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Molecular Cloning and Expression of Human Carnitine Octanoyltransferase: Evidence for Its Role in the Peroxisomal β-Oxidation of Branched-Chain Fatty Acids

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To study the putative role of human carnitine octanoyltransferase (COT) in the β-oxidation of branched-chain fatty acids, we identified and cloned the cDNA encoding human COT and expressed it in the yeast *Saccharomyces cerevisiae*. Enzyme activity measurements showed that COT efficiently converts one of the end products of the peroxisomal β-oxidation of pristanic acid, 4,8-dimethylnonanoyl-CoA, to its corresponding carnitine ester. Production of the carnitine ester of this branched/medium-chain acyl-CoA within the peroxisome is required for its transport to the mitochondrion where further β-oxidation occurs. In contrast, 4,8-dimethylnonanoyl-CoA is not a substrate for carnitine acetyltransferase, another acyltransferase localized in peroxisomes, which catalyzes the formation of carnitine esters of the other products of pristanic acid β-oxidation, namely acetyl-CoA and propionyl-CoA. Our results shed new light on the function of COT in fatty acid metabolism and point to a crucial role of COT in the β-oxidation of branched-chain fatty acids.

In recent years the involvement of carnitine in the peroxisomal β-oxidation of fatty acids has been clearly established in both higher (1, 2) and lower (*Saccharomyces cerevisiae*) eukaryotes (3). The exact mechanism and the nature of the enzymes involved, however, have not been completely elucidated (see 4 for review). There is growing evidence that the end products of the peroxisomal β-oxidation system, including acetyl-CoA, propionyl-CoA and medium-chain acyl-CoA esters, are converted into carnitine esters by carnitine acyltransferases before they are exported from the peroxisomes to the mitochondria where further β-oxidation occurs (2, 5). Mammalian peroxisomes contain at least three distinct carnitine acyltransferases, namely carnitine acetyltransferase (CAT) (6, 7), carnitine octanoyltransferase (COT) (8, 9) and a less well characterized medium/long-chain acyltransferase (10). While all three carnitine acyltransferases catalyse the reversible transfer of fatty acyl groups between CoA and carnitine, the individual enzymes differ in their particular substrate specificities. CAT has been reported to have a preference for short-chain acyl-CoAs (C2–C4) (6, 7), whereas COT is most active with medium-chain length substrates (C6–C10) (8, 9).

Thus far, all the substrate specificity studies for CAT and COT have been performed with straight-chain fatty acids. In addition to the β-oxidation of straight-chain fatty acids, however, peroxisomes also play a crucial role in the β-oxidation of branched-chain fatty acids, including pristanic acid (2,6,10,14-tetrapentadecanoic acid). Recent studies by Verhoeven *et al.* (2) indicated that peroxisomal β-oxidation of pristanic acid proceeds efficiently for three cycles yielding 4,8-dimethylnonanoyl-CoA (C11-CoA), which is then converted to its corresponding carnitine ester and exported from the peroxisome. Further β-oxidation occurs in the mitochondrion after import of the C11-carnitine ester by the carnitine-acylcarnitine translocase (CACT) localized in the mitochondrial inner membrane, followed by reconversion into C11-CoA by the mitochondrial carnitine palmitoyltransferase II (CPT II) (2). At the onset of this study, however, it was unknown which peroxisomal carnitine acyltransferase is responsible for the conversion of C11-CoA to its carnitine ester. We now report that this conversion is catalyzed by COT. To study this, we identified and cloned the human cDNA encoding COT and expressed

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TABLE 1

Primers Used for Amplification of Human COT cDNA by PCR

Primer name	Nucleotide sequence
−40COTf [1]	5′-[-21M13] TCATCTTCTTGGTGTACTGG-3′
1067COTr [2]	5′-[M13rev] GAACCCTTCCATCTTCCTTC-3′
965COTf [1]	5′-[-21M13] GCTGTAATTGTGATCATGCTCC-3′
2117COTr [2]	5′-[M13rev] GAGGCTTAAATACTGCATAGTTC-3′
1COTSalIf [3]	5′-tttttgtcgacATGGAAAATCAATTGGCTAAATCAAC-3′
1845COTSphIr [3]	5′-tttttgcatgcTCATCTCTAAAGATGAGTAGAGTTC-3′

[1] −21M13 extension: tgtaaaacgacggccagt.
[2] M13rev extension: caggaaacagctatgacc.
[3] The restriction sites for *Sal*I and *Sph*I are underlined.

it in the yeast *S. cerevisiae* followed by enzyme activity measurements.

