FRET microscopy demonstrates molecular association of non-specific lipid transfer protein (nsL-TP) with fatty acid oxidation enzymes in peroxisomes

Fred S.Wouters1,2,3, Philippe I.H.Bastiaens2,3, Karel W.A.Wirtz1 and Thomas M.Jovin2,4

1Centre for Biomembranes and Lipid Enzymology, Institute of Biomembranes, Utrecht University, Padaulaan 8, NL-3584 CH, Utrecht, The Netherlands and 2Department of Molecular Biology, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, D-37077, Göttingen, Germany
3Present address: Cell Biophysics Laboratory, Imperial Cancer Research Fund, P.O. Box 123, Lincoln’s Inn Fields, London WC2A 3PX, UK
4Corresponding author

The fate of fluorescently labeled pre-nsL-TP (Cy3-pre-nsL-TP) microinjected into BALB/c 3T3 fibroblasts was investigated by confocal laser scanning microscopy. The protein exhibited a distinct punctate fluorescence pattern and colocalized to a high degree with the immunofluorescence pattern for the peroxisomal enzyme acyl-CoA oxidase. Proteolytic removal of the C-terminal leucine of the putative peroxisomal targeting signal (AKL) resulted in a diffuse cytosolic fluorescence. These results indicate that microinjected Cy3-pre-nsL-TP is targeted to peroxisomes. The association of nsL-TP with peroxisomal enzymes was investigated in cells by measuring fluorescence resonance energy transfer (FRET) between the microinjected Cy3-pre-nsL-TP and Cy5-labeled antibodies against the peroxisomal enzymes acyl-CoA oxidase, 3-ketoacyl-CoA thiolase, bifunctional enzyme, PMP70 and catalase. The technique of photobleaching digital imaging microscopy (pbDIM), used to quantitate the FRET efficiency on a pixel-by-pixel basis, revealed a specific association of nsL-TP with acyl-CoA oxidase, 3-ketoacyl-CoA thiolase and bifunctional enzyme in the peroxisomes. These observations were corroborated by subjecting a peroxisomal matrix protein fraction to affinity chromatography on Sepharose-immobilized pre-nsL-TP. Acyl-CoA oxidase was retained. These studies provide strong evidence for a role of nsL-TP in the regulation of peroxisomal fatty acid β-oxidation, e.g. by facilitating the presentation of substrates and/or stabilization of the enzymes.

Keywords: acyl-CoA oxidase fluorescence resonance energy transfer/3-ketoacyl-CoA thiolase/ PMP70/sterol carrier protein-2

Introduction

The non-specific lipid transfer protein (nsL-TP, also called sterol carrier protein 2, SCP2) is a basic (pI 8.5–9) protein of 14 kDa which catalyzes the transfer in vitro of glycerophospholipids, glycolipids and sterols (Bloj and Zilversmit, 1977, 1981; Crain and Zilversmit, 1980; Chanderbhun et al., 1982; Gadella and Wirtz, 1991). By virtue of this transfer activity, nsL-TP stimulates a number of enzyme activities related to cholesterol metabolism (Billheimer and Reinhart, 1990; Ossendorp et al., 1994). nsL-TP is synthesized on cytoplasmic polyribosomes as a 15 kDa protein containing a 20-amino-acid pre-sequence (Trzcinski et al., 1987; Fujiki et al., 1989) and a C-terminal tripeptide Ala-Lys-Leu. This tripeptide corresponds to the PTS1 consensus sequence (Ser/Ala/Cys)-(Lys/Arg/His)-Leu for peroxisomal targeting (Gould et al., 1987, 1989, 1990) and accounts for the peroxisomal localization of nsL-TP established by various immunocytochemical and biochemical techniques (Van der Krift et al., 1990). Moreover, there does not seem to be any clear correlation between intracellular levels of nsL-TP and the intracellular lipid transport. This conclusion is supported by the lack of an involvement of nsL-TP in the intracellular trafficking of lysosomal cholesterol (Johnson and Reinhart, 1989). The function of the presequence remains unknown, but it has been presumed to be involved in mitochondrial targeting (Keller et al., 1989; Billheimer et al., 1990; Moncecechi et al., 1991). After synthesis in the cytoplasm, pre-nsL-TP is imported into the peroxisomes via a receptor-mediated process (reviewed by Subramani, 1998) and converted into nsL-TP by cleavage of the presequence (Fujiki et al., 1989; Suzuki et al., 1990).

