Changes in Lipid Distribution and Dynamics in Degranulated Rat Liver Rough Endoplasmic Reticulum Due to the Membrane Attachment of Polyribosomes†

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ABSTRACT: Binding of rat liver polyribosomes to homologous degranulated rough endoplasmic reticulum (dRER) labeled with 10-(pyren-1-yl)decanoic acid (PDA) was studied. As a consequence of the membrane association of polysomes, the excimer/monomer fluorescence intensity ratios (I_e/I_m) decreased, thus indicating alterations in the dynamics and organization of lipids. These fluorescence changes were complete within approximately 1 min, in accordance with the tight binding of ribosomes to RER. In order to characterize the changes in membrane lipid dynamics in more detail, polysomes were covalently labeled with trinitrobenzenesulfonic acid so as to allow their use as Förster-type resonance energy-transfer acceptors while utilizing PDA as a donor. Accordingly, assuming the binding of native and quencher-labeled ribosomes to the PDA-labeled membranes to be identical, we were able to discriminate fluorescence changes (a) in the proximity of the ribosome binding site from (b) those arising in the surrounding ribosome-free membrane and beyond the effective quenching radii of the TNP residues coupled to polysomes. Our data suggest that lipids in the polysome attachment site of dRER are less mobile than those in the remaining, ribosome-free membrane. In addition, there appears to be a relative enrichment of the PDA probe in the polyribosome membrane attachment sites.

The synthesis of secreted as well as integral membrane proteins of eukaryotes occurs perhaps exclusively in membrane-bound polyribosomes of rough endoplasmic reticulum (RER).[1] Membrane-associated polysomes are characteristically present as spiral aggregates (Palade, 1975). The signal recognition particle has been shown to cause, due to its binding to a specific receptor in the RER, attachment of the growing polypeptide chain to the membrane (Gilmore et al., 1982; Meyer et al., 1982). Specific ribosome receptor protein has recently been purified and reconstituted in liposomes (Savitz & Meyer, 1990).

Membrane-bound ribosomes differ functionally from those in the cytoplasm in their sensitivity to protein synthesis inhibitors (Glazer & Sartorelli, 1972). The membrane attachment of ribosomes is altered in transformed cells (Pryme, 1988). Of particular interest are the findings by Klein et al. (1988) on the translational arrest of mRNA coding for chlorophyll α-apoproteins of photosystem I when cells were maintained in dark. Yet dissociation of the polysomes from RER relieves this translational arrest and allows protein synthesis to proceed. Clearly, attachment of polysomes to membranes allows translational control employing a hitherto unknown mechanism.

The activation energy for translation on RER coincides with temperature-induced changes in membrane microviscosity determined by ESR for lipid probes, thus supporting a connection of the state of lipids with the translation process (Towers et al., 1972). Inhibition of membrane attachment of ribosomes to dRER treated with phospholipases, phospholipase C in particular, indicates an involvement of membrane lipids (Jothy et al., 1975). The role of lipids in ribosome function in RER is, however, unclear. In prokaryotes, the signal peptide–lipid interactions are currently being investigated [for a recent review, see De Vrije et al. (1990)]. In addition, in these cells, the phospholipid phase transition has been shown to lead to a translational arrest (Pages et al., 1978). As a first step in our own studies, we developed a method to monitor the binding of polysomes to degranulated RER.

Materials and Methods

Reagents. 10-(Pyren-1-yl)decanoic acid was from KSV-Chemicals (Helsinki, Finland) and 2,4,6-trinitrobenzenesulfonic acid from Serva. Other chemicals of analytical grade were from Sigma.