MATERIALS AND METHODS

Identification of the cDNA encoding human COT. The expressed sequence tags database (dbEST) of the National Center of Biotechnology Information was screened with the amino acid sequence of rat and bovine COT, and several partial human EST clones with high homology were identified. Based on the EST sequences two sets of primers with –21M13 or M13rev extensions were designed (Table 1) and used to amplify the entire open reading frame of COT cDNA in two overlapping fragments by RT-PCR. First strand cDNA was prepared from total RNA isolated from cultured human skin fibroblasts as described before (11) and used as template. PCR fragments were sequenced in both directions by means of –21M13 and M13rev fluorescent primers on an ABI 377A automated DNA sequencer according to the manufacturer's protocol (Perkin-Elmer).

Expression of COT cDNA in S. cerevisiae. The coding sequence of human COT cDNA was amplified by PCR using the cloning primers described in Table 1 and subsequently cloned into the yeast expression vector pEL26 under transcriptional control of the oleate-inducible *CTA1* promoter (12). The subcloned PCR fragment was sequenced to exclude errors introduced by *Taq* polymerase. *S. cerevisiae* strain BJ1991 in which the *YCAT* gene was disrupted (BJ1991 Δ*ycat::LEU2*) was transformed with the expression plasmid using the lithium acetate method (13). Transformants were selected and grown at 28°C on minimal medium containing 6.7 g/L yeast nitrogen base without amino acids, 30 g/L glucose and 20 mg/L of the appropriate amino acids. Induction was initiated by shifting the cells to a rich medium containing oleic acid (5 g/L potassium phosphate buffer, pH 6, 3 g/L yeast extract, 5 g/L peptone and 1 g/L oleic acid + 2 g/L Tween-40). The cells were harvested and resuspended in phosphate buffered saline (PBS) containing Complete protease inhibitor cocktail (1 tablet in 25 ml H_2O). To prepare cell lysates, 200 μl glass beads were added and the suspension was vortexed 11 times for 15 s with a 45 s interval at 4°C. The lysates were subsequently homogenized by sonication and cell debris was removed by centrifugation at 10,000 × g_{av} for 30 s. The supernatant was used for immunoblot analysis and enzyme activity measurements.

Immunoblot analysis. 40 μg of protein was subjected to electrophoresis on a 10% (w/v) SDS polyacrylamide gel essentially as described by Laemmli (14) and transferred to a nitrocellulose sheet. After blocking of non-specific binding sites with 50 g/L Protifar and 10 g/L BSA in PBS + 1 g/L Tween-20 for 1 h, the blot was incubated for 2 h with rabbit polyclonal antibodies raised against COT (prepared as described (8)) and diluted (1:2000) in 40 g/L normal goat serum. Goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase were used for detection according to the manufacturer's instructions (Bio-Rad).

Carnitine acyltransferase activity measurements. Carnitine acyltransferase activity was assayed in the direction of acylcarnitine formation. The incubations consisted of 1.3 μg lysate of yeast expressing COT or 5 ng purified CAT, 50 mM HEPES, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA, 1.3 mg/ml bovine serum albumine, 150 mM potassium chloride, and 0.5 mM L-carnitine (including [1-^{14}C]carnitine, 4 kBq) in a final volume of 250 μl. Reactions were initiated by the addition of 100 μM acyl-CoA. After an incubation period of 10 min at 37°C reactions were terminated by the addition of 250 μl of 1.2 M HCl. Acylcarnitines were extracted essentially as described by Solberg (15). In experiments where acetyl-CoA was used as substrate the incubations consisted of 50 mM HEPES, pH 7.4, 5 mM L-carnitine and 300 μM acetyl-CoA (including [1-^{14}C]-acetyl-CoA, 4 kBq) in a final volume of 100 μl. Reactions were initiated by the addition of 0.1 ng purified CAT and terminated after an incubation period of 60 min at 37°C by the addition of 100 μl ice cold 99% ethanol. Assay mixtures were applied to an AG 1-X8 (200–400 mesh, chloride form) column. Upon washing with ethanol, acetylcarnitine passed through the column, whereas acetyl-CoA remained bound. Radioactivity was determined by scintillation counting.

