High-affinity binding of very-long-chain fatty acyl-CoA esters to the peroxisomal non-specific lipid-transfer protein (sterol carrier protein-2)

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Binding of fluorescent fatty acids to bovine liver non-specific lipid-transfer protein (nsL-TP) was assessed by measuring fluorescence resonance energy transfer (FRET) between the single tryptophan residue of nsL-TP and the fluorophore. Upon addition of pyrene dodecanoic acid (Pyr-C12) and *cis*-parinaric acid to nsL-TP, FRET was observed indicating that these fatty acids were accommodated in the lipid binding site closely positioned to the tryptophan residue. Substantial binding was observed only when these fatty acids were presented in the monomeric form complexed to β-cyclodextrin. As shown by time-resolved fluorescence measurements, translocation of Pyr-C12 from the Pyr-C12–β-cyclodextrin complex to nsL-TP changed dramatically the direct molecular environment of the pyrene moiety: i.e. the fluorescence lifetime of the directly excited pyrene increased at least by 25% and a distinct rotational correlation time of 7 ns was observed. In order to evaluate the affinity of nsL-TP for intermediates of the β-oxidation pathway, a binding assay was developed based on the ability of fatty acyl derivatives to displace Pyr-C12 from the lipid binding site as reflected by the reduction of FRET. Hexadecanoyl-CoA and 2-hexadecenoyl-CoA were found to bind readily to nsL-TP, whereas 3-hydroxyhexadecanoyl-CoA and 3-ketohexadecanoyl-CoA bound poorly. The highest affinities were observed for the very-long-chain fatty acyl-CoA esters (24:0-CoA, 26:0-CoA) and their enoyl derivatives (24:1-CoA, 26:1-CoA). Binding of non-esterified hexadecanoic acid and tetracosanoic acid (24:0) was negligible.

Key words: beta-oxidation, fluorescence spectroscopy, pyrene dodecanoic acid.

INTRODUCTION

Mammalian non-specific lipid-transfer protein (nsL-TP) is a small (14 kDa), basic (pI = 8.5) protein which, *in vitro*, is able to mediate the transfer of a great variety of lipids between membranes [1]. Originally, this protein was identified by its ability to transport phosphatidylethanolamine [2,3]. Independently, nsL-TP was recognized as a protein (sterol carrier protein-2) capable of stimulating cholesterol synthesis *in vitro* [4]. In line with this activity, nsL-TP effectively stimulates the transfer of cholesterol between membranes [3]. These diverse transfer activities may be due to the fact that nsL-TP has a low-affinity lipid binding site which interacts with the membrane [5]. The physiologically relevant ligand of nsL-TP has been a matter of extensive research. Binding of fluorescently labelled phospholipids has been observed [6,7], but conflicting reports exist with regard to its ability to bind sterols [5,8,9]. More recently, nsL-TP was shown to bind fatty acids and fatty acyl-CoA esters [10,11].

nsL-TP is synthesized on free cytosolic polyribosomes as a precursor protein (pre-nsL-TP), which, by virtue of its C-terminal Ala-Lys-Leu tripeptide, is transported to peroxisomes [12,13]. The peroxisomal localization of nsL-TP was confirmed by immunogold labelling [14,15,16]. Evidence has been provided for the involvement of nsL-TP in peroxisomal fatty-acid oxidation, and it was shown that nsL-TP constitutes the C-terminal part of the peroxisomal 58 kDa protein (sterol carrier protein-x), which expresses branched-chain acetyl-CoA *C*-acetyltransferase (3-ketoacyl-CoA thiolase) and lipid transfer activity [17–19]. By fluorescence resonance energy transfer (FRET) experiments *in situ* using BALB/c 3T3 fibroblasts, nsL-TP was shown to be associated with the enzymes of the peroxisomal β-oxidation pathway [20]. Knock-out mice lacking nsL-TP and sterol-carrier protein-x showed an increased expression of mitochondrial and peroxisomal β-oxidation enzymes [21].

In the study by Frolov et al. [11], fluorescent fatty acyl analogues were used, which on binding to nsL-TP induced a change in the fluorescent characteristics of the lipid. In the present study, a novel fatty-acid binding assay is used based on FRET between the tryptophan residue (the donor) of nsL-TP and a fluorescent fatty acid (the acceptor) which binds to nsL-TP. This assay has as a major advantage in that the binding of naturally occurring fatty acyl species as well as that of other lipids can be estimated directly by measuring their ability to displace the fluorescent fatty acid, pyrene dodecanoic acid (Pyr-C12), from the lipid binding site. The efficiency of displacement as reflected by a decrease in FRET is taken as a measure of the affinity of nsL-TP for the non-fluorescent fatty acids. By making use of this assay we have shown that nsL-TP has a high affinity for binding the CoA esters of very-long-chain fatty acids (VLCFAs).

MATERIALS AND METHODS

Materials

Hexadecanoic acid, the corresponding CoA esters and β-cyclodextrin were from Sigma (St. Louis, MO, U.S.A.). Pyr-C12 and *cis*-parinaric acid were from Molecular Probes (Eugene, OR, U.S.A.). The β-oxidation intermediates α,β-unsaturated, 3-

Abbreviations used: nsL-TP, non-specific lipid-transfer protein; ACBP, acyl-CoA binding protein; FABP, fatty acid binding protein; Pyr-C12, pyrene dodecanoic acid; VLCFA, very-long-chain fatty acid; FRET, fluorescence resonance energy transfer; LTP, plant non-specific lipid-transfer protein.