Preparation of Rat Liver Rough and Smooth Endoplasmic Reticulum Membranes. Male rats were starved for 16–20 h prior to decapitation while anesthetized with ether. Livers were removed, weighed, and maintained on ice. All subsequent operations were performed at 4 °C. Endoplasmic reticulum membranes were prepared according to Gaetani et al. (1983) with minor modifications. Livers were minced and homogenized in 3 volumes (w/v) of 0.5 M sucrose. The homogenate was centrifuged in a Kontron A 8.24 rotor at 9500 rpm for 10 min, and the postmitochondrial supernatant (PMS) was recentrifuged at 9500 rpm for 20 min. Three-step discontinuous gradients were prepared by mixing different volumes of PMS with 2.5 M sucrose (Gaetani et al., 1983). To obtain the smooth endoplasmic reticulum fraction, the tubes were overlaid with 1 M sucrose. Membranes were collected by centrifugation for 8 h at 42000 rpm in a Beckman Ti60 rotor. RER is recovered at the boundary between 1.55 and 1.85 M

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[1] Abbreviations: PDA, 10-(pyren-1-yl)decanoic acid; RER, rough endoplasmic reticulum; dRER, degranulated rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; PMS, postmitochondrial supernatant; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; DOC, sodium deoxycholate; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNP, trinitrophenyl; ATA, aurintricarboxylic acid; RFI_m and RFI_e, relative intensities of pyrene monomer and excimer fluorescence, respectively; I_m and I_e, pyrene monomer and excimer fluorescence intensities, respectively.

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sucrose whereas SER flotates to the boundary between 1.0 and 1.35 M sucrose.

RER and SER fractions for polyribosome binding experiments were further subjected to gel filtration on a Sepharose CL-2B column (32 cm × 1.6 cm) equilibrated with HTKM (100 mM Tris-HCl, pH 7.5, 100 mM KCl, and 5 mM $MgCl_2$). The column was eluted with the same buffer at approximately 15 mL/h, and the material eluting in the void volume was collected. The RNA/protein ratios (w/w) in RER and SER membranes were 0.18 and 0.03, respectively. The phospholipid composition of rough endoplasmic reticulum membranes was in agreement with the published values (Colbeau et al., 1971).

Degranulation of Rough Endoplasmic Reticulum. RER was stripped of ribosomes by treatment with puromycin and KCl (Adelman et al., 1973) which were added to freshly prepared membranes to final concentrations of 1 mM and 0.5 M, respectively. The membrane suspension was thereafter incubated for 1 h at 4 °C, and 0.2 M EDTA was then added to a final concentration of 20 mM. Incubation was continued for 30 min at 4 °C. After treatment of the RER with the degranulating agents, the stripped membranes were isolated on a Sepharose CL-2B column (64 cm × 2.4 cm) (Hawkins & Freedman, 1979). Occasionally, the column procedure was omitted, and dRER was collected by centrifugation. This modification of the purification procedure did not change the behavior of dRER in polysome binding experiments. After removal of ribosomes from RER to yield dRER, the RNA/protein and phospholipid/protein ratios were 0.03–0.05 and 0.45, by weight, respectively. Samples were diluted with 1 volume of glycerol and stored at −70 °C.

Labeling of dRER Membranes by Pyrenyldecanoic Acid. Labeling of dRER membranes with pyrenyldecanoic was carried out by adding up to 10 μL of PDA (1 mg/mL in ethanol) to 1 mL of membrane suspension. Protein and phospholipid concentration ranges were 800–1000 and 350–450 μg/mL, respectively. The mixture was incubated for 30 min at 4 °C. The minimum phospholipid to PDA molar ratio in the labeled membranes was 15/1. Addition of PDA to dRER resulted in a complete incorporation of the probe into the membrane as revealed by gel filtration (Figure 1A). The incorporated PDA could be removed from dRER by fatty acid free albumin, thus indicating the absence of metabolic conversion of the probe into phospholipids (Figure 1B). Spin-labeled lipids incorporated in RER and dRER have revealed as a function of temperature a break in the lipid dynamics at approximately 22 °C (Towers et al., 1972). Similarly, upon monitoring I_e/I_m of PDA-labeled dRER, a change in the temperature dependency of pyrene fluorescence was observed at this temperature (data not shown).

