) ± SD of three independent experiments, and, when indicated, the coefficient of variation (V) was also calculated (SD/M^*100).

Concentrations of inhibitors and chemicals added in the assays are as indicated.

Protein was determined by the method of Lowry et al. (1951) or by the use of the Bio-Rad protein assay kit, using bovine serum albumin as a standard.

RESULTS

Live *Tetrahymena* biotransforms CHOL into the unsaturated derivatives, 7-DHC, 22-DHC, and 7,22-DHC, either if cells have been previously adapted to grow with sterols or not. However, the kinetics of product formation differed significantly with regard to previous culture conditions. Cultures pre-grown with CHOL display increased consumption rates of the latter and faster formation of the double unsaturated derivative 7,22-DHC, which is the final product accumulated in cells (Fig. 2). On the other hand, the mono-unsaturated derivatives, 7-DHC and 22-DHC, accumulated at low and relatively constant levels, mostly at an early stage of growth, displaying slightly higher yields in non-adapted cultures (Fig. 2).

Expression and isolation of cholesterol desaturases in *Tetrahymena*. In a previous study, *T. thermophila* cell-free extracts were incubated aerobically with CHOL in the presence of ATP and reduced cofactors, and, upon incubation, a $\Delta^{5,7}$ sterol metabolite was detected by HPLC analysis monitored at 205 and 285 nm, and unequivocally identified as 7-DHC (pro-vitamin D_3) by GC, HPLC, and MS (Valcarce, Florin-Christensen, and Nudel 2000). We have now completed the identification of another sterol metabolite, also originated upon incubation with CHOL, corresponding to 22-DHC. Both products, 7-DHC and 22-DHC, can be formed during incubation of *Tetrahymena* cell-free extracts with CHOL, indicating that C7(8) and C22(23)-cholesterol desaturase activities are present and active on cholesterol (Fig. 3). Formation of the double unsaturated derivative 7,22-DHC from cholesterol was seldom observed in vitro, indicating that the two reactions are less likely to proceed sequentially in cell-free preparations, possibly because of the large differences in concentrations between competing substrates present in the reaction mixture, namely CHOL and the two mono-unsaturated derivatives.

Directing enzyme production towards one specific sterol desaturase is best achieved by culturing *Tetrahymena* with selected sterols. As shown in Table 1, supplementing cultures with CHOL increased mostly C22(23) desaturase expression, while adding 22-DHC to the culture medium increased significantly C7(8) desaturase expression and C22(23) desaturase to a lower extent. Cultures supplemented with 7-DHC displayed both activities, although C22(23)-desaturase predominated, while those prepared from cultures with no sterols showed almost undetectable desaturase activity. Differences in yields between experiments may be attributed to variable amounts of sterols accompanying each preparation, thus affecting the calculations of enzyme activity.

These results strongly suggest that C(7)8 and C(22)23 cholesterol desaturases in *Tetrahymena* are inducible by sterols and that this induction depends on sterol concentration (Table 1). Because of their different specificity towards substrates and sensitivity to inhibitors (see below), they are, most probably, separate enzymes.