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hydroxy- and 3-oxo-hexadecanoyl-CoA were synthesized enzymically from hexadecanoyl-CoA using acyl-CoA oxidase, crotonase and 3-hydroxyacyl-CoA dehydrogenase (all obtained from Sigma), essentially using the method described by Seubert et al. [22]. The acyl-CoA esters were purified by HPLC on a reverse-phase C_{18}-column (Supercosil SPLC-18-DB, 250-mm × 10-mm; Supelco, Bellefonte, PA, U.S.A.) and eluted using an acetonitrile gradient [40–50 % (v/v)] in 16.9 mM sodium phosphate, pH 6.9. Acyl-CoA esters were stored at 4 °C in 20 mM Mes/NaOH, pH 6.0, in which they were stable for extended periods of time. Lignoceroyl-CoA (24:0-CoA) was obtained from Sigma. Cerotoyl-CoA (26:0-CoA) was prepared by the method of Rasmussen [23]. The product was purified by HPLC on an SPLC-8-DB column (Supelco) using a linear gradient of acetonitrile [58–70 % (v/v)] in 16.9 mM sodium phosphate, pH 6.9. Lignocerenoyl and cerotenoyl CoA esters (24:1-CoA and 26:1-CoA respectively) were prepared by incubation of the corresponding saturated fatty acyl-CoA esters (100 μM) with acyl-CoA oxidase (Sigma) (7.5 and 22.5 units respectively) for 1 h at 37 °C in 50 mM Tris/HCl, pH 8.0, containing 1 μM FAD. The reaction was stopped by adding HCl to a final concentration of 0.5 M. After cooling on ice for 1 h, the products were sedimented by centrifugation at 2700 ***g*** for 10 min at 4 °C. The pellet was taken up in 20 mM Mes buffer, pH 6.0, and the products were purified on HPLC using similar conditions as those used for the cerotoyl-CoA ester. Recombinant rat pre-nsL-TP was overexpressed and purified as described by Ossendorp et al. [24]. Bovine nsL-TP was purified using a modification of the procedure of [25]. Chicken egg-white lysozyme was from Sigma.

Preparation of fatty acid substrates

Pyr-C12 or *cis*-parinaric acid was diluted from a methanol stock solution to 75 μM in 17.5 mM β-cyclodextrin in PBS, vortex-mixed for 1 min and sonicated at 35 kHz for 15 min in a Branson ultrasonic waterbath. The molar excess of β-cyclodextrin (230-fold) was determined by titrating self-quenched Pyr-C12 micelles with β-cyclodextrin until no further increase in fluorescence intensity was observed. Under these conditions Pyr-C12 is taken up as monomer into the β-cyclodextrin [26]. This solution was diluted ten times in PBS to obtain a working solution. Non-fluorescent fatty acids from methanol and the fatty acyl-CoA esters from a 20 mM Mes (pH 6.0) stock solution were prepared in a similar way. The concentrations of the fluorescent fatty acids were determined by measuring the absorbance in methanol (ϵ_{341}, 44 $mM^{-1} \cdot cm^{-1}$ for Pyr-C12; ϵ_{303}, 76 $mM^{-1} \cdot cm^{-1}$ for *cis*-parinaric acid) and concentrations of the fatty acyl-CoA esters were determined by measuring the absorbance in 100 mM Mes, pH 6.0 (ϵ_{269} 16 $mM^{-1} \cdot cm^{-1}$).

Steady-state fluorescence measurements

Measurements were performed on a SLM-Aminco SPF-500C spectrofluorimeter equipped with a thermostatted cuvette holder and a magnetic stirring device. Tryptophan was excited at 280 nm (band width 4 nm), and the emission was measured from 300 to 550 nm (band width 10 nm) to obtain spectra or was set at 378 nm (Pyr-C12), 415 nm (*cis*-parinaric acid) or 335 nm (tryptophan) for single point measurements (averaged for 2 s). All measurements were performed at 25 °C with continuous stirring.

Time-resolved fluorescence measurements

Time-resolved fluorescence measurements were carried out using a time-correlated photon counting procedure as described previously [27]. The frequency doubled output of a mode-locked continuous-wave Nd:YLF laser (Coherent, Santa Clara, California) was used for the synchronous pumping of a cavity-dumped DCM [4-dimethylene-2-methyl-6-(*p*-dimethylaminostyryl)-4*H*-pyran] dye laser (repetition rate 475 kHz). A $LiIO_3$ crystal was used for frequency doubling of the output of the dye laser to obtain excitation pulses at 340 nm wavelength. The pulse duration was around 4 ps FWHM (full-width half-maximum) and the pulse energy in the tens of pJ range.

Fluid samples were contained in fused silica cuvettes (1.0-cm^3 with a 1-cm light path) in a thermostatted holder. The sample holder was placed in a housing containing as few detection optics as possible to yield maximum detection efficiency. The filters used for wavelength selection of emission were an interference filter (381.7-nm, 10.1-nm FWHM; Schott IL, Mainz, Germany) combined with an extra cut-off filter (model KV 370; Schott).

