Proofs should be sent to:

Dr. Tatsuyuki Kamiryo, Faculty of Integrated Arts and Sciences, Hiroshima University, Kagamiyama, Higashi-Hiroshima 739-8521, Japan.

Fax: +81 824 24 0759. E-mail: kamiryo@ipc.hiroshima-u.ac.jp.

New Aspects of Sterol Carrier Protein 2 (Non-specific Lipid-transfer Protein) in Fusion Proteins and in Peroxisomes

(Running title: New Aspects of SCP2)

Masanori Bun-ya, Yoshitaka Muro, Toshiro Niki, Jun Kondo, and Tatsuyuki Kamiryo†*

*Faculty of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima 739-8521, and *Yokohama Research Center, Mitsubishi Chemical Corporation, Yokohama 227-8502, Japan*

†Author to whom all correspondence and reprint request should be addressed.

E-mail: kamiryo@ipc.hiroshima-u.ac.jp.

ABSTRACT

Sterol carrier protein 2 (SCP2) is a 14-kDa peroxisomal protein, identical to non-specific lipid-transfer protein, and stimulates various steps of cholesterol metabolism *in vitro*. Although the name is reminiscent of acyl carrier protein involved in fatty acid synthesis, SCP2 does not bind to lipids specifically or stoichiometrically. This protein is expressed either as a small precursor or as a large fusion (termed SCPx) that carries at its C-terminal the complete sequence of SCP2. SCPx exhibits 3-oxoacyl-CoA thiolase activity as well as sterol-carrier and lipid-transfer activities. The N- and C-terminal part of SCPx are similar to the nematode protein P-44 and the yeast protein PXP-18, respectively. P-44, which has no SCP2 sequence, thiolyitically cleaved the side-chain of bile acid intermediate at a rate comparable to that of SCPx. This, together with the properties of other fusions with SCP2-like sequence, suggests that the SCP2 part of SCPx does not play a direct role in thiolase reaction. PXP-18, located predominantly inside peroxisomes, is similar to SCP2 in primary structure and lipid-transfer activity and protects peroxisomal acyl-CoA oxidase from thermal denaturation. PXP-18 dimerized at a high temperature, formed an equi-molar complex with the oxidase subunit, and released the active enzyme from the complex when the temperature went down. This article aims to get an insight into the role of SCP2 and to present a model where PXP-18, a member of the SCP2 family, functions as a molecular chaperone in peroxisomes.

Index Entries: Sterol carrier protein 2; thiolase; molecular chaperone; bile acid.

INTRODUCTION

Sterol carrier protein 2 (SCP2) was originally described as the cytosolic factor required for the microsomal conversion of lanosterol to cholesterol *(1)*. It stimulated various steps in cholesterol metabolism: the conversion of 7-dehydrocholesterol to cholesterol, cholesterol esterification, pregnenolone synthesis, 7α-hydroxycholesterol formation *(2)*. This protein was later found to be located in the matrix of peroxisomes *(3, 4)*, where a 58-kDa protein (termed SCPx) that carried at its C-terminal the complete sequence of SCP2 also resided. Seedorf and colleagues elucidated that SCPx was a thiolase and renamed it SCP2/3-oxoacyl-CoA thiolase *(5)*. Three research groups demonstrated that SCPx reacted with 2-methyl-branched 3-oxoacyl-CoAs as well *(6-8)*. However, the molecular function of the SCP2 part in SCPx is uncertain. Krisans and colleagues demonstrated the peroxisomal localization of many enzymes involved in cholesterol synthesis *(9)*, while Puglielli *et al.* revealed the SCP2-dependent transport of cholesterol from the endoplasmic reticulum to the plasma membrane *(10)*. Again, the cellular function of the free SCP2 remains unclear.

SCP2 is identical to mammalian non-specific lipid-transfer protein (nsLTP) *(1)*. This protein accelerates the transfer of cholesterol, glycolipids, and all common phospholipids between donor and acceptor membranes *in vitro (11)*. Unlike other phospholipid-transfer proteins, which bind to specific phospholipids tightly and exchange them between membrane bilayers, nsLTP binds to various membrane lipids with a low affinity and mediates a net mass transfer of them *(12)*. A currently accepted model for the action of nsLTP is that the two molecules interacting with each membrane form a dimer with the single cysteine residue, facilitate the transient contact of two lipid-bilayers, and enable lipids to move between the membranes *(13, 14)*. The non-specific lipid-transfer activity seems crucial to understand SCP2, though the

term nsLTP shall not be used in this article in order to avoid confusion by way of terminology.

Mammalian SCPx is a fusion protein *(15, 16)* coded by a single gene that has two distinct promoters *(17)*. The shorter mRNA expressed constitutively in liver cells encodes pre-SCP2, which has a cleavable pre-sequence of 20 amino acid residues at the N-terminal of 123-residue long SCP2 *(16)*. The longer mRNA inducible by a peroxisome proliferator encodes SCPx, which consists of the N-terminal part of 404 amino acid residues and pre-SCP2 *(16)*. The C-terminal tripeptide of these proteins is Ala-Lys-Leu, a peroxisomal targeting signal 1 *(18)*, being consistent with their subcellular localization *(3, 5)*. Although the non-mammalian counterpart of SCP2 was found in yeast species *(19, 20)*, that of SCPx has not been reported except in avian species *(21)*. Bun-ya *et al.* reported a protein highly similar to the N-terminal part of SCPx in the nematode *Caenorhabditis elegans (22)*. This *C. elegans* protein and the yeast SCP2 seem to be components of the fusion protein SCPx and reveal a new aspect of the role of SCP2.