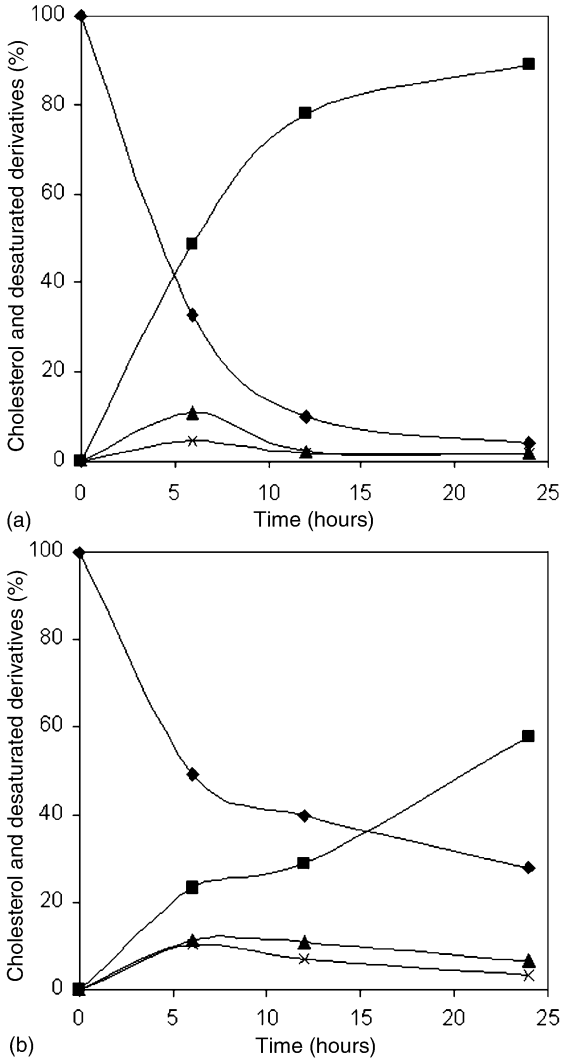

Fig. **2.** Biotransformation of cholesterol by live *Tetrahymena thermophila:* Cultures were pre-grown either with (a) or without (b) cholesterol for 24 h, transferred into cholesterol-containing medium, and analyzed for cholesterol consumption and product formation. Symbols: ◆, Cholesterol; ■, 7,22-*bis*, dehydrocholesterol (DHC); X, 7-DHC; ▲, 22-DHC.

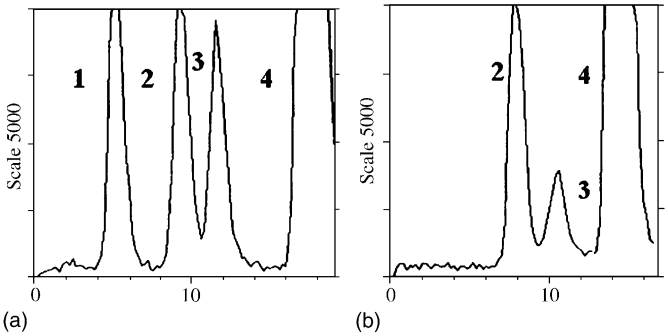

Fig. **3.** Product formation during the incubation of (a) live *Tetrahymena thermophila* and (b) cell-free extracts with radiolabeled [1,2 ^{3}H] cholesterol after 3-h incubation. Sterols were identified as: (1) 7,22-*bis*, dehydrocholesterol (DHC); (2) 22-DHC; (3) 7-DHC; (4) cholesterol.

Table **1.** Cholesterol desaturase activities in cell-free extracts and the influence of sterols added in *Tetrahymena thermophila* culture medium.

Cells grown in medium containing:	Product formation by microsomes, expressed as % of the total labeled cholesterol added[a]		
	7-dehydrocholesterol (%)	22-dehydrocholesterol (%)	7,22-*bis*, dehydrocholesterol (%)
No sterols[b]	<1	1–4	<1
Cholesterol (2 μg/ml)	2–3	7–8	<1
Cholesterol (5 μg/ml)	5–7	17–35	<1
Cholesterol (20 μg/ml)	5–7	19–32	<1
22-dehydrocholesterol (2 μg/ml)	4–6	5–7	<1
22-dehydrocholesterol (5 μg/ml)	9–12	14–20	<1
22-dehydrocholesterol (20 μg/ml)	16–25	14–22	<1
7-dehydrocholesterol (5 μg/ml)	5–9	13–21	<1

[a] The amount of each product was estimated with radiolabeled [1,2 ^{3}H] cholesterol (3 μCi/ml, final concentration) upon 3-h incubation at 30 °C with shaking. The range of activities recorded corresponds to not less than 20 independent microsomal preparations.
[b] Minor amounts of sterols may be present in PPY medium.

In all subsequent experiments in this study, cultures were supplemented either with 5 μg/ml CHOL for maximal C22(23)-desaturase expression or 5 μg/ml 22-DHC for maximal C7(8)-desaturase expression. All other procedures used for the isolation of desaturases in homogenates, MF, and detergent-solubilized microsomal preparations were similar, except for the addition of β-ME in all the steps concerned with the recovery of C7(8) desaturase with minimal competing C22(23) desaturase activity (see below). These procedures, outlined in Materials and Methods, yielded enzyme activities and protein concentrations as described in Table 2.

Solubilization and reconstitution of microsomal sterol desaturase activity with liposomes. Detergent-solubilized MFs showed almost no desaturase activity (2 ± 1%). However, addition of DLPC liposomes and elimination of the detergent restored partially the enzyme activity (25 ± 5% of the initial value). With the same procedure, asolectin or egg-PC liposomes restored a minor fraction of the desaturase activity (5 ± 3% and 7 ± 3%, respectively).