Detection electronics were time-correlated single photon counting modules. The start signal for the time-to-amplitude converter was generated by exciting a fast PIN photodiode with the 680-nm wavelength light omitted from the frequency-doubling procedure [28]. Single fluorescence photon responses from a proximity-focused microchannel-plate photomultiplier were amplified by a wide-band amplifier and used as a stop signal for the time-to-amplitude converter. The output pulses of the time-to-amplitude converter were analysed by an analogue-to-digital converter. The output of the analogue-to-digital converter was gathered into the 1024 channels of a multichannel analyser.

Pyrene fluorescence was sampled during 10 cycles of 10 s in alternating parallel and perpendicular directions. A maximum frequency of fluorescence photons of 23 kHz ($\approx$ 5 % of 475 kHz) in the parallel direction was chosen to prevent pile-up distortion [27]. Additionally, other instrumental sources of data distortion were minimized [29] to below the noise level of normal photon statistics. After measuring the fluorescence of the sample, the background emission of a sample with identical composition but excluding Pyr-C12 was measured for 2 cycles of 10 s and the result was subtracted from sample values (background subtraction).

To obtain a dynamic instrumental response as a reference for deconvolution, 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) (Eastman Kodak, Rochester, U.S.A.) in ethanol (exhibiting a single exponential fluorescence decay with $\tau = 1.35$ ns) was measured for 3 cycles of 10 s as a reference compound. Data analysis was performed (Indy Studio model, Silicon Graphics) using the maximum entropy method [30].

Binding of fluorescent fatty acids

Increasing amounts of fluorescent fatty acid in β-cyclodextrin were added to nsL-TP (0.5 μM) or pre-nsL-TP (0.5 μM) in 1 ml of PBS. As a measure of binding, the emission of Pyr-C12 (378 nm) or of *cis*-parinaric acid (415 nm) was determined upon excitation of tryptophan (280 nm). In the case of FRET, the excitation of tryptophan (donor) gives rise to sensitized emission of the fluorescent fatty acid (acceptor), provided that binding of fatty acids has occurred. Emissions were corrected for direct excitation of the fluorescent fatty acid at 280 nm in the absence of protein and for dilution. Given the low affinity of β-cyclodextrin for fatty acids (230-fold molar excess needed), this compound exerted a minimal effect on the binding of the different fatty acyl compounds to nsL-TP.

FRET between tryptophan and Pyr-C12 or *cis*-parinaric acid resulted in quenching of the tryptophan fluorescence, and this quenching could be used as a measure of fatty acid binding according to:

$$E = 1 - F_{DA}/F_D$$

where E is the energy transfer efficiency, F_{DA} is the fluorescence intensity of the donor (tryptophan) in the presence of acceptor (fluorescent fatty acid) and F_D is the fluorescence intensity of the donor in the absence of acceptor. In this case the fluorescent fatty acids were added to nsL-TP (0.5 μM) to enable accurate tryptophan fluorescence determination.

Binding of non-fluorescent fatty acids

Protein (0.6 μM nsL-TP or 0.5 μM pre-nsL-TP in PBS) was preincubated with Pyr-C12 (1.7 μM) for 5 min at 25 °C followed by titration with increasing amounts of non-fluorescent fatty acid substrates. The efficiency of competition was determined by measuring the decrease in Pyr-C12 (sensitized) fluorescence emission. After correction for direct excitation of Pyr-C12 (280 nm in the absence of protein) and for dilution, the decrease in fluorescence emission was normalized relative to the small decrease (max. 25%) observed with blank titrations (addition of β-cyclodextrin without competitor). The displacement of Pyr-C12 from nsL-TP by the fatty-acid substrates was analysed by fitting the normalized fluorescence decrease to a hyperbolic function.

RESULTS

Binding of fluorescent fatty acids

Upon addition of *cis*-parinaric acid or Pyr-C12 to nsL-TP, the emission of tryptophan (donor) at 338 nm was decreased (Figures 1A and 1B). Concomitantly, a sensitized emission of *cis*-parinaric acid (Figure 1A) and Pyr-C12 (Figure 1B) was observed, which was indicative of FRET. In agreement with this, a high correlation was observed between the increase in *cis*-parinaric acid emission (inset Figure 1A) or pyrene emission (inset Figure 1B) and the decrease in tryptophan emission. The occurrence of FRET demonstrated the very close proximity of pyrene or parinaric acid and the single tryptophan residue of nsL-TP, indicating that these fatty acids were bound to nsL-TP. As shown in Figure 1(B), the increase in Pyr-C12 fluorescence intensity was much more pronounced than the decrease in tryptophan emission. In addition to FRET, the increased pyrene fluorescence in the presence of nsL-TP was caused by an increased quantum yield (or fluorescence lifetime) of Pyr-C12 when compared with Pyr-C12 complexed to β-cyclodextrin (see below). To investigate the significance of the Pyr-C12 binding to nsL-TP, Pyr-C12 was also added to lysozyme. Lysozyme has a similar isoelectric point and size (pI = 9, molecular mass = 14.3 kDa) when compared with nsL-TP (pI = 8.5, molecular mass = 14 kDa). As is apparent from Figure 1(C), the intrinsic tryptophan fluorescence of lysozyme was unaffected by the presence of Pyr-C12 and, clearly, no sensitized Pyr-C12 emission was detected.