TYPE II THIOLASE: THE PHYSIOLOGICAL ROLE OF SCPx

The amino acid sequence of the *C. elegans* protein, named P-44 after its molecular mass of 44 kDa, was deduced from its full-length cDNA *(22)*. The 412-residue sequence was significantly similar to that of SCPx but terminated in the middle of the pre-sequence of SCP2 and thus lacked the entire SCP2 part. The possibility that alternative splicing generated SCPx-like fusion protein was excluded by both northern blot analysis and genomic DNA sequence. RNA prepared from a mixed-phase culture of nematode contained only a 1.4-kb RNA species; the size matches the length of the cDNA cloned (1371 bp) *(22)*. No sequence related to SCP2 was detected in the

corresponding region of the genome (EMBL/GenBank/DDBJ accession number Z81559). Therefore P-44 is independent of SCP2, though it resembles SCPx.

P-44 was more similar to SCPx than conventional thiolases in amino acid sequence. Unrooted neighbor-joining analysis with P-44 and 14 thiolases exhibited that distances from P-44 to three SCPx thiolases (from chicken, rat, and human) were nearly the same and about one-half of those to four acetoacetyl-CoA thiolases and seven conventional 3-oxoacyl-CoA thiolases (one bacterial, two mitochondrial, and four peroxisomal). A cysteine residue believed to be essential for the substrate binding was conserved in P-44 as well. Recombinant P-44 that was tagged with hexahistidine and expressed in bacterial cells displayed thiolytic cleavage of 3-oxoacyl-CoA with 8–16 carbon atoms in the acyl group; the highest activity was observed with 3-oxotetradecanoyl-CoA *(22)*. Kinetic analysis revealed that P-44 catalyzed thiolysis by the ping-pong bi-bi reaction mechanism and that the inhibition of P-44 by acetyl-CoA was competitive with CoA and non-competitive with 3-oxoacyl-CoA. This pattern of inhibition is shared with SCPx *(5)* but not with conventional thiolase, which is inhibited uncompetitively with respect to 3-oxoacyl-CoA *(23)*. Thus, P-44 and the thiolase of SCPx are similar to each other and distinct from conventional thiolase not only in sequence but also in kinetic property. They were categorized, therefore, as type II 3-oxoacyl-CoA thiolase *(22)*.

Although the term type II thiolase then did not imply the physiological role, studies on bile acid formation did so. The cleavage of the steroid side chain in bile acid formation occurs in peroxisomes *(24)*. The reaction proceeds in the form of CoA esters and resembles one cycle of fatty acid β-oxidation except the presence of a methyl group on the α-carbon. The enzymes involved in the side-chain cleavage are different from those responsible for the peroxisomal degradation of straight-chain fatty acids. In liver peroxisomes, there are multiple oxidases acting on 2-methyl branched-chain acyl-

CoA as well as straight-chain acyl-CoA *(25)*. The resulting CoA esters of enoyl-form intermediate with the branched-chain are converted to their oxo-form *via* the hydroxy-form of D-configuration by D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein (D-bifunctional protein, also called multifunctional protein 2) *(26, 27)*. D-bifunctional protein is identical to 17β-hydroxysteroid dehydrogenase type IV, which carries at its C-terminal an SCP2-like sequence *(28)*. In the final step of the oxidation it is SCPx that acted on the CoA esters of oxo-form including 2-methyl-branched fatty acids, pristanic acid, and bile acid intermediates *(6-8)*. P-44 formed cholic acid and chenodeoxycholic acid in the presence of D-bifunctionl protein from 3α, 7α, 12α-trihydroxycholest-24-enoyl-CoA and 3α, 7α-dihydroxycholest-24-enoyl-CoA, respectively *(8)*. ·SCPx formed bile acid under similar conditions. P-44 also acted on CoA esters of 2-methyl-2-hexadecenoic acid and stereoisomers of varanic acid and 12-deoxyvaranoic acid *(8)*. Thus, it becomes significant physiologically to categorize SCPx and P-44 as type II thiolase; no known conventional thiolase acts on branched-chain CoA esters. .

ROLE OF THE SCP2 PART IN THE SCPx MOLECULE

The high specific activity of P-44 toward various branched-chain substrates examined poses a question as to what is the role of the SCP2 part on type II thiolase. It is unlikely that the SCP2 part is essential for carrying the substrate because the activity of P-44, which lacks the SCP2 sequence, was comparable to, or even higher than, that of SCPx *(8)*. The term SCP2 resembles acyl carrier protein (ACP), which is a part of fatty acid synthase complex. However, ACP is essential for the catalytic activity of the enzyme, having the covalently bound prosthetic group phosphopantetheine, which accepts specific substrates, carries fatty acid intermediates

through thioester bonding, and keeps them reactive throughout the synthesis. On the contrary, SCP2 does not bind to lipids specifically or stoichiometrically. Therefore this part must not be involved in catalysis directly. Dispensability of the SCP2 part in catalysis was also suggested by the observation that active SCPx preparation from rat liver contained two polypeptides of 58 kDa and 46 kDa reactive with antibody against the N-terminal part of SCPx. The 46-kDa polypeptide was much more abundant than the other and presumably resulted from the C-terminal truncation *(6)*, being similar to P-44.

There are at least two proteins that carry an SCP2-like sequence at the C-terminal: D-bifunctional protein *(29, 30)* and UNC-24 *(31)*. D-bifunctional protein is tripartite; the N-terminal part (323 residues) is the dehydrogenase acting on 17β-hydroxysteoids and D-3-hydroxyacyl-CoAs with or without a 2-methyl branched chain, the central part (residues 324-596) is the dehydratase toward above substrates, and the 141-residue C-terminal part shares 36% sequence identity with pre-SCP2 *(29)*. Porcine D-bifunctional protein (*i. e.*, 17β-hydroxysteroid dehydrogenase type IV) and its C-terminal part exhibited sterol-carrier and phospholipid-transfer activities comparable to those of human SCPx and SCP2, respectively *(28)*. UNC-24 is bipartite and the product of the *C. elegans* gene *unc-24*, whose mutation brings the nematode into uncoordinated movement. The N-terminal part (272 residues) resembles the erythrocyte protein stomatin (also called band 7.2) and the C-terminal part (143 residues) has 23-24% sequence identity with respect to pre-SCP2 and the SCP2-like part of D-bifunctional protein. Since stomatin is the integral membrane phosphoprotein of red blood cells, fusion proteins with an SCP2-like sequence are not limited to those involved in lipid metabolism.