Characterization of C7(8) and C22(23) desaturases in microsomes

Requirement for cofactors and molecular oxygen. Several lines of evidence obtained in studies with sterol desaturases from various sources suggested that cholesterol desaturases in *Tetrahymena* might also be mixed-function oxidases that consume both oxygen and reduced pyridine nucleotide (Honjo, Ishibashi, and Ima 1985; Kelly et al. 1995). For this reason, the requirements for cofactors and molecular oxygen were investigated (Table 3). In the absence of any added pyridine nucleotide, the desaturation reactions were almost negligible. The addition of exogenous NADH strongly promoted cholesterol desaturation, and the addition of NADPH was in the same order of magnitude as NADH. These data indicate a requirement for a reducing equivalent in the Δ7(8) and Δ22(23) desaturation fulfilled by NADH or NADPH, and that the NADH-supported electron transport system predominates.

In addition, NAD+ could also be used as a cofactor, albeit less efficiently, while supplementing with an external NADH regenerating system did not further improve desaturation rates. The absolute requirement for oxygen in both reactions was also well established (Table 3).

There was a difference with respect to NADPH- and NADH-supported desaturation rate for both desaturases presented in this study. Indeed, while C22(23) sterol desaturase was fully active with any cofactor (NADH, NADPH, or NAD), C7(8) desaturase was only partially active with the last two (Table 3).

Based on these results, MF prepared from sterol-containing *Tetrahymena* cultures were tested for C7(8) and C22(23) cholesterol desaturase activity, and investigated with regard to the presence of *b*-type CYT and CRP's. Extinction maxima at 428 nm and the peak at 560 nm in a difference spectrum (dithionite reduced minus oxidized) confirmed the presence of CYT-b_5 in these microsomes (Fig. 4).

Cytochrome reductases, both CYT-b_5 and CYT P-450, were assayed by the change in absorbance of cytochrome *c* (at 550 nm) upon addition of NADH or NADPH. Calculated activities, following the procedure outlined in Materials and Methods, were 120 nmol/min/mg for NADH-cytochrome *c* reductase (CYT b_5-CRP) and 55.2 nmol/min/mg for NADPH-cytochrome *c* oxidoreductase (CYT P-450-CRP).

Effect of chemicals and inhibitors. Addition of β-ME to MF inhibited selectively C22(23) desaturase activity in a concentration-dependent manner (data not shown). This unusual

Table **2.** Localization and partial purification of C7(8) and C22(23) cholesterol desaturases from *Tetrahymena thermophila*.

Material	Protein (mg/ml)	C7(8) and C22(23) desaturase activity[a] (pmol/mg/min)	
		Rate of 7-dehydrocholesterol formation	Rate of 22-dehydrocholesterol formation
Homogenate	10.5 ± 1.5	0.8 ± 0.15	1.2 ± 0.26
Mitochondrial pellet (M)	3.3 ± 0.3	0.1 ± 0.06	0.2 ± 0.05
Post-mitochondrial supernatant (MS)	7.2 ± 1.0	1.2 ± 0.01	1.5 ± 0.20
Microsomal fraction (MF)	0.8 ± 0.2	1.0 ± 0.10	1.7 ± 0.11
Microsomal supernatant (MFS)	6.5 ± 0.5	0.3 ± 0.10	0.6 ± 0.10

* Results are expressed as mean ± SD of three independent experiments.
[a] Cholesterol desaturase activities were measured in 1 ml reaction mixture also containing 0.02 mM cholesterol, 3 μCi/ml (final concentration) radiolabeled [1,2 ^{3}H] cholesterol and 1 mM NADH in 0.1 M NaH_2PO_4 buffer with 0.004% (w/v) EDTA (pH 7.4). The activity was calculated from the percentage of radiolabeled cholesterol transformed into 7-dehydrocholesterol (column 1) or 22-dehydrocholesterol (column 2) after 2 h incubation at 30 °C with shaking. For maximal induction of each enzyme, cultures were supplemented with 5 μg/ml cholesterol or 22-dehydrocholesterol, as described in Materials and Methods.

Table **3.** Molecular oxygen and cofactor requirements for C7(8) and C22(23) cholesterol desaturases in *Tetrahymena thermophila* microsomal fraction.

Addition to microsomes	Rate of 7-DHC formation (A)[a] pmol/mg/min (%)	Relative % (A)	Rate of 22-DHC formation (B)[a] pmol/mg/min (%)	Relative % (B)
None	0.001 (0.7)	3.8	0.005 (2.1)	10
NADH 1 mM	0.032 (18.4)	100	0.050 (21.7)	100
NADH 1 mM/Nitrogen	0.0 (0)	0	0.0 (0)	0
NADH 1 mM+RS[b]	0.026 (15.0)	82	0.042 (18.3)	84
NADPH 1 mM	0.021 (12.5)	68	0.048 (20.9)	96
NAD 1 mM	0.012 (6.7)	36	0.047 (20.4)	94

* Results shown are mean values (M) of three independent experiments. Coefficient of variation (V = SD/M*100) was less than 5% between experiments.