Figure 2 shows the spectra of pyrene-sensitized emission upon titration of nsL-TP (0.5 μM) with Pyr-C12. As shown in Figure 2 insert, binding of Pyr-C12 to nsL-TP was saturable, giving a K_d of 0.24 μM. In comparison, the K_d of *cis*-parinaric acid was 0.18 μM [11]. Under comparable conditions, pre-nsL-TP (0.5 μM) gave a similar titration curve, indicating that the presequence does not affect the affinity for Pyr-C12.

As shown in Figure 3, fast binding kinetics were observed when Pyr-C12 was presented to nsL-TP in monomeric form, i.e. solubilized in β-cyclodextrin (trace A). Equilibration was complete within 1 min. If Pyr-C12 was added directly to nsL-TP without prior complexation to β-cyclodextrin, binding was much slower (trace B). This observation underlines the importance of presenting the fatty acids and fatty-acyl-CoA esters to be tested

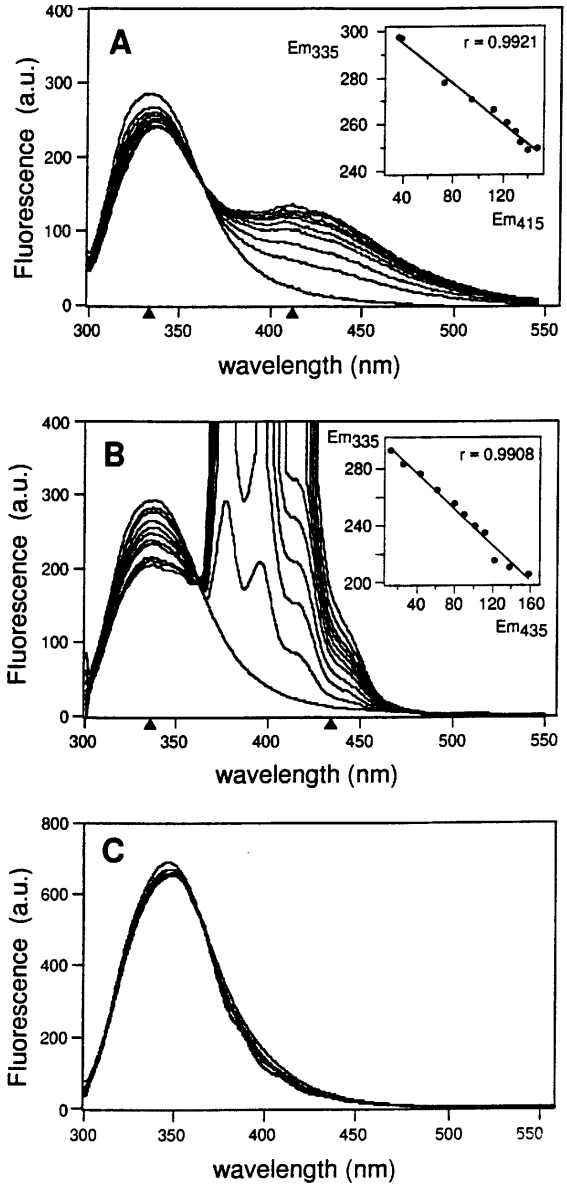

Figure 1 FRET as a function of fluorescent fatty acids bound to nsL-TP

Emission spectra upon titration of nsL-TP with *cis*-parinaric acid (**A**) and with Pyr-C12 (**B**) (excitation wavelength 280 nm). The correlation between quenched tryptophan emission (335 nm) and sensitized emission of *cis*-parinaric acid (415 nm) is shown in insert (**A**) and of Pyr-C12 (435 nm) is shown in insert (**B**). Emission spectra upon titration of lysozyme with Pyr-C12 (**C**). Fluorescence intensities are given in arbitrary units (a.u.). Spectra were corrected for direct excitation of fatty acid in the absence of protein and for dilution.

in monomeric form in order to eliminate the effect of different substrate solubilities on the interaction with nsL-TP. Since the increase in Pyr-C12-fluorescence emission was much larger than the decrease in tryptophan emission, the pyrene emission was used for estimating the affinities of the fatty-acyl substrates in the displacement binding assay (see below).

Time-resolved fluorescence analysis of Pyr-C12 binding

To obtain additional information on how Pyr-C12 is accommodated in nsL-TP, time-resolved fluorescence measurements were carried out on the Pyr-C12–nsL-TP complex. The results of

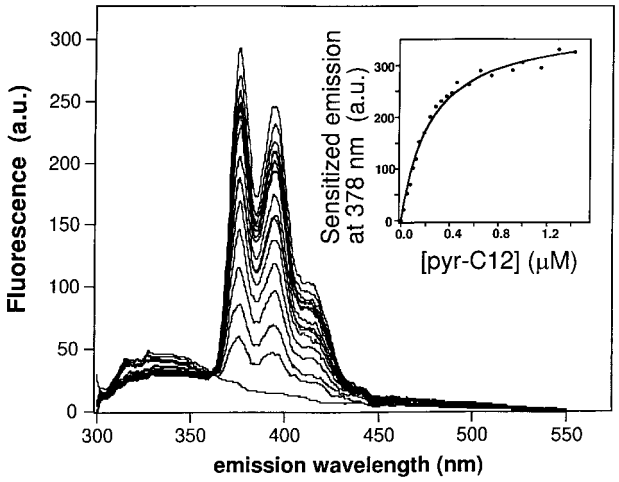

Figure 2 Sensitized emission spectra of Pyr-C12 bound to nsL-TP

The increase in pyrene fluorescence emission (378 nm) as a function of Pyr-C12 concentration (0–1.4 μM) with 0.5 μM nsL-TP is shown in the insert (excitation wavelength 280 nm). Spectra were corrected for direct excitation of Pyr-C12 in the absence of protein and for dilution.