SCP2 shows some analogies with ubiquitin, a 76-residue stress protein. Firstly, ubiquitin exists both in an independent form and in a fusion form. Secondly, the

ubiquitin part seems not essential for the function of the rest of the fusion protein. Thirdly, the fusion proteins are often found in organisms with higher phylogenic complexity. Ribosomal proteins S27a and L40 carry at its N-terminal the single copy of ubiquitin *(32)* and the ubiquitin part is split off during or after ribosome formation by a specific protease. It is similar to the truncation of the SCP2 part from SCPx discussed above *(6)*. The mutation *ubi3Δub-1* that eliminates the ubiquitin part from yeast S27a affected the processing of 20S pre-rRNA, reduced the rate of cell growth, but was not lethal *(33)*. Another ribosomal protein S30 is fused with a 74-residue ubiquitin-like protein in mammals but is unfused in yeast *(34)*, being reminiscent of mammalian SCPx and nematode P-44. These findings suggest that the SCP2 part on type II thiolase facilitates the formation of a specific multienzyme complex. This indirect, molecular chaperone-like role was postulated for the ubiquitin part during ribosome biogenesis *(33)*. In this context, both SCP2 *(35)* and ubiquitin *(36)* mainly consist of a three-stranded β-sheet faced on one α-helix.

IN VITRO ACTIVITY OF YEAST SCP2

The non-mammalian counterpart of SCP2 found thus far is the yeast protein PXP-18, which is the 18th-largest peroxisomal polypeptide of the yeast *Candida tropicalis (37)*. The related yeast *Candida maltosa* has the ortholog *(20)*. In these yeast cells, peroxisomes and about 20 abundant polypeptides including PXP-18 are markedly induced when cells are grown on oleate or *n*-alkanes (C_{10-13}) as a sole source of carbon and energy, but hardly detected in glucose-grown cells. This inducible nature is similar to SCPx but not SCP2 *(16)*. PXP-18 consisting of 126 residues shared 33-35% sequence identity with SCP2 and showed a non-specific lipid-transfer activity comparable to that of rat SCP2 *(19)*. The lipid-transfer activity of PXP-18 was

detected only after the lysis of peroxisomes, suggesting that it was located inside peroxisomes. Intensive analyses by means of biochemical and cytological techniques indicated that PXP-18 was present in peroxisomal matrix but no other cellular compartment *(38)*. Import experiments in heterologous yeast cells proved its C-terminal tripeptide Pro-Lys-Leu was essential for peroxisomal targeting *(38)*. PXP-18 was synthesized without any pre-sequence except the N-terminal methionine. The genomic sequence surrounding the gene, *POX18 (19, 20)*, revealed no possibility to code any fusion protein. Thus, PXP-18 is similar to SCP2 in structure and lipid-transfer activity but independent of type II thiolase.

The principal role of PXP-18 must not be to carry sterols or to transfer lipids. If it is necessary for sterol synthesis, it should be constitutively expressed because yeast membranes require much ergosterol regardless of growth condition. If it transfers membrane lipids to proliferating peroxisomes, it should be present on the surface of organelles. The expression pattern and subcellular localization of PXP-18 suggest that it functions solely in the peroxisomes of yeast cells assimilating fatty acids. The major activity in these peroxisomes is fatty acid degradation, where the rate-limiting enzyme is acyl-CoA oxidase (ACO). PXP-18 protected peroxisomal ACO from acid inactivation *(19)* and thermal denaturation *(39) in vitro*. It also facilitated the reactivation of urea-treated ACO and protected urate oxidase, another peroxisomal enzyme, from thermal inactivation *(39)*.

PXP-18 protected ACO from thermal denaturation through a reversible interaction with the enzyme. Yeast ACO, which consists of two homo-octamers with similar but not identical subunits, PXP-4 and PXP-5, encoded by distinct genes *(40)*, was completely inactivated when heated alone at 70°C for 15 min. When heated with PXP-18, however, up to 60% of the initial activity was retained in a dose-dependent manner. Circular dichroism measurement demonstrated that, at 70°C, ACO was

irreversibly denatured, while PXP-18 underwent a little conformational change that was perfectly reversible *(39)*; SCP2 is also thermostable *(1, 41)*. After the thermal treatment of the mixture, free ACO and the large aggregates composed of both ACO and PXP-18 were detected by sucrose density gradient centrifugation. When isolated the free ACO was hardly distinguishable from the native ACO in sedimentation behavior, specific activity, and secondary structure. The aggregates exhibited ACO activity, being different from inactive aggregates generated with ACO alone. Aggregates produced in the presence of either lysozyme or cytochrome *c*, each of which is basic and small like PXP-18 were also inactive. The active aggregate was named near-stoichiometric complex because the molar ratio of PXP-18 to the ACO subunit in the aggregates was 1.1 to 1.2, even if the ratio in the initial mixture was changed from 1.0 to 4.0 *(39)*. This constant ratio suggested a fairly specific interaction between ACO and PXP-18. When the near-stoichiometric complex was isolated, suspended, and kept at 30°C, ACO activity was released into supernatant (Fig. 1A). Sedimentation experiment proved that this activity was due to free ACO (Fig. 1B).