Preparation of Polyribosomes. Rats were starved for 16–20 h prior to sacrifice in order to remove glycogen. Polyribosomes were prepared as described by Ogata and Terao (1979). The livers were minced and homogenized in 2 volumes (v/w) of ice-cold STKM (0.25 M sucrose, 25 mM Tris-HCl, pH 7.6, 25 mM KCl, and 5 mM $MgCl_2$). Homogenate was centrifuged at 9500 rpm for 10 min in a Kontron A 8.24 rotor. The postmitochondrial supernatant was collected. The precipitate was dispersed into 1 volume (v/w) of STKM, homogenized, and recentrifuged at 9500 rpm for 10 min. Both supernatant fractions were combined and centrifuged in a Beckman 60Ti rotor at 42000 rpm for 1 h, 40 min. The resulting pellet was homogenized in 8.75 mL of 35 mM Tris-HCl buffer, pH 7.8, containing 0.15 M sucrose, 25 mM KCl, and 10 mM $MgCl_2$. To the microsomal suspension were added 2.5 mL of 2.5 M KCl and 10 mM $MgCl_2$ solution and 1.25 mL of 10% DOC.

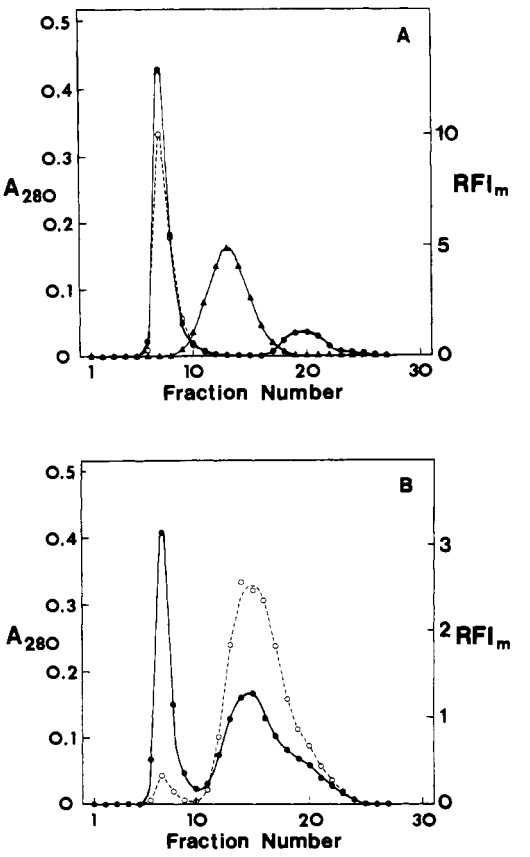

FIGURE 1: Incorporation of pyrenyldecanoic acid (PDA) into degranulated rough endoplasmic reticulum membranes. (Panel A) PDA-labeled dRER (1 mg of dRER protein in 0.5 mL of TKM) was eluted on a CL-6B column (25 cm × 0.8 cm) with HTKM at 4 °C. One-milliliter fractions were collected, and the absorbance at 280 nm (●) and the fluorescence at 400 nm (RFI_m, ○) were measured. Fatty acid free albumin (1 mg in 0.5 mL of HTKM) was separately eluted through the same column (A_{280}, ▲). (Panel B) Albumin (1 mg) was added to the PDA-labeled dRER suspension (1 mg of dRER protein in 0.5 mL of TKM) and incubated for 5 min at 4 °C. The mixture was thereafter chromatographed on a CL-6B column as in panel A. Closed circles (●) represent the combined absorbance of dRER and albumin; open circles (○) show the relative intensity of the pyrene monomer fluorescence (RFI_m).