The in vivo function of nsL-TP has been a matter of extensive research and contention. The predominantly peroxisomal localization seems to exclude a role in intracellular lipid transport. This conclusion is supported by the lack of an involvement of nsL-TP in the intracellular trafficking of lysosomal cholesterol (Johnson and Reinhart, 1994). Moreover, there does not seem to be any clear correlation between intracellular levels of nsL-TP and the metabolism of cholesterol in situ (Van Heusden et al., 1985, 1992; Geelen et al., 1987). In contrast, there are various indications for a role of nsL-TP in peroxisomal fatty acid β-oxidation. The 58 kDa protein (also called SCPx) containing the complete sequence of pre-nsL-TP at the C-terminus is 50% homologous to peroxisomal 3-ketoacyl-CoA thiolase (Ossendorp et al., 1987, 1989, 1991). Recently, it has been shown that SCPx possesses a 3-ketoacyl-CoA thiolase activity operating on medium-chain fatty acids (octanoyl-CoA) (Seedorf et al., 1994) and, in contrast to the peroxisomal Type I thiolase, branched chain intermediates (Antonenkov et al., 1997; Wanders et al., 1997). It has been suggested that the presence of 3-ketoacyl-CoA thiolase and lipid transfer activity in a single protein results from gene fusion (Baker et al., 1991) and may denote an interdependence of the two activities. Disruption of the nsL-TP gene encoding both nsL-TP and SCPx leads to a severe deficiency of the peroxisomal β-oxidation of pristanic acid in mice (Seedorf et al., 1998). The yeast analog of nsL-TP, PXP18, is expressed in peroxisomes provided the cells are grown on oleic acid (Tan et al., 1994). Recently, Niki et al. (1994) demonstrated an interaction of PXP18 in vitro with yeast
poxisomal acyl-CoA oxidase. PXP18 protected acyl-CoA oxidase against heat-induced denaturation, suggesting that PXP18 might function as a molecular chaperone maintaining the integrity of peroxisomal proteins.

In the present study, we assessed the peroxisomal import of microinjected fluorescently labeled rat liver pre-nsL-TP (Cy3-pre-nsL-TP) by confocal laser scanning microscopy (CLSM) in the living cell. Proteolytic removal of the C-terminal leucine established the functionality of the putative peroxisomal targeting. The association of Cy3-nsL-TP imported into the peroxisomes with enzymes of the fatty acid β-oxidation pathway was determined by fluorescence resonance energy transfer (FRET) between Cy3-nsL-TP and Cy5-labeled antibodies raised against the various enzymes. These interactions were investigated in parallel by affinity chromatography of peroxisomal matrix proteins on columns of Sepharose-bound pre-nsL-TP.

Results

**Fluorescent labeling and characterization**

Purified pre-nsL-TP was labeled with the sulfoindocyanine dye Cy3 and the various antibodies to peroxisomal enzymes were labeled with Cy5. Under the conditions of labeling, a Cy3:pre-nsL-TP molar ratio of 0.7 and Cy5:antibody molar ratios of 5–7.5 were obtained. Cy3-pre-nsL-TP was incubated with a rat liver M-fraction to remove the pre-sequence by proteolysis (Ossendorp et al., 1992). As a result of this treatment, the molecular mass of Cy3-pre-nsL-TP shifted from 15 kDa (Figure 1, lane 1) to 14 kDa (Figure 1, lane 2), a position corresponding to nsL-TP. Transillumination with UV demonstrated that the Cy3 fluorescence was restricted to pre-nsL-TP and nsL-TP (Figure 1, lanes 3 and 4). In the latter case, no fluorescence was observed in the front, indicating that free dye was absent and the pre-sequence was unlabeled.

**Targeting of Cy3-pre-nsL-TP to peroxisomes**

After microinjection of Cy3-pre-nsL-TP in BALB/c 3T3 cells, CLSM images revealed a punctate fluorescence pattern distributed throughout the cytoplasm and indicative of peroxisomes (Figure 2A). Upon import of pre-nsL-TP in peroxisomes the presequence is removed; processing is substantial at 17 min and complete within 45 min (Fujiki et al., 1989; Suzuki et al., 1990). Since the Cy3 probe was located on the mature part of the protein (Figure 1), we infer that the fluorescence signals associated with peroxisomes corresponded to processed Cy3-nsL-TP.

The peroxisomal localization of the microinjected Cy3-labeled pre-nsL-TP was further confirmed by digestion with carboxypeptidase A to remove leucine from the C-terminal alanine-lysine-leucine (AKL), the putative peroxisomal targeting sequence. Digestion of unlabeled pre-nsL-TP under these conditions was assessed by determination of the molecular mass using electrospray mass spectrometry (Figure 3). The peak shifted from 15 053 mass units for pre-nsL-TP (Figure 3A) to 15 053 mass units for the treated protein (Figure 3B), indicating that the digestion was complete and specific for the C-terminal leucine. Upon microinjection, the truncated Cy3-pre-nsL-TP exhibited a diffuse labeling in the cell (Figure 2B), attesting to the essential role of the C-terminal AKL sequence in pre-nsL-TP as a peroxisomal targeting signal. The Cy3

![Image](https://example.com/image.png)
donor–acceptor pair is <10% beyond 7.2 nm. The rationale of the experiments was that FRET between Cy3-nsL-TP and a Cy5-labeled antibody would occur with detectable efficiency only for those cases in which intimate interactions between the respective proteins were present.

The first enzyme tested by FRET for its association with Cy3-nsL-TP was acyl-CoA oxidase using the combined approach of acceptor and donor pbDIM (Bastiaens et al., 1996; Bastiaens and Jovin, 1998; see Materials and methods). Cells microinjected with Cy3-pre-nsL-TP were incubated with Cy5-anti-acyl-CoA oxidase antibodies, and the corresponding FRET efficiencies determined by the CLSM acceptor photobleaching technique (Figure 4A).