PXP-18 forms dimer at a high temperature. When the purified PXP-18 (14 kDa) was heated at 70°C for 15 min, a new entity appeared at the position of 29 kDa. Since the new entity reacted with anti-PXP-18 antibody and resulted from only PXP-18 (Fig. 2A) depending on time and temperature (Fig. 2B), it is certain that PXP-18 forms a dimer. Mammalian SCP2 is reported to have a tendency to dimerize supposedly by way of forming disulfide bond *(13)* with the single cysteine residue (Cys^{71}), which was thought to be essential for the lipid-transfer activity *(13, 41)*. However, the sequence of PXP-18 deduced from the gene lacks the cysteine residue, which was replaced with valine (Val^{73}). Although it is possible that PXP-18 expressed in *C. tropicalis* cells has a cysteine residue because of the non-universal coding in *Candida* species *(42)* or the preferential expression of the allelic PXP-18 *(19)*, this possibility was excluded

experimentally. The sequencing of peptides derived from the purified PXP-18 covered 77 amino acid residues out of 126, verified valine instead of cysteine at residue 73, and revealed three residues inconsistent with those deduced from the allele reported previously (*POX18-1*) *(19)*. The amino acid sequence deduced from *POX18-2*, the newly sequenced allele (DDBJ/EMBL/GenBank accession number D86472), confirmed the absence of cysteine in PXP-18, showed four amino-acid replacements between the two allelic PXP-18, and proved that the three inconsistent residues were encoded by *POX18-2*. These results imply the predominant expression of *POX18-2* over *POX18-1*, indicate that the PXP-18 dimerizes by interaction other than disulfide bond, and agree with the observation that the lipid-transfer activity of PXP-18 was insensitive to *N*-ethylmaleimide *(19)*. Mammalian SCP2 also seems to dimerize without cysteine residue because Cys^{71} was recently shown to be dispensable for its lipid-transfer activity *(43)*. Molecular characteristics responsible for the dimerization of PXP-18 and SCP2 need to be clarified.

HYPOTHESIS

How does PXP-18 prevent the thermal denaturation of ACO? A model was constructed (Fig. 3) on the basis of the properties of PXP-18: the strong conformational reversibility, the dimerization at a high temperature, and the formation of near-stoichiometric complex, from which free ACO was released. At 70°C, PXP-18 changes its conformation slightly and interacts with an exposed hydrophobic region of the partially denatured ACO in a fairly specific manner, and forms a large complex that is reminiscent of antibody-antigen complexes owing to the divalent characteristic of the dimerized PXP-18. The tight, regular configuration prevents the further denaturation of ACO, preserves the octameric structure of the enzyme, and allows the

spontaneous release of the intact ACO accompanied by the conformational change of PXP-18. The large complex that still remains after cooling is the near-stoichiometric complex detected by sucrose density gradient centrifugation (Fig. 1B). Free ACO can be released from this complex slowly. The hydrophobic interaction mediated by a dimeric protein reminds us of the action of SCP2 in the non-specific lipid-transfer between membranes *(14)*. The role of PXP-18 in this model is partly similar to that of molecular chaperones *(44)* and compatible with the indirect role of the SCP2 part on type II thiolase.

Some well-known proteins exhibit an unexpected activity. Lens crystallins, whose primary function is to ensure the transparency and integrity of the lens, are revealed to have metabolic or chaperone activity *(45)*. Yeast phosphatidylinositol-transfer protein (identical to Sec14p) *(12, 14)*, which catalyzes the exchange of phosphatidylinositol and phosphatidylcholine between membrane bilayers, was shown to play an essential role in linking phospholipid metabolism with vesicle trafficking by way of maintaining the pools of diacylglycerol *(46)*. These and other instances encourage us to speculate that SCP2 or PXP-18 acts as a peroxisomal molecular chaperone as well. This speculation is plausible because no molecular chaperon has been identified in peroxisomes in spite of intensive search, though a plant hsp70 is imported into either glyoxysomes or plastids depending on the pre-sequence produced by alternative transcription initiation *(47)*. Common chaperones may not function under a severe oxidative-stress condition in peroxisomes. More information on the three-dimensional structure of proteins of SCP2 family is necessary to understand their physiological role.

REFERENCES

1. Noland, B. J., Arebalo, R. E., Hansbury, E., and Scallen, T. J. (1980) Purification and properties of sterol carrier protein$_2$. *J. Biol. Chem.* 255, 4282-4289.
2. Scallen, T. J., Pastuszyn, A., Noland, B. J., Chanderbhan, R., Kharroubi, A., and Vahouny, G. V. (1985) Sterol carrier and lipid transfer proteins. *Chem. Phys. Lipids* 38, 239-261.
3. Tsuneoka, M., Yamamoto, A., Fujiki, Y., and Tashiro, Y. (1988) Nonspecific lipid transfer protein (sterol carrier protein-2) is located in rat liver peroxisomes. *J. Biochem. (Tokyo)* 104, 560-564.
4. Keller, G. A., Scallen, T. J., Clarke, D., Maher, P. A., Krisans, S. K., and Singer, S. J. (1989) Subcellular localization of sterol carrier protein-2 in rat hepatocytes: Its primary localization to peroxisomes. *J. Cell Biol.* 108, 1353-1361.
5. Seedorf, U., Brysch, P., Engel, T., Schrage, K., and Assmann, G. (1994) Sterol carrier protein x is peroxisomal 3-oxoacyl coenzyme A thiolase with intrinsic sterol carrier and lipid transfer activity. *J. Biol. Chem.* 269, 21277-21283.
6. Antonenkov, V. D., Van Veldhoven, P. P., Waelkens, E., and Mannaerts, G. P. (1997) Substrate specificities of 3-oxoacyl-CoA thiolase A and sterol carrier protein 2/3-oxoacyl-CoA thiolase purified from normal rat liver peroxisomes. Sterol carrier protein 2/3-oxoacly-CoA thiolase is involved in the metabolism of 2-methyl-branched fatty acids and bile acid intermediates. *J. Biol. Chem.* 272, 26023-26031.
7. Wanders, R. J. A., Denis, S., Wouters, F., Wirtz, K. W. A., and Seedorf, U. (1997) Sterol carrier protein x (SCPx) is a peroxisomal branched-chain β-ketothiolase specifically reacting with 3-oxo-pristanoyl-CoA: A new, unique role for SCPx in branched-chain fatty acid metabolism in peroxisomes. *Biochem. Biophys. Res. Commun.* 236, 565-569.