Synthesis of 2,6-dimethylheptanoyl-CoA and 4,8-dimethylnonanoyl-CoA. 2,6-Dimethylheptanoyl-CoA was synthesized as described before (16). 4,8-Dimethylnonanoic acid was prepared from 3,7-dimethyloctanol by a one carbon chain elongation. 3,7-Dimethyloctanol was first reacted with methanesulfonylchloride and triethylamine to form the methanesulfonate (17) which was purified by a silica gel chromatography with hexane diethylether (8:2 v/v). The methylsulfonate was subsequently converted to 4,8-dimethylnonanonitrile (18) with potassium cyanide in dry dimethylsulfoxide. The 4,8-dimethylnonanonitrile was extracted from the reactionmixture with hexane and further purified on a silica gel column with hexane-ethylacetate (9:1 v/v). Finally, the nitrile was hydrolized with 1 M sodiumhydroxide in ethanol-H_2O to yield 4,8-dimethylnonanoic acid. The acid was purified by columnchromatography on silica gel with hexane-diethylether 98:2 and 95:5 (v/v). Gas liquid chromtography-mass spectrometry of the methylester showed one homogeneous peak with a molecular ion at m/z 200. Mass spectrometry of the unesterified acid: m/z 186 (M+ 4,3%), 171 (8,7%), 143 (15,0%), 115 (38,0%), 110 (18,0%), 74 (100%), 55 (22,0%), 43 (25,0%). Overall molar yield from 3,7-dimethyloctanol to 4,8-dimethylnonanoïc acid was 58%. The CoA ester of 4,8-dimethylnonanoïc acid was prepared by the method of Rasmussen *et al.* (19).

Materials. Acetyl-CoA, octanoyl-CoA and L-carnitine were purchased from Sigma Chemicals (St. Louis, MO). [1-^{14}C]carnitine was obtained from NEN (Hertogenbosch, The Netherlands). CAT purified from pigeon breast (80 U/mg) and Complete protease inhibitor were