The first acquired image (D1) is the fluorescence intensity distribution of the donor Cy3-nsL-TP directly excited at 543 nm. The second image (A1) represents the fluorescence intensity distribution of the acceptor Cy5-anti-acyl-CoA oxidase excited at 633 nm. The acceptor fluorophore was subsequently photobleached in part of the field (demarcated by a white rectangle in A2) by repeated scanning with the 633 nm laser line, thereby abolishing FRET. A second donor fluorescence image (D2) was obtained with 543 nm excitation. An increase of donor fluorescence intensity would be expected in the region of acceptor photobleaching only in those cellular structures exhibiting FRET. This effect was apparent exclusively in the peroxisomal punctate structures containing Cy3-nsL-TP (Figure 4B, donor difference image D2–D1), indicating the existence of a complex between nsL-TP and acyl-CoA oxidase in peroxisomes. The FRET efficiency throughout
the cell was calculated by a simple image arithmetic operation (Figure 4B, [D2–D1]/D2). As expected, the FRET efficiency outside the white rectangle (Figure 4B, upper panel) was near zero.

A second independent assessment of FRET in the cells was made from the donor photobleaching kinetics (Figure 4C). The part of the cell in which the acceptor was photodestroyed (Figure 4D, white rectangle) and thus FRET was abolished served as the reference. The average donor photobleaching time (τ) in the reference region was used to calculate FRET elsewhere in the image. That is, the donor photobleaching time increased at sites where FRET was operative (Bastaens and Jovin, 1996). This effect is seen in Figure 4D, which depicts the computed time constants τ for every pixel in pseudocolor. Noteworthy are the longer τ values indicative of FRET in punctate structures outside the reference region. The τ values for the non-imported Cy3-nsL-TP, observed as a diffuse staining in the cytoplasm and nucleus, were comparable to those within the reference region, demonstrating that FRET was only evident for Cy3-nsL-TP imported into peroxisomes.

The FRET efficiency was calculated for every pixel outside the reference region according to Equation 2 (Materials and methods; Figure 4E). The highest efficiencies were restricted to punctate structures. The specificity of the phenomenon was evident from the saturation of the FRET efficiency with increasing amounts of Cy5-labeled antibody (Figure 4E). At the highest dilutions (3200- and 1600-fold) no FRET was evident, while at a dilution of 600-fold, the FRET efficiency was substantial, ~30%, a value which did not increase further at still lower dilutions (higher concentrations). This finding confirmed that the observed complexes were specific, as was also apparent from the relative distribution of τ values (and the corresponding FRET efficiencies), within and outside the reference regions. Based on these results, the 600-fold dilution was used in all further experiments. The concentrations of the other antibodies were adjusted so as to obtain Cy5-fluorescence intensities equivalent to that of the 600-fold diluted anti-acyl-CoA oxidase.

The same technique was used to investigate possible associations of the other peroxisomal proteins to nsL-TP (Figure 5). Column 1 depicts the donor (Cy3-nsL-TP) fluorescence intensity distribution in the cells, and column 2 the corresponding acceptor (Cy5-labeled antibody) signal; the white rectangle denotes the location of the acceptor-free region after Cy5 photodestruction. The FRET efficiencies were calculated from the acceptor photobleaching method by division of the donor fluorescence intensity images before and after Cy5 photodestruction (Equation 1, Materials and methods; Figure 5, column 3) and by analyzing the donor photobleaching kinetics (Figure 5, column 4). FRET was restricted to punctate structures and was substantial with antibodies raised against bifunctional enzyme, 3-ketoacyl-CoA thiolase and PMP70, but negligible with the antibodies against catalase (Figure 5, row 4), the values for which were comparable to those in the absence of antibody. From these observations we conclude that acyl-CoA oxidase, 3-ketoacyl-CoA thiolase, bifunctional enzyme and PMP70 are in close molecular proximity to nsL-TP, but not to catalase. The structures exhibiting FRET matched the peroxisomal staining by Cy3-nsL-TP. That is, the extraperoxisomal Cy3-pre-nsL-TP did not produce a finite FRET signal, as already noted above.

Statistical evaluations were made of the FRET data in the various confocal images (Table 1; Materials and methods). The mean ± SD estimations from the donor and acceptor photobleaching experiments on regions from single cells were consistent (within experimental error). The combined mean FRET efficiencies for the various peroxisomal target proteins increased in the order: catalase < α < bifunctional enzyme (0.13) < PMP70 (0.24) < acyl-CoA oxidase (0.24) < 3-ketoacyl-CoA thiolase (0.33). The projected area/peroxisome was quite similar for all the target proteins.

During fluorescent detection of microinjected and endogenous nsL-TP and peroxisomal marker enzymes it was observed that nsL-TP and the marker enzymes did not invariably display the same pattern. nsL-TP was always detected in globular peroxisomes in both live and fixed cells, whereas the marker enzymes were also detected in elongated peroxisomes (Figure 5, columns 1 and 2). Such a heterogeneity in morphology and protein composition has been reported previously (Schrader et al., 1995, 1996; Wilcke et al., 1995).

**Affinity chromatography of peroxisomal matrix proteins**

The complexation of peroxisomal β-oxidation enzymes with nsL-TP was further investigated by affinity chromatography of rat liver peroxisomal matrix proteins on a pre-nsL-TP–Sepharose column. As a control for nonspecific binding, the matrix proteins were also applied to bovine serum albumin (BSA)- and lysozyme–Sepharose columns. Lysozyme was selected because the protein is small and basic, like nsL-TP.