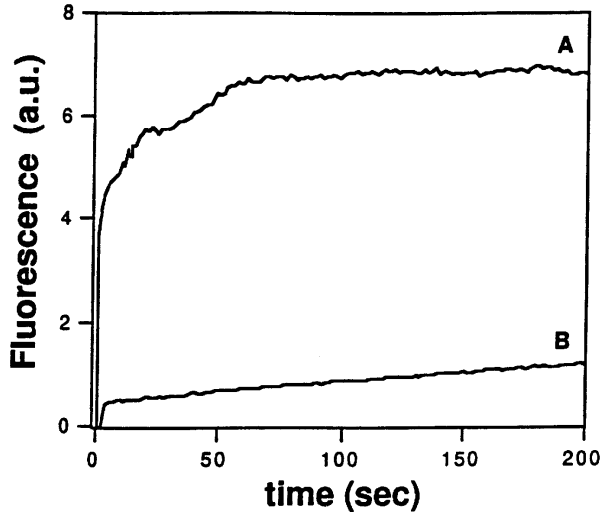

Figure 3 Binding velocity of Pyr-C12 to nsL-TP

Time-dependent binding of Pyr-C12 to nsL-TP measured by sensitized emission at 378 nm (excitation wavelength 280 nm). Curve (A), Pyr-C12 associated with β-cyclodextrin; curve (B), Pyr-C12 in buffer.

the experiments are shown in Figure 4, and Table 1 shows the parameters describing the best fits to the experimental data. From Figure 4(C), it can be inferred that upon addition of nsL-TP to a solution of Pyr-C12–cyclodextrin complex, the pyrene fluorescence decays more slowly (compare curves 1 and 2), reflecting an increase in the average pyrene-fluorescence lifetime from 111.9 to 139.4 ns (see Table 1). This indicates a substantial change in the molecular environment of the pyrene moiety when Pyr-C12 is bound to nsL-TP. Figure 4(D) reveals that, in contrast to Pyr-C12–β-cyclodextrin (curve 1), Pyr-C12 bound to nsL-TP shows a distinct anisotropy decay with a rotational correlation time of 6.9 ns (Table 1). This correlation time closely resembled that of 1-hexadecanoyl-2-[3-(diphenylhexatrienyl)propionyl]-*sn*-3-phosphocholine (DPH-PC) bound to nsL-TP [5] or of parinaric acid bound to recombinant nsL-TP [31]. In addition to this nsL-TP-related rotational mobility, there was also a very fast anisotropy decay (sub-ns timescale) of pyrene (0.1–0.15 ns) (Table 1), which could represent free rotational motion of pyrene. Despite the excellent fits to the data (χ^2 close to 1.0), the slow timescale of the time-resolved experiments (0.609 ns/channel, necessary for accurate determination of the long pyrene lifetime) prohibits analysis of this fast depolarization process with reasonable accuracy.

Addition of hexadecanoyl-CoA (1.5 μM final concentration) to the Pyr-C12–nsL-TP complex clearly reduces the Pyr-C12 fluorescence lifetime (curve 3, Figure 4C) to 126.3 ns (Table 1), and also diminishes the initial anisotropy of the component with a rotational correlation time of 7.6 ns to 0.032. Subsequent addition of a 4-fold excess of hexadecanoyl-CoA over Pyr-C12 further reduced the fluorescence lifetime (to 120.9 ns) and the initial anisotropy of the slow-rotating component to 0.014. It is of note that, under these conditions, the fluorescence and the anisotropy decay were still slower than the control situation without nsL-TP (compare curves 1 and 4), reflecting incomplete competition by hexadecanoyl-CoA (see Figure 5).

Binding of non-fluorescent fatty acids

The displacement of Pyr-C12 bound to nsL-TP by non-fluorescent fatty-acid derivatives is taken as a measure of the affinity of nsL-TP for that particular compound. As shown in Figure 5, the addition of the VLCFA-CoA esters to nsL-TP pre-equilibrated with Pyr-C12, almost eliminated the sensitized-pyrene fluorescence. In fact, given the amount of nsL-TP present (0.6 μM) it appeared that, at low concentrations ($< 0.5\ \mu$M), 26:0-CoA and 24:1-CoA bound completely (results not shown). Addition of the non-esterified tetracosanoic acid (24:0) had virtually no effect on the pyrene emission, indicating that nsL-TP had a greater preference for the CoA ester. This was also observed for hexadecanoic acid. As for hexadecanoyl-CoA, the displacement was less efficient over the concentration range tested. The relative decrease in the normalized fluorescence (Figure 5) correlated well with the results of the time-resolved fluorescence experiments (Figure 4), thereby validating the accuracy of the competition binding assay.