The mixture was incubated on ice for 15 min. The suspension was layered over 15 mL of 35 mM Tris-HCl, pH 7.8, containing 0.3 M sucrose, 0.6 M KCl, and 10 mM $MgCl_2$, and centrifuged in a Beckman 60Ti rotor at 42000 rpm for 2 h. The pellet was rinsed with STKM and then suspended in 2 mL of this buffer. The suspension was clarified by centrifugation at 13000 rpm in a Kontron A 8.24 rotor, and the supernatant was eluted on a Sepharose CL-6B column (18 cm × 0.8 cm) with HTKM.

The protein/RNA ratio (w/w) of collected polysomes was slightly under 1 (0.93) (Wool, 1979). A_{260}/A_{280} and A_{260}/A_{235} ratios for polyribosomes were 1.80–1.85 and 1.60–1.65, respectively. Polysomal proteins were run on SDS–PAGE (Laemmli, 1970) and stained with Coomassie Blue. The protein band pattern complied with those published in the literature [e.g., see Ogata and Terao (1979)]. The isolated polysomes were stored at −70 °C in HTKM.

Labeling of Polyribosomes by TNBS. To 2 mL of polysomes in HTKM (10–12 mg/mL) was added 250 μL of TNBS in H_2O (10 mg/mL). The mixture was incubated for 2 h at room temperature protected from light and occasionally mixed. One milliliter of sample was eluted with HTKM on a Sepharose CL-6B column (11 cm × 0.8 cm) equilibrated with the same buffer. The most concentrated fractions (1 mL) were

collected. A_{260}/A_{280} and A_{260}/A_{235} ratios of the pooled TNBS-labeled ribosomal fractions were approximately 1.85 and 1.50, respectively. The efficiency of labeling was estimated by measuring the absorbance difference for equal concentrations of nonlabeled polysomes and TNP-polysomes at 345 nm and using a millimolar absorption coefficient of 14.5 mM^{-1} cm^{-1} (Goldfarb, 1966). Approximately 1 out of 5 lysine residues in ribosomal proteins was labeled, thus corresponding to ≈200 TNP moieties per ribosome. Judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970), this covalent modification of ribosomes did not change their protein composition (not shown).

Fluorescence Measurements. Fluorescence measurements were carried out with a Kontron SFM 23 spectrofluorometer or with an SLM 4800S instrument interfaced to a Hewlett-Packard 85 computer. Excitation was at 343 nm, and emission intensities were measured at 400 nm (I_m) and 480 nm (I_e); 1.6 mL of TKM buffer (50 mM Tris-HCl, pH 7.6, 25 mM KCl, and 5 mM $MgCl_2$) and 50 μL of PDA-labeled dRER (1–1.5 mg of protein/mL) were placed sequentially into a magnetically stirred four-window quartz cuvette in a holder thermostated with a circulating water bath. The cuvette contents were allowed to equilibrate for 1–2 min prior to the fluorescence measurements. Aliquots of 10–100 μL of polysomes (6–12 mg/mL in HTKM) or TNP-labeled polysomes (3–4 mg/mL in HTKM) were then added while monitoring changes in pyrene fluorescence. Unless otherwise stated, the experiments were carried out at 25 °C.

Correction for the inner filter effect for the fluorescence data recorded with TNP-labeled polysomes was made as follows. TNP-lysine was prepared as described, and the absorbance for the TNP-substituted amino groups was measured at 345 nm (Goldfarb, 1966). No evidence for membrane association of TNP-Lys could be demonstrated either by gel filtration or by ultracentrifugation used for the isolation of dRER while monitoring the presence of TNP-Lys spectrophotometrically (data not shown). Changes in pyrene fluorescence were recorded after the addition of this water soluble quencher to PDA-labeled dRER preparations in TNP concentrations equimolar to those used with the TNP-labeled polysomes. The decrease in both I_m and I_e was linear as a function of increasing [TNP-Lys], again indicating a lack of specific binding to dRER. The values measured for TNP-polysomes were then corrected for quenching due to the inner filter effect (i.e., the degree of quenching due to TNP-Lys was added to the values measured with TNP-labeled polysomes) so as to yield fluorescence changes due to the specific association of polysomes to dRER. The maximal concentration of TNP added in labeled polysomes was 2.2 μM. When added to PDA-labeled dRER under conditions similar to those used when the effects of TNP-polysome binding were studied, 2.2 μM TNP-Lys decreased T_m and T_e by 8.3 and 9.7%, respectively.