8. Bun-ya, M., Maebuchi, M., Kamiryo, T., Kurosawa, T., Sato, M., Tohma, M., Jiang, L. L., and Hashimoto, T. (1998) Thiolase involved in bile acid formation. *J. Biochem. (Tokyo)* 123, 347-352.

9. Krisans, S. K. (1996.) Cell compartmentalization of cholesterol biosynthesis. *Ann. N Y Acad. Sci.* 804, 142-164

10. Puglielli, L., Rigotti, A., Greco, A. V., Santos, M. J., and Nervi, F. (1995) Sterol carrier protein-2 is involved in cholesterol transfer from the endoplasmic reticulum to the plasma membrane in human fibroblasts. *J. Biol. Chem.* 270, 18723-18726.

11. Bloj, B. and Zilversmit, D. B. (1977) Rat liver proteins capable of transferring phosphatidylethanolamine.. Purification and transfer activity for other phospholipids and cholesterol. *J. Biol. Chem.* 252, 1613-1619.

12. Wirtz, K. W. A. (1991) Phospholipid transfer proteins. *Annu. Rev. Biochem.* 60, 73-99.

13. Poorthuis, B. J. H. M. and Wirtz, K. W. A. (1983) Nonspecific lipid transfer protein from rat liver. *Methods Enzymol.* 98, 592-596.

14. Wirtz, K. W. A. (1997) Phospholipid transfer proteins revisited. *Biochem. J.* 324, 353-360.

15. Seedorf, U. and Assmann, G. (1991) Cloning, expression, and nucleotide sequence of rat liver sterol carrier protein 2 cDNAs. *J. Biol. Chem.* 266, 630-636.

16. Mori, T., Tsukamoto, T., Mori, H., Tashiro, Y., and Fujiki, Y. (1991) Molecular cloning and deduced amino acid sequence of nonspecific lipid transfer protein (sterol carrier protein 2) of rat liver: A higher molecular mass (60 kDa) protein contains the primary sequence of nonspecific lipid transfer protein as its C-terminal part. *Proc. Natl. Acad. Sci. USA* 88, 4338-4342.

17. Ohba, T., Holt, J. A., Billheimer, J. T., and Strauss III, J. F. (1995) Human sterol

carrier protein x/sterol carrier protein 2 gene has two promoters. *Biochemistry* 34, 10660-10668.

18. Gould, S. J., Keller, G.-A., and Subramani, S. (1987) Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase. *J. Cell Biol.* 105, 2923-2931.

19. Tan, H., Okazaki, K., Kubota, I., Kamiryo, T., and Utiyama, H. (1990) A novel peroxisomal nonspecific lipid-transfer protein from *Candida tropicalis*. Gene structure, purification and possible role in β-oxidation. *Eur. J. Biochem.* 190, 107-112.

20. Hwang, C. W., Yano, K., and Takagi, M. (1991) Sequences of two tandem genes regulated by carbon sources, one being essential for *n*-alkane assimilation in *Candida maltosa*. *Gene* 106, 61-69.

21. Pfeifer, S. M., Sakuragi, N., Ryan, A., Johnson, A. L., Deeley, R. G., Billheimer, J. T., Baker, M. E., and Strauss III, J. F. (1993) Chicken sterol carrier protein 2/sterol carrier protein x: cDNA cloning reveals evolutionary conservation of structure and regulated expression. *Arch. Biochem. Biophys.* 304, 287-293.

22. Bun-ya, M., Maebuchi, M., Hashimoto, T., Yokota, S., and Kamiryo, T. (1997) A second isoform of 3-ketoacyl-CoA thiolase found in *Caenorhabditis elegans*, which is similar to sterol carrier protein x but lacks the sequence of sterol carrier protein 2. *Eur. J. Biochem.* 245, 252-259.

23. Miyazawa, S., Furuta, S., Osumi, T., Hashimoto, T., and Ui, N. (1981) Properties of peroxisomal 3-ketoacyl-CoA thiolase from rat liver. *J. Biochem. (Tokyo)* 90, 511-519.

24. Pedersen, J. I. (1993) Peroxisomal oxidation of the steroid side chain in bile acid formation. *Biochimie* 75, 159-165.

25. Mannaerts, G. P. and Van Veldhoven, P. P. (1996) Functions and organization of

peroxisomal β-oxidation. *Ann. N Y Acad. Sci.* 804, 99-115.

26. Jiang, L. L., Miyazawa, S., and Hashimoto, T. (1996) Purification and properties of rat D-3-hydroxyacyl-CoA dehydratase: D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein. *J. Biochem. (Tokyo)* 120, 633-641.

27. Dieuaide-Noubhani, M., Novikov, D., Baumgart, E., Vanhooren, J. C. T., Fransen, M., Goethals, M., Vandekerckhove, J., Van Veldhoven, P. P., and Mannaerts, G. P. (1996) Further characterization of the peroxisomal 3-hydroxyacyl-CoA dehydrogenases from rat liver. Relationship between the different dehydrogenases and evidence that fatty acids and the C_{27} bile acids di- and tri-hydroxycoprostanic acids are metabolized by separate multifunctional proteins. *Eur. J. Biochem.* 240, 660-666.

28. Leenders, F., Tesdorpf, J. G., Markus, M., Engel, T., Seedorf, U., and Adamski, J. (1996) Porcine 80-kDa protein reveals intrinsic 17β-hydroxysteroid dehydrogenase, fatty acyl-CoA-hydratase/dehydrogenase, and sterol transfer activities. *J. Biol. Chem.* 271, 5438-5442.

29. Leenders, F., Husen, B., Thole, H. H., and Adamski, J. (1994) The sequence of porcine 80 kDa 17β-estradiol dehydrogenase reveals similarities to the short chain alcohol dehydrogenase family, to actin binding motifs and sterol carrier protein 2. *Mol. Cell. Endocrinol.* 104, 127-131.

30. Jiang, L. L., Miyazawa, S., Souri, M., and Hashimoto, T. (1997) Structure of D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein. *J. Biochem. (Tokyo)* 121, 364-369.