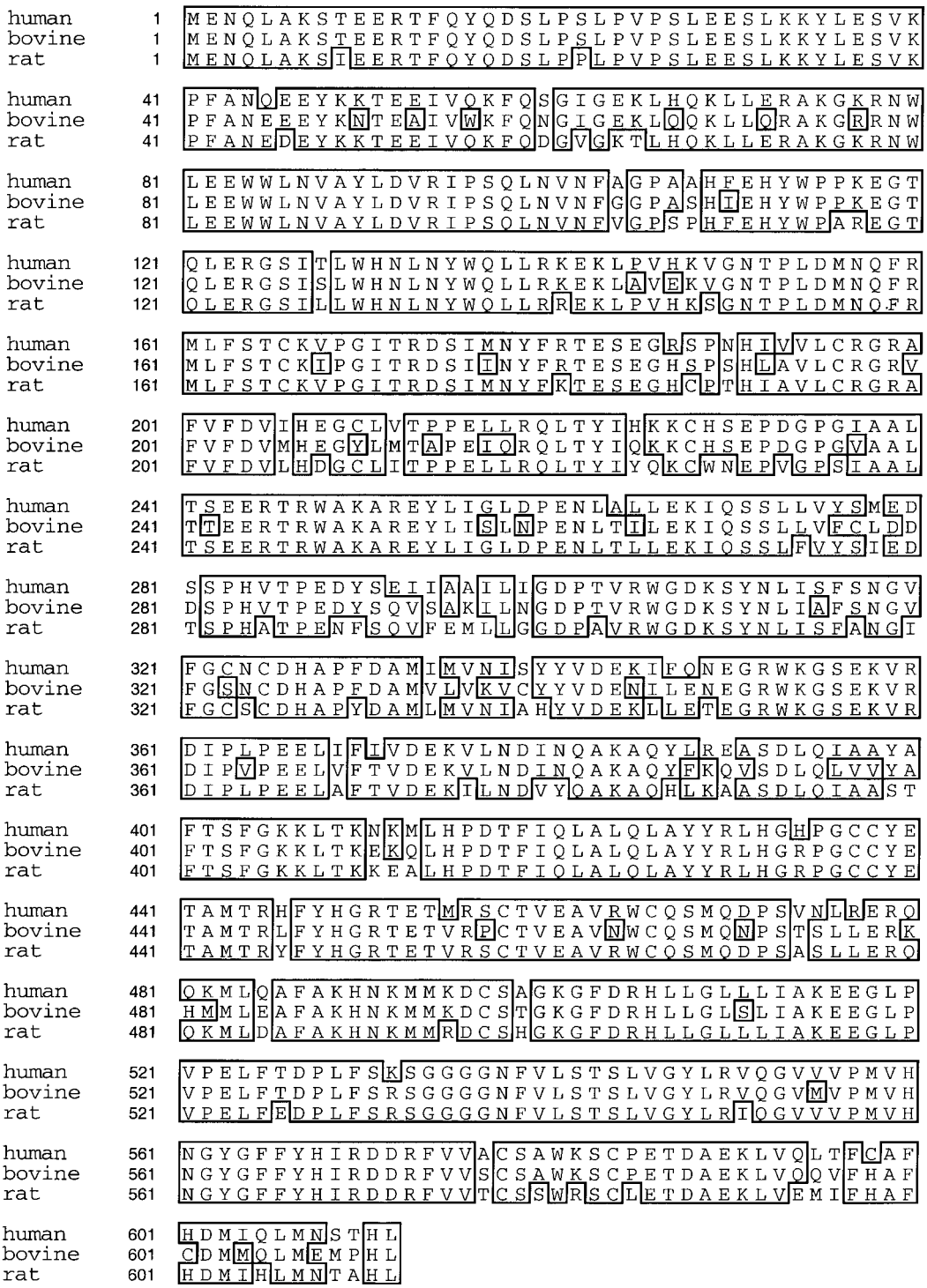

FIG. 1. Alignment of the amino acid sequences of human, bovine and rat COT. The boxes represent the identical amino acids. The human amino acid sequence shares 85% identity with both the bovine (20) and rat (21) COT sequence. As in the bovine and rat sequences, the human sequence contains a putative peroxisomal targeting signal type 1 at the carboxy terminus (-THL).

purchased from Boehringer Mannheim (Mannheim, Germany). Yeast nitrogen base and amino acids were obtained from Difco Laboratories Inc. (Detroit, MI), Profitar from Nutricia (Zoetermeer, The Netherlands) and AG 1-X8 and goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase from Bio-Rad Laboratories (Richmond, CA). Antibodies raised against COT were a kind gift from Prof. Dr. T. Hashimoto (Shinshu University, Matsumoto, Japan).

GenBank accession numbers. Rat COT U26033; bovine COT U65745; human COT AF168793.

TABLE 2

Enzyme Activity of Human COT Expressed in *S. cerevisiae* and CAT Purified from Pigeon Breast

Substrate	COT (nmol/min/mg)	CAT (μmol/min/mg)
C8-CoA	118[a]	6[b]
C2-CoA	—	90[b]
C11-CoA	99[a]	ND
C9-CoA	ND	ND

Note. —, not measured. ND, not detectable.
[a] Each value represents the mean of four experiments.
[b] Each value represents the mean of two experiments.

RESULTS

Cloning of Human COT cDNA and Its Expression in S. cerevisiae

The EST database at the NCBI was searched with the amino acid sequences of rat and bovine COT for cDNA sequences encoding the human homologue. Several partial human cDNA clones with high homology were found. Primers were designed based on the cDNA sequences to amplify the complete coding sequence from cDNA prepared from human skin fibroblasts. The nucleotide sequence was determined and revealed an open reading frame of 1839 bp encoding a polypeptide of 612 amino acids with a calculated molecular weight of 70 kDa. The deduced amino acid sequence showed 85% identity with both the bovine (20) and rat (21) COT sequence (Fig. 1). As in the bovine and rat sequences, a putative peroxisomal targeting signal type 1 was identified at the carboxy terminus (-THL). Subsequently, the entire coding sequence of human COT was expressed in an *S. cerevisiae* strain with a targeted disruption of the *YCAT* gene encoding the yeast carnitine acetyltransferase. Expression of the protein was confirmed by immunoblot analysis (data not shown). In yeast cells transformed with the expression vector containing the coding sequence for COT, a protein with an estimated size of 66 kDa was expressed after induction with oleic acid. This is in accordance with the molecular weight of COT determined by SDS gel electrophoresis as reported in literature (8). No cross-reactive material with α-COT antibody was observed in yeast cells transformed with the expression vector without insert.