Under high salt conditions (120 mM KCl), acyl-CoA oxidase eluted from the nsL-TP column in two peaks (Figure 6A). The immunoreactive bands at 71 and 52 kDa corresponded to the A and B subunits of acyl-CoA oxidase, respectively (Osumi et al., 1980). The first peak (fractions 3–12) was recovered in the run-through. The second peak (fractions 17–26) was retained on the column, showing an interaction of acyl-CoA oxidase with nsL-TP. The biphasic elution pattern was also observed when smaller amounts of matrix proteins were applied to the column.
Association of nsL-TP with β-oxidation enzymes

Fig. 4. Interaction between nsL-TP and acyl-CoA oxidase monitored by FRET between Cy3-nsL-TP and Cy5-labeled anti-acyl-CoA oxidase antibodies. Cy3-pre-nsL-TP microinjected BALB/c 3T3 fibroblasts were incubated with Cy5-labeled α-acyl-CoA oxidase antibodies. (A) Sequence of events to obtain FRET efficiency maps from acceptor photobleaching. A donor image D1 (Cy3-nsL-TP fluorescence intensity image) and acceptor image A1 (Cy5-labeled α-acyl-CoA oxidase fluorescence intensity image) are acquired. The acceptor is photobleached in a specific portion of the cell by continuous scanning with the 633 nm laser line (A2). The intracellular reference regions created by Cy5 photodestruction are enclosed in white rectangles. After photodestruction of Cy5 a second donor image D2 is obtained. (B) Calculated FRET efficiency maps ([D2–D1]/D2) and difference image (D2–D1). The upper image shows the FRET efficiency calculated for the whole field, and the lower image an enlarged area of the difference image (top half) and FRET efficiency (bottom half) where Cy5 was photobleached. (C) First nine images of a donor photobleaching series with the same cell as in (A). (D) Calculated photobleaching time map (τ) and photobleaching time histogram with the reference region in which the acceptor Cy5 was photodestructed marked by a white rectangle (ref), and reference photobleaching time (τr) marked by a white arrow. The red curve in the histogram provides the mapping from τ values to FRET efficiencies. Bar: 10 μm. (E) Lower panel, FRET efficiency map outside reference region calculated from donor photobleaching kinetics; upper panel, average FRET efficiency in peroxisomes as a function of Cy5-labeled α-acyl-CoA oxidase antibody dilution.
indicating that the two pools may have represented different forms of acyl-CoA oxidase. In support of this conclusion, acyl-CoA oxidase present in the run-through was not retained when again applied to the nsL-TP–Sepharose column. The acyl-CoA oxidase bound to neither the lysozyme- nor the BSA–Sepharose control columns (Figure 6B and C). A further finding was that 3-ketoacyl-CoA thiolase (Figure 6D) and catalase (Figure 6E) were not retained on the nsL-TP–Sepharose column. The affinities of β-oxidation enzymes for nsL-TP were also determined upon application of the peroxisomal matrix proteins to the affinity columns under low salt conditions (25 mM KCl). Acyl-CoA oxidase was quantitatively retained, whereas 3-ketoacyl-CoA thiolase or bifunctional enzyme showed no interaction. Under these low salt conditions, the bulk of acyl-CoA oxidase again exhibited no binding to the lysozyme– or BSA–Sepharose columns.

**Discussion**

We have demonstrated that the targeting sequence AKL in pre-nsL-TP is active in living BALB/c 3T3 fibroblasts (Figure 2A). Since the protein is synthesized on free polyribosomes in the cytoplasm, we expected that fluores-
cently labeled pre-nsL-TP (Cy3-pre-nsL-TP) microinjected in the cytoplasm would be likely to follow the same import route as natively expressed protein. This expectation was fulfilled. The destruction of the peroxisomal targeting signal resulted in abolishment of peroxisomal import.

Using a similar approach, it was shown that firefly luciferase containing the C-terminal PTS1 SKL motif and human serum albumin conjugated to a peptide carrying SKL are transported to peroxisomes upon microinjection into BALB/c 3T3 cells (Walton et al., 1992). Coinjection of SKL-bearing peptides prevented peroxisomal import of firefly luciferase, demonstrating that the process was saturable. Interestingly, microinjected protein started to appear in peroxisomes 2 h post-injection and reached maximal import at 18 h. This time scale of import was significantly longer than the 30 min which we observed in the case of pre-nsL-TP, for which there was no further increment at longer times. Furthermore, distinct structures were absent after carboxypeptidase treatment of the protein (Figure 2B). We obtained no evidence (in mouse fibroblasts) for a function of the pre-sequence in mitochondrial targeting (Keller et al., 1989; Billheimer et al., 1990; Moncecchi et al., 1991).