31. Barnes, T. M., Jin, Y., Horvitz, H. R., Ruvkun, G., and Hekimi, S. (1996) The *Caenorhabditis elegans* behavioral gene *unc-24* encodes a novel bipartite protein similar to both erythrocyte band 7.2 (stomatin) and nonspecific lipid transfer

protein. *J. Neurochem.* 67, 46-57.

32. Chan, Y.-L., Suzuki, K., and Wool, I. G. (1995) The carboxyl extensions of two rat ubiquitin fusion proteins are ribosomal proteins S27a and L40. *Biochem. Biophys. Res. Commun.* 215, 682-690.

33. Finley, D., Bartel, B., and Varshavsky, A. (1989) The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. *Nature* 338, 394-401.

34. Baker, R. T., Williamson, N. A., and Wettenhall, R. E. H. (1996) The yeast homolog of mammalian ribosomal protein S30 is expressed from a duplicated gene without a ubiquitin-like protein fusion sequence. Evolutionary implications. *J. Biol. Chem.* 271, 13549-13555.

35. Szyperski, T., Scheek, S., Johansson, J., Assmann, G., Seedorf, U., and Wüthrich, K. (1993) NMR determination of the secondary structure and the three-dimensional polypeptide backbone fold of the human sterol carrier protein 2. *FEBS Lett.* 335, 18-26.

36. Vijay-Kumar, S., Bugg, C. E., and Cook, W. J. (1987) Structure of ubiquitin refined at 1.8 Å resolution. *J. Mol. Biol.* 194, 531-544.

37. Kamiryo, T. and Okazaki, K. (1984) High-level expression and molecular cloning of genes encoding *Candida tropicalis* peroxisomal proteins. *Mol. Cell. Biol.* 4, 2136-2141.

38. Tan, H., Bun-ya, M., Hirata, A., and Kamiryo, T. (1994) Predominant localization of non-specific lipid-transfer protein of the yeast *Candida tropicalis* in the matrix of peroxisomes. *Yeast* 10, 1065-1074.

39. Niki, T., Bun-ya, M., Hiraga, Y., Muro, Y., and Kamiryo, T. (1994) Near-stoichiometric interaction between the non-specific lipid-transfer protein of the yeast *Candida tropicalis* and peroxisomal acyl-coenzyme A oxidase prevents the

thermal denaturation of the enzyme *in vitro*. *Yeast* 10, 1467-1476.

40. Okazaki, K., Takechi, T., Kambara, N., Fukui, S., Kubota, I., and Kamiryo, T. (1986) Two acyl-coenzyme A oxidases in peroxisomes of the yeast *Candida tropicalis*: Primary structures deduced from genomic DNA sequence. *Proc. Natl. Acad. Sci. USA* 83, 1232-1236.
41. Bloj, B. and Zilversmit, D. B. (1983) Nonspecific lipid transfer protein from rat and beef liver: Purification and properties. *Methods Enymol.* 98, 574-581.
42. Ohama, T., Suzuki, T., Mori, M., Osawa, S., Ueda, T., Watanabe, K., and Nakase, T. (1993) Non-universal decoding of the leucine codon CUG in several *Candida* species. *Nucleic Acids Res.* 21,4039-4045.
43. Seedorf, U., Scheek, S., Engel, T., Steif, C., Hinz, H.-J., and Assmann, G. (1994) Structure-activity studies of human sterol carrier protein 2. *J. Biol. Chem.* 269, 2613-2618.
44. Hartl, F. U. and Martin, J. (1992) Protein folding in the cell: The role of molecular chaperones Hsp70 and Hsp60. *Annu. Rev. Biophys. Biomol. Struct.* 21, 293-322.
45. Piatigorsky, J. and Wistow, G. J. (1989) Enzyme/crystallins: Gene sharing as an evolutionary strategy. *Cell* 57, 197-199.
46. Kearns, B. G., McGee, T. P., Mayinger, P., Gedvilaite, A., Phillips, S. E., Kagiwada, S., and Bankaitis, V. A. (1997) Essential role for diacylglycerol in protein transport from the yeast Golgi complex. *Nature* 387, 101-105.
47. Wimmer, B., Lottspeich, F., Van der Klei, I., Veenhuis, M., and Gietl, C. (1997) The glyoxysomal and plastid molecular chaperones (70-kDa heat shock protein) of watermelon cotyledons are encoded by a single gene. *Proc. Natl. Acad. Sci. USA* 94, 13624-13629.