Enzyme Activity Measurements with Human COT Expressed in S. cerevisiae

Activity measurements showed that human COT expressed in *S. cerevisiae* was active with C8-CoA as substrate, which was in line with literature data showing that COT is most active with medium-chain acyl-CoAs (C6–C10) (Table 2). The aim of this study, however, was to investigate whether COT is responsible for the conversion of C11-CoA, one of the main end products of the peroxisomal β-oxidation of pristanic acid, into its carnitine ester. Activity measurements in yeast lysates with C11-CoA as substrate showed a high rate of C11-carnitine formation, comparable to the activity measured with C8-CoA. Remarkably, 2,6-dimethylheptanoyl-CoA (C9-CoA), which is derived from C11-

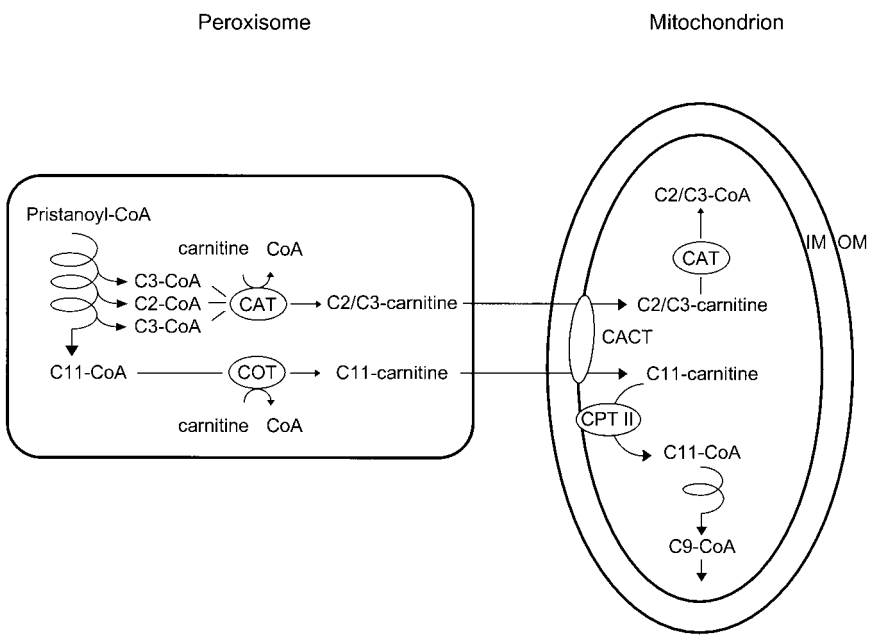

FIG. 2. Schematic presentation of the organization of the pristanic acid β-oxidation and the role of COT and CAT in this process. See main text for detailed information.

CoA after one cycle of β-oxidation, was not handled by COT. This is in agreement with the earlier observation by Verhoeven *et al.* (2) that peroxisomal β-oxidation does not proceed beyond C11-CoA. No activity could be measured with any of these substrates in wild-type yeast transformed with the expression vector without insert.

Next, we compared the activities of COT and CAT, which is also localized in peroxisomes, with these branched-chain substrates. Initial experiments to clone and express human CAT in *S. cerevisiae* with a targeted disruption of the *YCAT* gene were not successful because a second CAT protein, responsible for about 5% of the cellular CAT activity of wild-type yeast (22), interfered with activity measurements when acetyl-CoA was used as substrate. Since it has been demonstrated that CATs from different tissues and species have comparable enzymatic properties (23), we decided to use commercially available CAT purified from pigeon breast for this study. As shown in Table 2, CAT was active with C2-CoA and slightly active with C8-CoA, but not able to convert the branched-chain substrates C11-CoA and C9-CoA to their corresponding carnitine esters.

DISCUSSION

In 1998, Verhoeven *et al.* (2) showed that peroxisomal β-oxidation of pristanic acid proceeds for three cycles, after which C11-CoA is exported from the peroxisome to the mitochondrion for further oxidation. In addition, they showed that this transport occurs as carnitine ester. They performed tandem mass spectrometric analysis of acylcarnitine intermediates in intact human fibroblasts from control subjects and from patients with established deficiencies of either carnitine palmitoyltransferase I (CPT I), CPT II, or CACT. In CACT-deficient cell lines and CPT II deficient cell lines they observed an increased amount of C11-carnitine and either a decreased amount of C9-carnitine or a total absence of C9-carnitine, respectively. These results showed that CACT and CPT II are indispensable for further oxidation of C11-CoA to C9-CoA. Hence, it was concluded that C11-CoA is formed in peroxisomes, after which it is converted to its carnitine ester. C11-carnitine is then imported into the mitochondrion by CACT and reactivated by CPT II to C11-CoA, which can be further degraded by the mitochondrial β-oxidation system. The results obtained with the CPT I-deficient cell lines indicated that CPT I is not involved in the conversion of C11-CoA to its carnitine ester, but it remained unclear which carnitine acyltransferase did catalyze this reaction (2). In this paper we showed that COT is responsible for the peroxisomal conversion of C11-CoA to its corresponding carnitine ester. In order to determine whether the activity was specific for C11-CoA, activity was also measured using C9-CoA as substrate. C9-CoA is produced from C11-CoA after one cycle of β-oxidation and is also a branched/medium-chain fatty acyl-CoA. COT was found to be inactive with this substrate. This finding stresses the functional significance of the measured COT activity towards C11-CoA.