[a] Enzyme activities were calculated from the percentage of radiolabeled [1,2 ^{3}H] cholesterol (3 μCi/ml, final concentration) transformed after 3-h incubation at 30 °C with shaking.

[b] RS: NADH regenerating system, consisted in galactose+galactose dehydrogenase.

7-DHC, 7-dehydrocholesterol; 22-DHC, 22-dehydrocholesterol.

behavior was not observed with another thiol reducing reagent, dithiotreitol, in the range of concentrations tested (1–10 mM), and suggests a specific role for β-ME.

Microsomal C7(8) and C22(23) cholesterol desaturases are also cyanide- and azide-sensitive factors, as shown by their concentration-dependent inhibition profiles (Table 4). On the other hand, azole compounds, including itraconazole, ketoconazole, and fluconazole, tested up to 1 mM concentration each, showed no inhibitory effect. The addition of ethanol to the reaction mixture significantly improved C22(23) sterol desaturase activity, while no effect was observed on C7(8) desaturase (Table 4).

Kinetics and substrate reactivity for C7(8) cholesterol desaturase. The saturation curve (Fig. 5) for cholesterol as the substrate for C7(8) cholesterol desaturase permitted estimation of reaction parameters from a Lineweaver–Burke plot: $K_m = 30\ \mu M$ and $V_{max} = 0.087$ nmol/min/mg of microsomal protein.

DISCUSSION

To our knowledge, this is the first report of a direct induction of desaturation of cholesterol in cell-free fractions from *Tetrahymena* cells. The Δ7 and Δ22 desaturase activities could be induced by sterols added previously to the cultures, thus re-routing the pathways.

So far, the basic mechanisms of electron transport that have been identified in sterol oxidation pathways make use of reduced nucleotides, NADH or NADPH, and the corresponding electron carriers, CYT and CRP's, either NADH-CYT b_5 reductase or NADPH-CYT P-450-reductase, for electron transfer to the (final) sterol oxidase. In our case, NAD(P)H supported cholesterol desaturase activities, suggesting that both reductases may function as electron carriers for CYT b_5. A similar behavior has been described for microsomal Δ-7 sterol C5(6) desaturases isolated from maize (Rahier, Smith, and Taton 1997) and rat liver (Kawata, Trzaskos, and Gaylor 1985) as well as for a rat liver stearyl-CoA desaturase (Oshino and Omura 1973). In these systems both reduced pyridine nucleotides are active, albeit with different efficiency, and both reductases can serve as electron carriers for CYT b_5, a cytochrome that has been shown to be essential for the desaturation reaction. As reported, a NADH or NADPH-supported desaturase activity is consistent with an electron transfer through NADH-CYT b_5 reductase and through NADPH-CYT P-450 reductase to the desaturase (Rahier, Smith, and Taton 1997).

Cholesterol desaturases from *Tetrahymena* require reduced cofactors, molecular oxygen, CYT b_5, and eventually also phospholipids. These features are shared by other desaturases, such as fatty acid desaturases present in the same organism (Bertram and Erwin 1981; Sasaki et al. 1984), 4-methyl sterol oxidase (Fukushima et al. 1983), and lathosterol 5-desaturase of various sources (Ishibashi 2002).

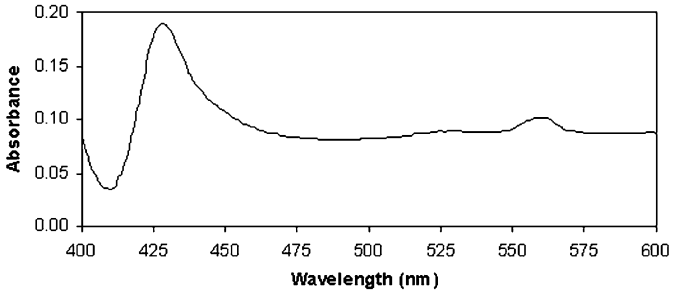

Fig. **4.** Difference spectrum (reduced minus oxidized) of CYT b_5 from *Tetrahymena thermophila*. Microsomal preparations contained 3.5 mg/ml protein in 0.1 M NaH_2PO_4 buffer (pH 7.4) and were reduced with the addition of sodium dithionite crystals.