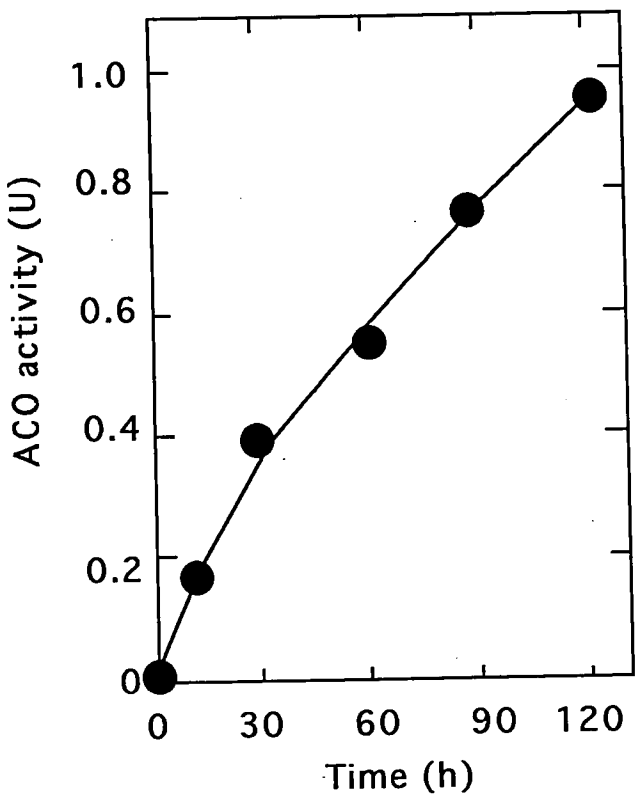
LEGENDS FOR FIGURES

Fig. 1. Release of free ACO from the near-stoichiometric complex. (A) A 100-μl mixture of 30 μM ACO and 120 μM PXP-18 was heated at 70°C for 15 min and centrifuged at 15,000 x *g* for 20 min to collect the near-stoichiometric complex. The precipitate, washed in buffer G *(39)*, was suspended in 100-μl buffer G containing 7.4 μM serum albumin and kept at 30°C. At each time indicated, an aliquot of the suspension was centrifuged as above, and the ACO activity in the supernatant was assayed. (B) The near-stoichiometric complex (a) and the supernatants obtained at 120 h (b) were analyzed by sucrose density gradient (42-60%) centrifugation as described *(39)*.

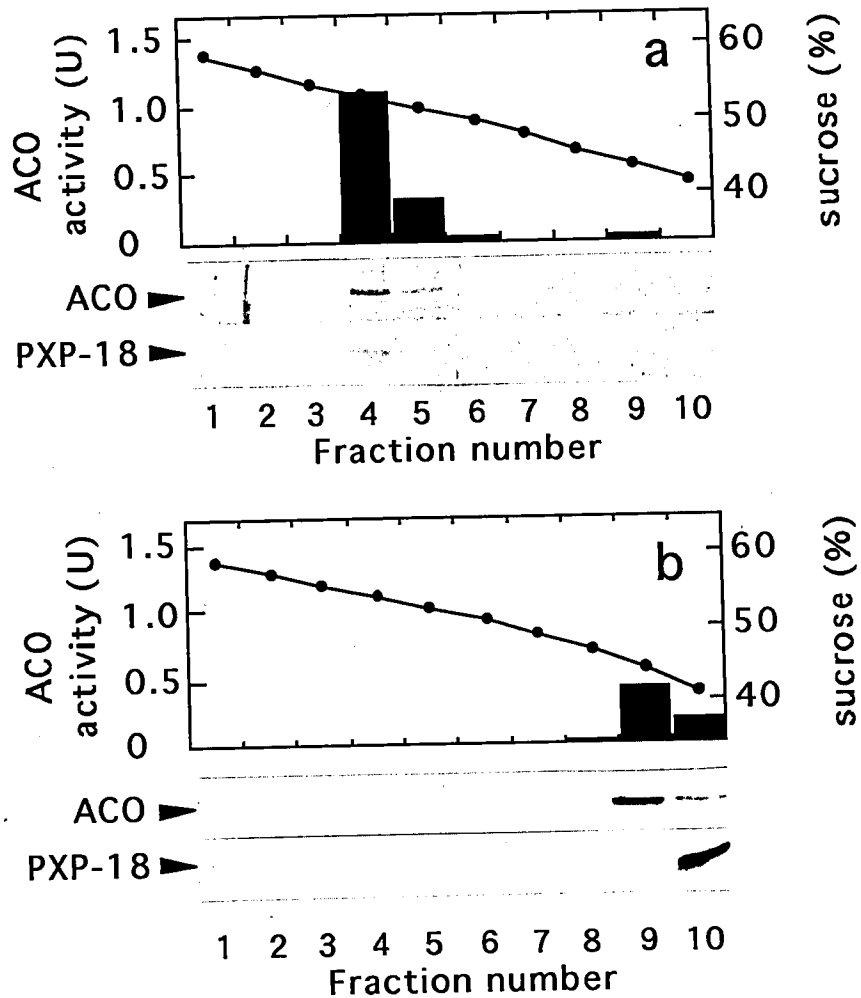
Fig. 2. The 29-kDa material produced at a high temperature. (A) The PXP-18 preparation after Phenyl-Sepharose chromatography *(39)* was heated at 70°C for 15 min and centrifuged at 15,000 x *g* for 15 min. The supernatant was passed through a Superdex 75 HR 10/30 column equilibrated with 20 mM sodium phosphate, pH 7.4, 200 mM NaCl and 1 mM EDTA. The resulting homogeneous PXP-18 was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) *(39)* before (lanes 1 and 3) or after (lanes 2 and 4) thermal treatment at 70°C for 15 min. Proteins were detected by silver stain (lanes 1 and 2) or anti-PXP-18 antibody (lanes 3 and 4). (B) The PXP-18 preparation was heated under conditions indicated, separated by SDS-PAGE, and stained with Coomassie brilliant blue. The arrow and arrowhead indicate PXP-18 and the 29-kDa material, respectively.