Extraction of Rough Endoplasmic Reticulum Lipids. Rough endoplasmic reticulum lipids were extracted with chloroform/methanol (2/1, by volume) according to Colbeau et al. (1971). The extract was then fractionated into neutral lipids and phospholipids by column chromatography on silicic acid eluted first with chloroform and then with methanol. The eluates were evaporated to dryness under nitrogen, dissolved in chloroform, and stored at −20 °C. The polar lipids were further separated by thin-layer chromatography on silica gel. Identification of lipid spots was accomplished by staining with iodine vapor and comparison with lipid standards. For quantitation of phospholipids, the phosphorus content of each spot was determined. The phospholipid composition of rat liver rough endoplasmic reticulum membranes was in agreement with the results published by Colbeau et al. (1971) (data not shown).

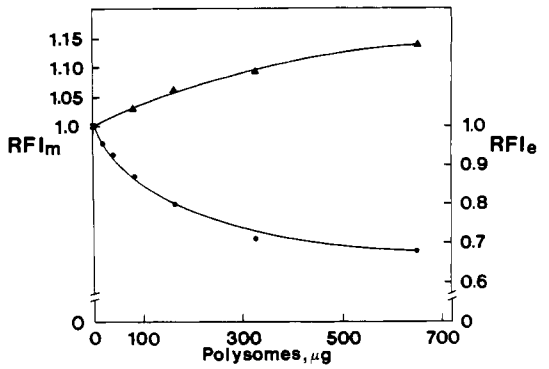

FIGURE 2: Polysome binding induced changes in the pyrene monomer (RFI_m, ▲) and excimer (RFI_e, ●) fluorescence intensities of PDA-labeled dRER membranes. Polysomes were added to the dRER suspension (65 μg of dRER protein in 1.6 mL of TKM) at 25 °C. The numerical values are normalized with respect to the fluorescence of PDA-labeled dRER measured prior to the addition of polysomes.

Preparation of Liposomes. Extracted RER phospholipids in chloroform were labeled with pyrenyldecanoic acid (PDA/phospholipid molar ratio of approximately 1/30) whereafter the solvent was removed under a stream of nitrogen. Phospholipids (0.5 mg) were hydrated with TKM buffer (1 mL), and the dispersion was irradiated in a Branson bath-type sonicator for 30 min at room temperature.

Analytical Methods. The preparations were analyzed for protein by the method of Lowry et al. (1951) with bovine serum albumin as a standard. RNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1, by volume) and determined by the orcinol method (Miller et al., 1951). Ribosome concentrations were also determined by optical absorption using $E^{1\%}_{1cm}$ = 123 at 260 nm (Nieuwenhuysen et al., 1978). Both methods gave consistent results. Polysome concentrations are given in micrograms, consisting of approximately 50% protein and 50% RNA. For phospholipid determination, membrane lipids were extracted with chloroform/methanol (2/1, by volume; Folch et al., 1957) and assayed for phosphorus (Bartlett, 1959).

RESULTS

Binding of Polysomes to dRER. Binding of polysomes to PDA-labeled dRER caused rapid changes in pyrene fluorescence. More specifically, polyribosome induced an increase in pyrene monomer emission and a decrease in excimer fluorescence (Figure 2). The features of the emission spectra remained normal. The magnitudes of the fluorescence changes increased with increasing polysome concentrations and were complete within 2 min (data not shown).