Using this assay, various fatty-acyl compounds were tested for binding to nsL-TP and its precursor, pre-nsL-TP, and their IC_{50} values were determined. As shown in Table 2, hexadecanoyl-CoA had an approx. 10-fold greater affinity for nsL-TP when compared with hexadecanoic acid. The introduction of a double bond into hexadecanoyl-CoA (16:1-CoA) did not affect the binding affinity. However, a significant (about 5-fold) decrease in the affinity was observed when a 3-hydroxy or a 3-oxo group was introduced into hexadecanoyl-CoA. Given that peroxisomes are the intracellular sites of VLCFA degradation [32], the affinity of nsL-TP for this class of fatty acids was also assessed. As was observed for hexadecanoic acid, nsL-TP had a very low affinity for 24:0 fatty acid but a 55-fold greater affinity for the 24:0-CoA ester (IC_{50} 0.27 μM). The introduction of a double bond had a significant effect and increased by a factor of 3 the affinity of nsL-TP for 24:1-CoA. By comparison, 26:0-CoA had an IC_{50} of 0.13 μM and 26:1-CoA of 0.44 μM. Each of these substrates was also tested for binding to pre-nsL-TP. As shown in Table 2, pre-nsL-TP possessed a 2- to 5-fold lower affinity than nsL-TP. Using this assay, nsL-TP failed to show an affinity for cholesterol, which supports the idea that nsL-TP probably has no function in cholesterol metabolism [21].

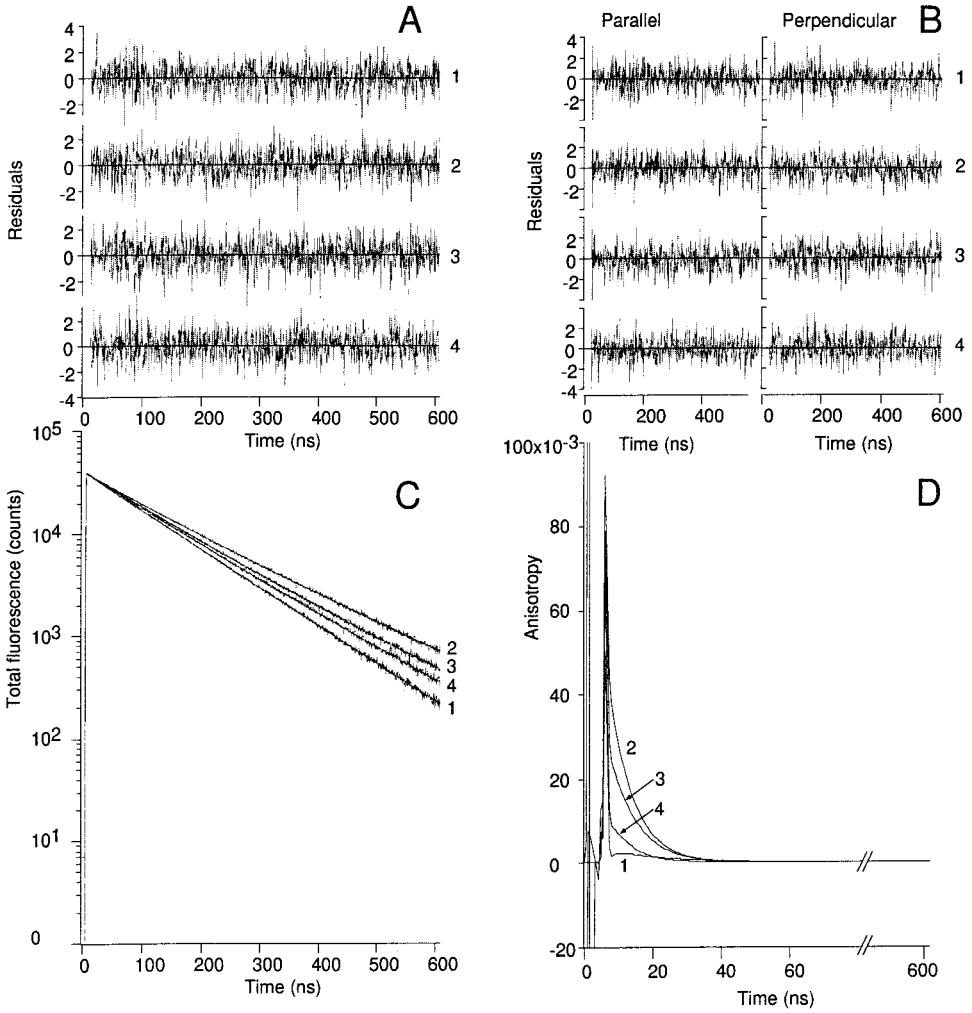

Figure 4 Time-resolved fluorescence analysis of Pyr-C12 binding to nsL-TP

(**A**, **C**) Fluorescence decay analysis. (**B**, **D**) Anisotropy decay analysis. All four experiments were carried out at room temperature in 1 ml cuvettes containing 1.5 μM Pyr-C12 bound to β-cyclodextrin in PBS. Subsequent additions: 1, no addition; 2, addition of 0.75 μM nsL-TP; 3, addition of 1.5 μM hexadecanoyl-CoA; 4, addition of 4.5 μM hexadecanoyl-CoA (total 6 μM). (**C**) Experimental and calculated fluorescence decays of the four experiments and the decay of POPOP in ethanol ($\tau = 1.35$ ns), which was used as a reference compound for deconvoluting the instrumental response. The weighted residuals for the four fits are shown in (**A**). The anisotropy decays of the four experiments are shown (**D**). For reasons of clarity, only the calculated curves are indicated. In (**B**), the weighted residuals for both the parallel and perpendicular experimental fluorescence decays defining the anisotropy decay are shown for all four experiments.