There was a punctate distribution of fluorescence intensity in the cells after microinjection of Cy3-pre-nsL-TP (Figure 2A) and after incubation of cells with Cy5-labeled antibodies against nsL-TP, i.e. the endogenous protein (data not shown). Using the Cy5-labeled antibodies against the other peroxisomal enzymes, elongated bodies were observed next to the punctate structures. The existence of a heterogeneous peroxisomal morphology was reported in HepG2 cells fixed with formaldehyde (Schrader et al., 1995, 1996), and found to be dependent on the degree of confluency of the cells. In subconfluent cells, peroxisomes were almost exclusively elongated. Although the fibroblasts in our study were maintained subconfluent in order to facilitate microinjection, we never observed elongated peroxisomes containing nsL-TP. Different peroxisomal shapes in primary Leydig cells have been reported using immunofluorescence specific for catalase, acyl-CoA oxidase and 3-ketoacyl-CoA thiolase (Litwin and Bilinska, 1995). Irregular shapes are also present in regenerating rat liver (Yamamoto and Fahimi, 1987).

Using pHbDIM, we were able to detect association of nsL-TP and the peroxisomal fatty acid oxidation enzymes acyl-CoA oxidase, 3-ketoacyl-CoA thiolase and bifunctional enzyme (Figures 4 and 5, Table I). The mean values of the FRET efficiencies for the various target proteins were in the range 0.13–0.33. The variations might have reflected: (i) inherent differences in the three-dimensional structure of the individual complexes; (ii) different labeling and binding stoichiometries of the antibodies; and/or (iii) differential accessibility of the antibodies. We attribute the spread in the FRET efficiency values, indicated by the SD values (Table I), to an actual distribution of values within the peroxisomes and not merely to measurement error. Data analysis based on two-dimensional histograms (not shown) demonstrated no correlation between the FRET values and the fluorescence intensities of the donor or the acceptor, with the exception of PMP70 for which the FRET efficiency increased with acceptor concentration. The latter phenomenon is consistent with the planar distribution of PMP70. On the basis of the FRET and biochemical data we propose that the direct peroxisomal protein partners of nsL-TP are present in the form of a multi-enzyme complex, or alternatively, that nsL-TP forms complexes with the individual enzymes as free species in the peroxisomal matrix. These two options could be addressed by a FRET approach using pairs of specific antibodies or Fabs directed against the individual β-oxidation enzymes labeled with donor/acceptor fluoro-
phores. However, this strategy is disadvantageous due to the anticipated large size of the resultant complex, leading to the separation between donors and acceptors exceeding the critical Förster distance, $R_0$.

The specific nature of the energy transfer signal was confirmed by the fact that the FRET efficiency was saturable with increasing amounts of Cy5-labeled antibodies. This was checked for the Cy5-acyl-CoA oxidase antibody by donor photobleaching kinetic analysis (Figure 4E). That the observed associations were specific was also apparent from the distribution of $\tau$ values within and external to the control region of the FRET experiments.

Unexpectedly, an apparent complexion of PMP70 to nsL-TP was detected using the two independent pbDIM techniques. It is known that nsL-TP can occur in a membrane-bound form (m-nsL-TP) (Van Heusden et al., 1990; Van Haren et al., 1992), especially in extrahepatic tissues. Thus, a complex with PMP70 might be explained by a membrane-association of Cy3-nsL-TP upon import. On the other hand, we note that PMP70 is the only protein tested that is geometrically restricted within the peroxisome, i.e. is confined to the peroxisomal membrane. The sixth-order dependency of FRET on the separation distance holds for a single donor–acceptor pair. In the case of PMP70, the acceptor is distributed in a plane and the distance dependency of FRET would be reduced to fourth order were this protein to function as an acceptor (Bastiaens et al., 1990). Thus, the observed ‘association’ might reflect this reduced distance dependency and/or the (small) dimensions of the peroxisome. Other proteins known to be localized in the peroxisomal matrix, presumably in a random distribution, should not exhibit this phenomenon.

The identification of an nsL-TP–protein complex inferred from the FRET measurements was confirmed biochemically by nsL-TP-affinity chromatography of a rat liver peroxisomal matrix preparation. Using this technique, specific binding of acyl-CoA oxidase to nsL-TP was detected (Figure 6). However, binding of 3-ketoacyl-CoA thiolase and of bifunctional enzyme to nsL-TP was not demonstrated. The apparent discrepancy between the FRET measurements and the results of affinity chromatography may indicate that 3-ketoacyl-CoA thiolase and bifunctional enzyme bind to nsL-TP indirectly via another protein, possibly acyl-CoA oxidase. The distance between nsL-TP and these enzymes would still fall within the limits of FRET detection, i.e. $\gtrsim R_0$. Affinity chromatography is not ideally suited for detecting indirect interactions (Phizicky and Fields, 1995). It is conceivable that the particular binding conditions of the affinity chromatography experiments (ionic, concentrations, absence of other factors) were unfavorable for the binding of 3-ketoacyl-CoA thiolase and bifunctional enzyme. In short, negative results in such experiments are not conclusive.