In contrast to COT, CAT was not able to convert either of these two branched-chain substrates. It is clear, however, that CAT is also indispensable for the peroxisomal β-oxidation of pristanic acid because it is responsible for the transport of acetyl-CoA and propionyl-CoA to the mitochondrion as carnitine esters (24). In Fig. 2 the proposed organization of the β-oxidation of pristanic acid, including the interaction between peroxisomes and mitochondria, is depicted. This figure demonstrates the crucial role of COT in the β-oxidation of pristanic acid as indicated by the results presented in this paper.

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REFERENCES

1. Jakobs, B. S., and Wanders, R. J. A. (1995) *Biochem. Biophys. Res. Commun.* **213,** 1035–1041.
2. Verhoeven, N. M., Roe, D. S., Kok, R. M., Wanders, R. J. A., Jakobs, C., and Roe, C. R. (1998) *J. Lipid Res.* **39,** 66–74.
3. Elgersma, Y., Van Roermund, C. W. T., Wanders, R. J. A., and Tabak, H. F. (1995) *EMBO J.* **14,** 3472–3479.
4. Wanders, R. J. A., and Tager, J. M. (1998) *Mol. Aspects Med.* **19**(2), 69–154.
5. Farrell, S. O., and Bieber, L. L. (1983) *Arch. Biochem. Biophys.* **222,** 123–132.
6. Fritz, I. B., Schultz, S. K., and Srere, P. A. (1963) *J. Biol. Chem.* **238,** 2509–2517.
7. Chase, J. F. A., and Tubbs, P. K. (1966) *Biochem. J.* **99,** 32–40.
8. Miyazawa, S., Ozasa, H., Osumi, T., and Hashimoto, T. (1983) *J. Biochem.* **94,** 529–542.
9. Farrell, S. O., Fiol, C. J., Reddy, J. K., and Bieber, L. L. (1984) *J. Biol. Chem.* **259,** 13089–13095.
10. Singh, H., Beckman, K., and Poulos, A. (1996) *J. Lipid Res.* **37,** 2616–2626.
11. IJlst, L., Wanders, R. J. A., Ushikubo, S., Kamijo, T., and Hashimoto, T. (1994) *Biochim. Biophys. Acta* **1215,** 347–350.
12. Elgersma, Y., Van den Berg, M., Tabak, H. F., and Distel, B. (1993) *Genetics* **135,** 731–740.
13. Becker, D. M., and Guarente, L. (1991) *in* Methods in Enzymology (Guthrie, C., and Fink, G. R., Eds.), pp. 186–187, Academic Press, San Diego, CA.
14. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
15. Solberg, H. E. (1972) *Biochim. Biophys. Acta* **280,** 422–433.
16. Wanders, R. J. A., Denis, S., Ruiter, J. P. N., IJlst, L., and Dacremont, G. (1998) *Biochim. Biophys. Acta* **1393,** 35–40.
17. Richter, T., and Mangold, H. K. (1973) *Chem. Phys. Lipids* **11,** 210–214.

18. Parlos, A., Bakone, E., and Johnson, D. W. (1980) *Lipids* **15,** 19–21.
19. Rasmussen, J. T., Börchers, T., and Knudsen, G. (1990) *Biochem. J.* **265,** 849–855.
20. Cronin, C. N. (1997) *Eur. J. Biochem.* **247,** 1029–1037.
21. Choi, S. J., Oh, D. H., Song, C. S., Roy, A. K., and Chatterjee, B. (1995) *Biochim. Biophys. Acta* **1264,** 215–222.
22. Schmalix, W., and Bandlow, W. (1993) *J. Biol. Chem.* **268,** 27428–27439.
23. Edwards, Y. H., Chase, J. F. A., Edwards, M. R., and Tubbs, P. K. (1974) *Eur. J. Biochem.* **46,** 209–215.
24. Bloisi, W., Colombo, I., Garavaglia, B., Giardini, R., Finocchiaro, G., and Didonato, S. (1990) *Eur. J. Biochem.* **189,** 539–546.