Table 1 Fluorescence and anisotropy decay parameters for Pyr-C12 under various conditions

The conditions of the experiments are described in the legend to Figure 4. Additions: L, Pyr-C12 (1.5 μM in cyclodextrin); P, 0.75 μM nsL-TP; C, hexadecanoyl-CoA.

		Fluorescence decay		Anisotropy decay				
Experiment	Addition	τ (ns)	χ^2	Φ_1 (ns)	(β_1)	Φ_2 (ns)	(β_2)	χ^2
1	L	111.9 ± 0.3	0.97	0.10 ± 0.05	(0.36 ± 0.24)	16 ± 10	(0.003 ± 0.002)	1.05
2	L + P	139.4 ± 0.4	1.03	0.15 ± 0.09	(0.24 ± 0.17)	6.9 ± 0.6	(0.049 ± 0.023)	1.03
3	L + P + C (1.5 μM)	126.3 ± 0.8	1.12	0.10 ± 0.05	(0.34 ± 0.25)	7.6 ± 0.9	(0.032 ± 0.016)	1.12
4	L + P + C (6.0 μM)	120.9 ± 1.0	1.12	0.10 ± 0.05	(0.4 ± 0.3)	6.1 ± 1.0	(0.014 ± 0.008)	1.12

DISCUSSION

In the present report, a simple and sensitive assay for measuring the binding of fatty-acyl compounds to nsL-TP is presented. In this assay, use is made of the presence of a single tryptophan residue and a fluorescent reporter fatty acid, which, because of the occurrence of FRET between these two, allows binding to be monitored directly. The FRET signal directly represents binding of the fatty acid, since this process is extremely dependent on the distance between the fluorescent fatty acid and nsL-TP. In addition, both the time-resolved fluorescence and anisotropy

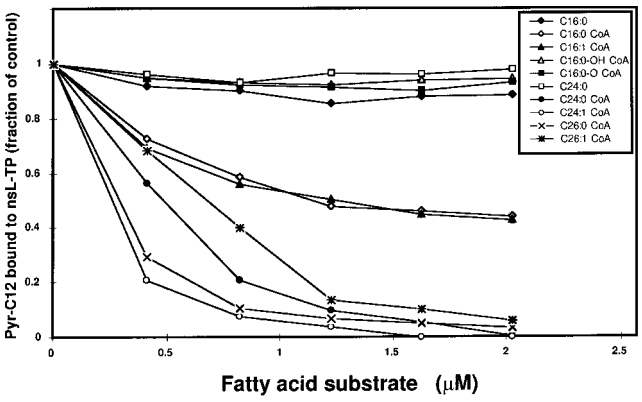

Figure 5 Displacement of Pyr-C12 bound to nsL-TP by fatty-acid substrates

The substrates (shown in the inset) were added to 0.6 μM nsL-TP pre-equilibrated with 1.7 μM Pyr-C12. Each point is the average of at least four independent measurements. S.D.s were between 0.2–4% for each point.

Table 2 Relative affinity of fatty-acid substrates for nsL-TP and pre-nsL-TP

The IC_{50} values represent the concentrations of fatty-acid substrates required to displace half of the amount of Pyr-C12 from the lipid-binding site of the protein. For incubation conditions, see the legend to Figure 5. Values are the means $\pm$ S.D. ($n = 5$) and were estimated from representative fits through the points shown in Figure 5. n.d., not determined.

Fatty-acid substrate	IC_{50} for nsL-TP (μM)	IC_{50} for pre-nsL-TP (μM)
16:0	10.7 $\pm$ 1.9	34.9 $\pm$ 12.5
16:0 CoA	1.28 $\pm$ 0.08	3.75 $\pm$ 0.37
16:1 CoA	1.16 $\pm$ 0.08	n.d.
16:0-OH-CoA	9.8 $\pm$ 2.2	n.d.
16:0-O-CoA	7.6 $\pm$ 0.9	n.d.
24:0	14.9 $\pm$ 5.0	22.4 $\pm$ 7.2
24:0 CoA	0.27 $\pm$ 0.07	0.89 $\pm$ 0.16
24:1 CoA	0.10 $\pm$ 0.03	0.42 $\pm$ 0.04
26:0 CoA	0.13 $\pm$ 0.02	0.40 $\pm$ 0.08
26:1 CoA	0.44 $\pm$ 0.11	0.90 $\pm$ 0.16

analysis showed a major change in the molecular environment of Pyr-C12 when added to nsL-TP, indicative of the incorporation of the pyrene moiety into the lipid binding site of nsL-TP. The maximal entropy fit of the time-resolved fluorescence decay of Pyr-C12 in the presence of nsL-TP (curve 2, Figure 4C) indicated a lifetime component centred at 162 ns in addition to a lifetime component centred at 112 ns (results not shown). The 162 ns component represented the Pyr-C12 population that is bound to nsL-TP. Hence, the quantum-yield increase of Pyr-C12 upon translocation from β-cyclodextrin to nsL-TP may amount to 44% which exceeds the increase of 25% calculated from the average lifetime parameters (112 ns versus 139 ns). This underestimation is a consequence of the simultaneous presence of nsL-TP-bound and β-cyclodextrin-bound Pyr-C12 populations in the experiments. Similar quantum-yield changes have been observed for translocation of pyrene-labelled phospholipids from nsL-TP to membranes [7]. The quantum-yield increase greatly enhanced the sensitivity of the competition binding assay as, in addition to the sensitized emission, it contributed to a net increase of pyrene-fluorescence emission in the presence of nsL-TP (Figure 2).