Our findings offer support for the notion that nsL-TP is involved in peroxisomal fatty acid $\beta$-oxidation rather than acting as a lipid-transfer (sterol carrier) protein. One can reconcile the earlier observations of lipid-binding exhibiting low affinity and broad specificity with a presumed role in fatty acid oxidation by invoking a shuttle function for nsL-TP. The protein would serve to transport fatty acyl-CoA through the chain of fatty acid oxidizing enzymes, presenting the substrate to the respective active sites and thereby transiently increasing the local concentration. The binding of fatty acyl-CoA to nsL-TP has been reported (Frolov et al., 1996). We have also demonstrated the high-affinity binding of CoA esters of very long fatty acids (C24:0, C24:1, C26:0, C26:1) to this protein (unpublished data) and the binding of fatty acid $\beta$-oxidation intermediates and branched-chain fatty acyl substrates, with affinities decreasing in the order palmitoyl-CoA $>$ palmitenoyl-CoA $>$ 3-hydroxyacyl-CoA $>$ 3-oxoacyl-CoA, phytanoyl-CoA $>$ pristanoyl-CoA (Wouters, 1997). Fast binding kinetics were only observed when the substrates were presented as monomers, i.e. in $\beta$-cyclodextrin, corresponding to a function in fatty acid uptake from a $\beta$-oxidation enzyme hydrophobic pocket. In the case of SCPx containing the complete sequence of pre-nsL-TP at the C-terminus and shown by Seedorf et al. (1994) to contain both a 3-ketoacyl-CoA thiolase activity and lipid transfer activity, the interaction between a lipid-metabolizing enzyme and nsL-TP was achieved physically by a fusion of the two genes. The findings of in vitro stimulation of several cholesterol-metabolizing activities by nsL-TP would implicate the low-affinity lipid-binding site in the presentation of the lipid substrate. In a recent report, the yeast analogue of nsL-TP, PXP18, was shown to protect acyl-CoA oxidase against heat-induced denaturation in vitro (Niki et al., 1994). The authors suggested that nsL-TP might help to stabilize acyl-CoA oxidase and 3-ketoacyl-CoA thiolase, thereby increasing the lifetime of these enzymes. Furthermore, a novel peroxisomal non-specific lipid transfer protein was shown to co-purify with peroxisomal acyl-CoA oxidase in yeast (Ceolotto et al., 1996). The finding that nsL-TP is associated with acyl-CoA oxidase, 3-ketoacyl-CoA thiolase and bifunctional enzyme raises the possibility that the $\beta$-oxidation enzymes in peroxisomes are organized in a functional complex, leading to an efficient transfer of the lipid intermediates between the enzymes. Functional compartmentalization would seem to be a prevailing principle of intracellular organelles.

**Materials and methods**

**Materials**

Recombinant rat pre-nsL-TP was expressed and purified to homogeneity as described in Osendarp et al. (1992). Polyclonal rabbit antisera against rat liver acyl-CoA oxidase, rat liver 3-ketoacyl-CoA thiolase, bovine liver catalase and the C-terminal 26 kDa ABC-binding cassette fragment of recombinant rat liver PMP70 were kindly provided by Prof. Dr H.F.Tabak (Dept of Biochemistry, Academical Medical Centre, Amsterdam, The Netherlands) and against rat liver bifunctional enzyme by Prof. Dr W.W.Just (Institut für Biochemie I, University of Heidelberg, Germany). Monofunctional succinimide esters of sulfoindocyanine fluorescent dyes Cy3.29 OSu (Cy3) and Cy5.29 OSu (Cy5) (Southwick et al., 1990) were from Amersham (Buckinghamshire, UK), Microcon and Centricron concentration units from Amicon (Beverly, MA), carboxypeptidase A from Sigma (St Louis, MO) and P10DG Econopac columns and protein assay kit from Bio-Rad (Hercules, CA). Protilfar non-fat dried milk powder was from Nutricia (Zoetermeer, The Netherlands), and Protran nitrocellulose (0.45 µm) from Schleicher & Schuell (Dassel, Germany). Horseradish peroxidase conjugated goat-anti-rabbit antibodies (GARPO) were from Nordic Immunology (Tilburg, The Netherlands). Renaissance enhanced chemoluminescence reagents were from Dupont NEN (Boston, MA). CNBr-activated Sepharose 4B was from Pharmacia (Uppsala, Sweden).

**Preparation of subcellular fractions**

Mitochondrial (M) and light mitochondrial (L) fractions were isolated from a rat liver homogenate by differential centrifugation according to...

---

F.S.Wouters et al.
Völk and Fahimi (1985). An enriched peroxisomal fraction was prepared by loading the L fraction onto a 30% Nycodenz cushion followed by centrifugation at 45 min at 132,000 g. The pellet enriched in peroxisomes was gently resuspended in a buffer consisting of 0.25 M sucrose, 5 mM MOPS–NaOH pH 7.2, 1 mM EDTA and 0.1% ethanol, and stored at −80°C. The matrix proteins (0.17 mg) were isolated from this peroxisomal fraction using the Triton X-114 phase separation method (Bordier, 1981).