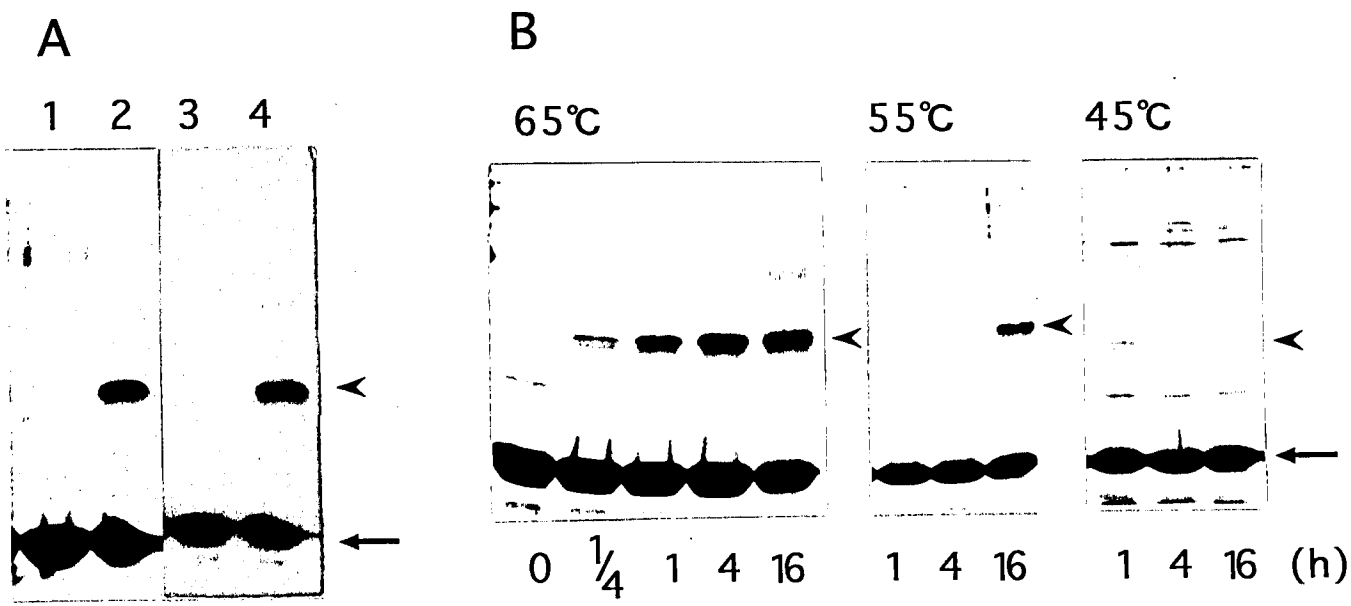
Fig. 3. A model for the interaction between PXP-18 and ACO.

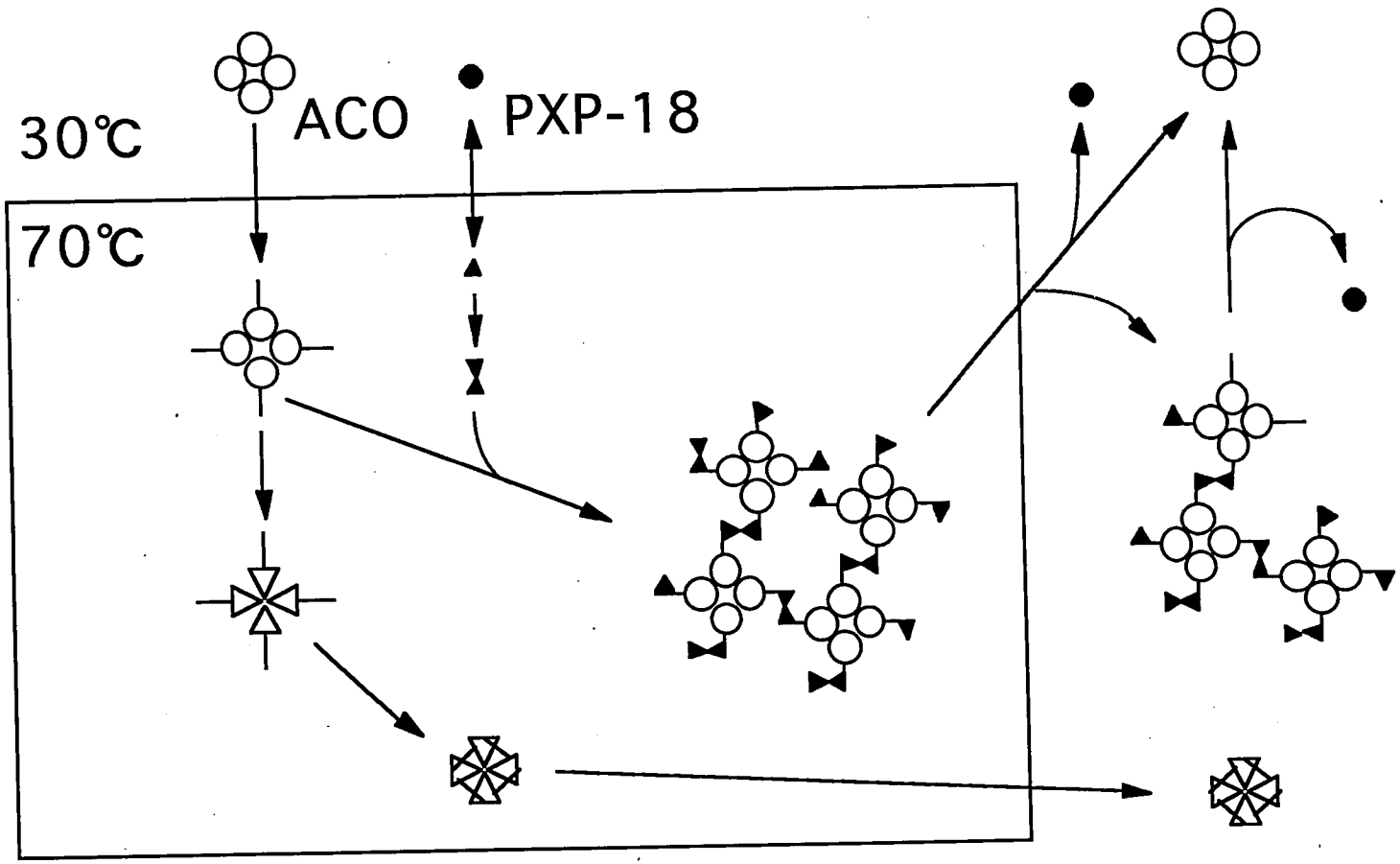
A


ACO activity (U)
1.0
0.8
0.6
0.4
0.2
0
0
30
60
90
120
Time (h)

B


a
ACO activity (U)
1.5
1.0
0.5
0
60
50
40
sucrose (%)
ACO
PXP-18
1 2 3 4 5 6 7 8 9 10
Fraction number
b
ACO activity (U)
1.5
1.0
0.5
0
60
50
40
sucrose (%)
ACO
PXP-18
1 2 3 4 5 6 7 8 9 10
Fraction number


A
1 2 3 4
B
65℃
55℃
45℃
0 1/4 1 4 16
1 4 16
1 4 16 (h)


30℃
ACO
PXP-18
70℃