Using this displacement assay, it was shown that nsL-TP can specifically bind fatty-acyl compounds, with highest affinity for hexadecanoyl-CoA, 2-hexadecenoyl-CoA and very-long-chain fatty-acyl-CoA. The other substrates tested, 3-hydroxyhexadecanoyl-CoA, 3-oxohexadecanoyl-CoA, even though structurally very similar, bound with relatively low affinity, suggesting that the physical constraints of the lipid binding were very distinct. It is striking that nsL-TP shows a high affinity for CoA esters and a relatively low affinity for the corresponding non-esterified fatty acids. Frolov et al. [11] reported a 300-fold increase in affinity of nsL-TP for *cis*-parinaroyl-CoA when compared with *cis*-parinaric acid, and the results of our study are in agreement. In the latter study [11], 16:0-CoA effectively displaced *cis*-parinaric acid from nsL-TP but 20:0-CoA failed to do so. In contrast to the high affinities for 24:0-CoA and 26:0-CoA observed in the present study, Frolov et al. [11] concluded that nsL-TP binds saturated fatty-acyl-CoAs only over a narrow acyl-chain length, from 10 to 18 carbon atoms.

Given the interaction of nsL-TP with acyl-CoA oxidase and the bifunctional enzyme in peroxisomes [20], it may well be that this protein is involved in the presentation of very-long-chain acyl-CoA and the enoyl derivatives of these enzymes. The finding that the binding of fatty acids is very fast when presented in the form of monomers complexed to β-cyclodextrin supports the idea that nsL-TP may function in the regulation of peroxisomal oxidation by transporting fatty-acyl intermediates to and from the hydrophobic binding sites in oxidation enzymes. In accordance with this proposed function, the functional homologue of nsL-TP in the yeast *Candida tropicalis*, PXP18, was shown to bind to peroxisomal acyl-CoA oxidase *in vitro* [33] and a novel nsL-TP in the yeast *Saccharomyces cerevisiae* was shown to co-purify with peroxisomal acyl-CoA oxidase [34].

Since pre-nsL-TP undergoes rapid proteolytic processing to nsL-TP upon import into the peroxisome, the 20 amino acid presequence is not likely to contribute to the fatty-acyl binding activity of nsL-TP. In the present work, we report a 2- to 4-fold lower binding affinity for pre-nsL-TP when compared with nsL-TP. Apparently, the presequence interferes with fatty acid binding, probably by destabilizing the tertiary structure of the hydrophobic fatty acid binding pocket and might function as an modulator for strict intraperoxisomal fatty acid binding activity.

The binding of fatty acids and CoA esters to nsL-TP has a cytosolic counterpart in the fatty acid binding protein (FABP) and acyl-CoA binding protein (ACBP) [35] The FABP family comprises several proteins (molecular mass 14–15 kDa) that differ in their substrate specificity and tissue abundance. These proteins are present in high amounts in the cytosol and show high affinity for fatty acids and in some cases (e.g. liver FABP) for fatty-acyl-CoAs [36,37] A function for FABP in delivering fatty acids to the outer membrane of mitochondria [38,39] and to peroxisomes [40,41] for subsequent β-oxidation has been suggested. In addition, the cytosol contains a 10 kDa ACBP with high affinity towards fatty-acyl-CoA esters. ACBP is thought to bind fatty-acyl-CoA esters formed by activation at the mitochondrial outer membrane and thus to 'take over' the role of FABP. The ACBP-bound fatty-acyl-CoA can be used for β-oxidation or for the biosynthesis of phospholipids and triacylglycerols. In this respect, nsL-TP may be considered to be the peroxisomal counterpart of ACBP. The dissociation constants of the FABPs ranged from 2–1000 nM, depending on the FABP and the type of fatty acid used [42]. The affinities reported in the present study for nsL-TP are within this range. Interestingly, many of the features described here for nsL-TP are very similar to those described for plant non-specific lipid-transfer proteins (LTPs). LTPs (reviewed in [43]) have a molecular mass of

approx. 10 kDa and a basic pI (8.8). Upon binding of pyrene-labelled lipids to LTPs, a similar pyrene quantum yield increase to that of nsL-TP is observed [44]. It has been shown that plant LTPs bind fatty-acyl-CoA esters in a 1:1 molar ratio [44–47]. Furthermore, immunolocalization indicated that LTPs are enriched in glyoxysomes (the plant peroxisomal counterpart) and enhance acyl-CoA oxidase activity [46]. This suggests that LTPs and nsL-TP have common physiological functions in plant and mammalian organisms despite having a completely different amino-acid sequence. This might be a case of evolutionary convergence similar to that proposed for yeast sec14p and mammalian phosphatidylinositol transfer protein [1,48].

Taken together, the data presented in the present study support a role for nsL-TP in peroxisomal fatty-acid oxidation rather than in intracellular lipid transfer and biosynthesis. In this process nsL-TP might function both by supplying the peroxisomal β-oxidation enzymes with fatty-acyl-CoA esters and by removing the oxidized or chain-shortened products. Currently, we are investigating further the role of nsL-TP in the peroxisomal β-oxidation pathway.

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