Fluorescent labeling of pre-nsL-TP and antibodies
Pre-nsL-TP (4.2 mg/ml) stored at −20°C in 25 mM Tris–HCl pH 7.5, 2.5 mM EDTA, 5 mM 2-mercaptoethanol, 50% glycerol. An aliquot (250 μg) was transferred to labeling buffer [50 mM Bicine–NaOH pH 8.0, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM 2-mercaptoethanol, 120 mM KCl, 10% (v/v) glycerol] by four cycles of concentration and subsequent redilution in a Centricon YM10 concentrator at 4°C up to a final concentration of 1 mg/ml. The labeling reaction was initiated by adding 1 μl of Cy3 reagent in dry DMF (60 mM) to 0.1 ml of pre-nsL-TP (0.1 mg) in labeling buffer. The reaction mixture was incubated for 30 min at room temperature and the reaction was stopped by the addition of glycine (final concentration of 10 mM). The labeled protein was separated from the unconjugated dye by gel permeation chromatography on a PD10 column equilibrated in the buffer used for microinjection [50 mM Tris–HCl pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM 2-mercaptoethanol, 120 mM KCl, 10% (v/v) glycerol]. IgG fractions of polyclonal rabbit antisera were obtained by Protein-G affinity chromatography and were transferred to 0.1 M bicine–NaOH pH 9.0 using a Centricron YM30 concentrator. The IgG fractions were labeled with the Cy5 reagent (10- to 30-fold molar excess) for 30 min at room temperature as described for pre-nsL-TP Unconjugated dye was removed by chromatography on a PD10 column equilibrated in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.3 mM Na2HPO4, 1.5 mM KH2PO4).

Dye to protein molar ratios were determined spectrophotometrically from $E_{528} = 150$ μM/cm for Cy3 and $E_{488} = 250$ μM/cm for Cy5, and protein concentrations by the Bio-Rad protein assay kit with BSA as standard.

Proteolytic removal of the presequence
The presequence of Cy3-pre-nsL-TP (1 μg) was proteolytically removed by overnight incubation with a rat liver M fraction (75 μg) at 37°C in 0.1 ml 50 mM Tris–HCl pH 7.0, 2 mM EDTA, 10 mM 2-mercaptoethanol as described by Osseendorp et al. (1992). The cleavage products were identified by applying the total reaction mixture to a 15% SDS–polyacrylamide gel. The gel was transilluminated by UV to detect Cy3 and stained for total protein by Coomassie Brilliant Blue.

Proteolysis of the C-terminus by carboxypeptidase A
Proteolysis of Cy3-pre-nsL-TP by carboxypeptidase A (10 units/μmol) was performed in 6.25 mM Tris–HCl pH 7.4, 0.6 mM EDTA, 12.5% (v/v) glycerol at 30 min at 25°C. Carboxypeptidase A was added from a crystalline stock solution and subsequently removed by filtration on an YM10 concentrator. After the incubation, the treated Cy3-pre-nsL-TP was transferred to the buffer used for microinjection as described in the Fluorescent labeling section.

The extent and specificity of carboxypeptidase A proteolysis was assessed by using unlabeled pre-nsL-TP. The characterization of the product was carried out by Dr W.D. van Dongen (Department of Analytical Molecule Spectrometry, Utrecht University, Utrecht, The Netherlands) using electrospray mass spectrometry.

Microinjection and immunolabeling
Microinjection was carried out on BALB/c 3T3 mouse fibroblasts grown at 37°C on Eppendorf Cell locator coverslips in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum at 100,000 g for 5 min, and the solution containing Cy3-pre-nsL-TP was centrifuged for 5 min at 100,000 g in a Beckman Airfuge. Cells were microinjected using an Eppendorf microinjection equipped with Eppendorf femtotip glass capillaries at an injection pressure of 80 hPa for 0.3 s. After injection the cells were allowed to recover for 30 min at 37°C in a 5% CO2 atmosphere. At this point the cells were either prepared for live observation or fixed and prepared for immunofluorescence. Coverslips with live cells were mounted in a temperature-controlled cell chamber and observed by CLSM under a continuous flow of DMEM without the pH indicator phenol red and supplemented with 10% FCS and 20 mM HEPES–NaOH pH 7.4.

For immunofluorescence, the cells were fixed with 4% w/v paraformaldehyde in PBS for 10 min at room temperature, washed in PBS, quenched in 100 mM Tris–HCl pH 7.5, 50 mM NaCl for 5 min, permeabilized in 0.1% (v/v) Triton X-100 for 5 min and after washing with PBS incubated for 1 h with Cy5-labeled antibody in PBS containing 1% (w/v) BSA. After incubation, the cells were washed four times in PBS and mounted on glass slides using Mowiol 481 ( Hoechst). All incubations were performed at room temperature. The antibodies used were anti-acyl-CoA oxidase (0.6 μg/ml) anti-bifunctional enzyme (0.3 μg/ml), anti-3-ketoacyl-CoA thiolase (0.3 μg/ml) and anti-catalase (1.8 μg/ml). At these concentrations, the fluorescence signals were comparable.

Imaging techniques
Fluorescence images of cells were acquired on a confocal laser scanning microscope (CLSM; Zeiss LSM 310). Cy3 was excited with a 543 nm external He–Ne laser and detected using a 575 nm long-pass filter or, if incu bated with Cy5-antibodies, using an Omega 590 ± 30 nm band pass filter. Cy5 was excited with a 633 nm He–Ne laser and detected using a 665 nm long-pass filter. The images were taken with a 40×1.3 NA oil immersion Plan-Neofluar objective.