Table **4.** Effect of chemicals and inhibitors on cholesterol desaturase activities in *Tetrahymena thermophila* microsomes.

Condition	C7(8) or C22(23) cholesterol desaturase activity, expressed as percentage of total cholesterol transformed into product[a]	
	7-dehydrocholesterol (%)	22-dehydrocholesterol (%)
No addition	100	100
β-Mercaptoethanol (700 μl/l)	87 ± 3	6 ± 2
Ethanol (4%)	88 ± 7	137 ± 7
Dithiotreitol (5 mM)	91 ± 5	94 ± 6
Cianide (1 mM)	62 ± 8	65 ± 9
Cianide (2 mM)	26 ± 5	35 ± 6
Cianide (5 mM)	10 ± 4	15 ± 5
Azide (1 mM)	77 ± 7	85 ± 1
Azide (10 mM)	47 ± 4	48 ± 6
Azide (15 mM)	33 ± 8	36 ± 6
Azide (50 mM)	ND	10 ± 4

* Results are expressed as mean ± SD of three independent experiments.

[a] Radiolabeled [1,2 ^{3}H] cholesterol (3 μ Ci/ml, final concentration) was added to all samples and incubated for 3 h at 30 °C with shaking.

ND, not detected.

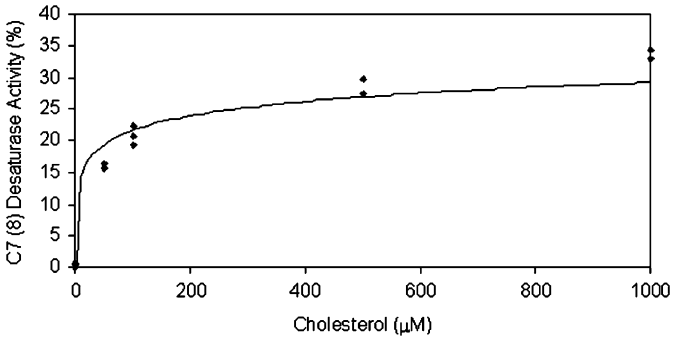

Fig. **5.** Substrate saturation plot for C7(8) cholesterol desaturase in the microsomal fraction derived from *Tetrahymena thermophila*. Radiolabeled [1,2 ^{3}H] Cholesterol (3 μCi/ml, final concentration) was added for quantitation. Activity was calculated from the percentage of radiolabeled cholesterol transformed in 2-h incubation at 30 °C with shaking. Protein concentration in each assay was 1.5 mg/ml.

Cytochrome reductases (b_5 and P450) were shown to be present and active in *Tetrahymena* MF prepared from cultures containing added sterols. The range of their activities was similar to those measured in microsomes isolated from cultures with no sterols (Sasaki et al. 1984). Studies with other desaturases (i.e. lathosterol desaturase) showed little effect of sulfhydryl regents on the catalytic role of the enzyme (Honjo, Ishibashi, and Ima 1985). In this respect, the inhibition of C22(23) desaturase by β-ME that we observed is an unusual feature for this enzyme.

Azide and cyanide are known as CYT b_5 inhibitors (Oshino, Imai and Sato 1966) and their inhibitory effect on C22(23) and C7(8) desaturases clearly indicated the requirement of this cytochrome in both reactions. This stringent requirement for CYT b_5 and the lack of effect of all tested azoles ruled out the possibility that these desaturases may belong to the P450 superfamily. This feature is a clear difference with respect to yeast and fungus C22(23) sterol desaturase involved in ergosterol biosynthesis (Kelly et al. 1997; Lamb et al. 1999). As in studies (Fukushima et al. 1983) of *Tetrahymena*, microsomal CYT consist mainly of *b*-type protein but not CYT P-450, although both NADH and NADPH can serve as reduced cofactors, presumably delivered by CYT b_5-CRP and CYT P-450-CRP indistinctly.

In conclusion, although we have previously demonstrated and preliminarily characterized a microsomal cholesterol desaturase activity (Valcarce, Florin-Christensen, and Nudel 2000), this is the first report to describe conditions for increased differential expression, solubilization, and reconstitution of C7(8) and C22(23) cholesterol desaturases in *Tetrahymena* cells. Moreover, the selective increase of $\Delta^{5,7}$ cholesterol desaturase activity achieved by the addition of 22-DHC in culture medium, and the inhibition of competing $\Delta^{5,22}$ desaturase activity by addition of β-ME, are encouraging results towards a practical application of the system for the biotransformation of cholesterol into pro-vitamin D_3, either in foodstuffs or as an alternative vitamin D_3 production process.

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