FRET in donor-labeled cells was determined in a microscope by the combined acceptor and donor photobleaching methods (pHDM) (Jovin and Arndt-Jovin, 1989; Gadella and Jovin, 1995; Bastaens and Jovin, 1996, 1998; Bastaens et al. 1996) according to the following sequence. (i) A pre-photobleach Cy3 (donor) image was acquired by scanning with 543 nm light in the CLSM. (ii) An intracellular region of interest (ROI) was selected and rendered free of Cy5 (acceptor) by repeated scanning with the 532 laser until all Cy5 was photodestroyed. (iii) A second post-photobleach Cy3 image was acquired by scanning with a 543 nm laser. (iv) After correction for image registration (Bastaens et al., 1996), the FRET efficiencies ($E_F$) in pixel $i$ of the ROI were calculated from image arithmetic of the two (pre-photobleach, $I_{pre,i}$, and post-photobleach, $I_{post,i}$) Cy3 images.

$$E_F = 1 - \frac{I_{post,i}}{I_{pre,i}}$$

A second FRET determination in the complementary part of the ROI in the cells was performed by time-resolved donor photobleaching on a Zeiss Axiosplan microscope equipped with a 100 W Hg lamp. The images were taken with a 40×1.3 NA oil immersion Plan-Neofluar objective. The fluorescent Cy3 label on the pre-nsL-TP was photobleached by excitation with 546 nm light (Zeiss 546B12 bandpass filter) for 30 min. At 30 s intervals Cy3 fluorescence images were obtained by 10–25 s exposure with a cooled CCD camera (Photometrics Series 200) using FITC-dichroic (Zeiss) and 600/62 bandpass filters. Images were recorded until the signal was ~80% bleached and the sequence was fitted pixel-by-pixel to a single exponential decay and offset model by the program DECAY (Gadella and Jovin, 1995, 1997). The latter yielded the photobleaching time constant $τ_c$, amplitude $a_i$ and offset $c_i$ for each pixel $i$. FRET efficiencies for every pixel $i$ ($E_F$) were then calculated from the ratio of photobleaching time ($τ_i$) and the average photobleaching time ($τ_c$) in the ROI in which Cy5 was photodestroyed.

$$E_F = 1 - \frac{〈τ_i〉}{τ_c}$$

Statistical analyses averaged over the peroxisomal compartment of single cells were obtained by a sequence of image processing operations using IPLab (Signal Analytics), as follows. The local background contributions around the individual peroxisomes were estimated by applying a morphological filter to the confocal gray value data (erosion, i.e. minimum estimation, over a 5×5 neighborhood), and subtracting the result from the original images. A global threshold was used to generate binary masks for isolating the peroxisomes. The estimated parameters are defined and given in Table I.

Affinity chromatography
Pre-nsL-TP, lysozyme and BSA were dissolved in coupling buffer (100 mM Bicine–NaOH pH 8.3, 500 mM NaCl, 1 mM EDTA) at a concentration of 5 mg/ml. The protein solutions (1 ml) were added to CNBr-activated Sepharose 4B (0.7 ml) and allowed to react by incubation overnight at 4°C. Coupling efficiency exceeded 95% as estimated by protein determination. After coupling, the beads were incubated for 1 h at room temperature in 100 mM Tris–HCl pH 8.0, 1 mM EDTA to block the reactive groups and were washed by four cycles of 100 mM sodium.
acetate pH 4.0, 1 mM EDTA and coupling buffer to remove unconjugated protein. The beads were loaded in glass columns (0.5 × 4 cm) and equilibrated in elution buffer (20 mM K-phosphate pH 6.4, 120 mM KCl, 1 mM EDTA). Non-specific protein binding sites were blocked by washing the affinity columns with 1 ml elution buffer containing 4 mg BSA.

The peroxisomal matrix fraction was transferred to elution buffer and concentrated to 0.4 mg/ml using an YM20 Centricon. Aliquots of these fractions (30 μg of protein) were applied to the column and 0.23 ml fractions were collected. Fractions were precipitated by trichloro-acetic acid, heated at 95°C for 5 min in sample buffer and subjected to SDS–polyacrylamide electrophoresis on 12.5% acrylamide gels according to Laemmli (1970).

Gels were electroblotted to nitrocellulose according to Kyhse-Andersen (1984) for 1 h at 1 mA/cm2. After transfer, the nitrocellulose was stained for total protein with Coomassie Brilliant Blue. The blots were washed for 5 min with PBS and blocked in 2% (w/v) skimmed milk powder in PBS-Tween (PBS supplemented with 0.5% Tween-20) for 1 h. The blots were then incubated with primary antisemir diluted 1:2000 in 0.2% (w/v) non-fat dried milk powder in PBS-Tween for 1 h, washed three times for 10 min in 0.2% milk powder in PBS-Tween, incubated with GARPO diluted 1:15 000 in 0.2% milk powder in PBS-Tween for 1 h and washed three times for 10 min in 0.2% milk powder in PBS-Tween. Enhanced chemiluminescence (ECL) immunodetection was performed according to the protocol provided by Dupont NEN. Routinely, 5 min exposures were required to detect the specific protein.

Acknowledgements

We thank Teunis B.H. Geijtenbeek for providing purified recombinant pre-nsL-TP. This research was carried out under auspices of The Netherlands Foundation for Chemical Research (SON) and with financial aid from The Netherlands Organization for Scientific Research (NWO) and a grant from the Federation of European Biochemical Sciences (FEBs).

References


Association of nsL-TP with β-oxidation enzymes


Received May 13, 1998; revised August 27, 1998; accepted October 12, 1998