To check if the partitioning of the PDA label between dRER and the aqueous phase was altered by the membrane association of polysomes, dRER membranes were pelleted by centrifugation before and after the addition of increasing amounts of polysomes (Schroeder, 1983). The monomer fluorescence of the supernatants varied between 1.6 and 4.3% of the total monomer fluorescence measured prior to the removal of dRER. No excimer fluorescence could be detected in the aqueous phase. There was a tendency of the monomer fluorescence in the aqueous phase to decrease due to the reassociation of polysomes (data not shown). Accordingly, the partitioning of PDA between the aqueous phase and dRER is slightly altered toward the latter by the addition of polysomes. This change in the PDA distribution does not jeop-

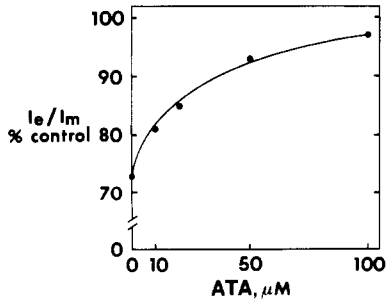

FIGURE 3: Inhibitory effect of aurintricarboxylic acid (ATA) on polyribosome binding to degranulated rough endoplasmic reticulum membranes. dRER was incubated with the indicated concentrations of ATA for 5 min at 25 °C. Fifty-microliter samples of dRER (75 μg of protein) were thereafter taken and mixed with TKM buffer (1.6 mL) in the fluorescence cuvette. After 1 min, the same amount of polysomes (117 μg) was added, and the relative fluorescence intensities were measured at 480 nm (I_e) and 400 nm (I_m). The control is the I_e/I_m fluorescence ratio of PDA-labeled membranes measured prior to polysome addition.

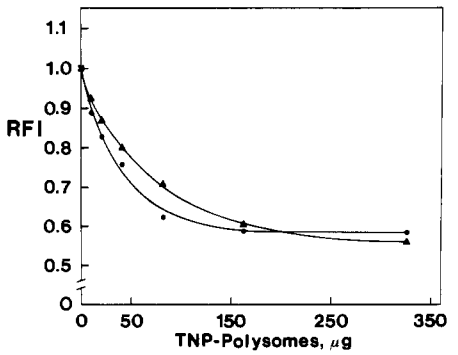

FIGURE 4: TNP-polysome-induced changes in the normalized pyrene monomer and excimer fluorescence intensities of PDA-labeled dRER membranes. TNP-polysomes were added to dRER suspension (60 μg of dRER protein in 1.6 mL of TKM) at 25 °C. Closed triangles (▲) show pyrene monomer and closed circles (●) excimer fluorescence (RFI). The values are corrected for the inner filter effect of the TNP label.

ardize the results or the conclusions of the present study. As a control, the effects of polysomes on the fluorescence of PDA-labeled liposomes made from RER lipids were studied. Ribosomes up to a concentration of 200 μg/mL added to liposomes (35 μg of phospholipid phosphorus/mL) in TKM buffer and containing 4 μg of PDA had essentially no influence on pyrene emission (data not shown). Similarly, addition of polysomes produced essentially no changes in the pyrene fluorescence of SER labeled with PDA in a manner identical with that described above for dRER, thus indicating lack of binding (data not shown). As a third control, binding of polysomes to dRER was blocked by aurintricarboxylic acid (ATA, Figure 3). Again, increasing concentration of ATA abolished polysome-induced changes in I_e/I_m nearly quantitatively (by approximately 98%), in agreement with previous studies (Borgese et al., 1974).

Reconstituted RER (i.e., dRER with reattached polysomes) was also subjected to centrifugation on sucrose gradients. As expected, dRER with added polysomes was recovered floating on the top of the gradient (Borgese et al., 1974). When polysomes were added to dRER digested with trypsin and I_e/I_m was measured for this membrane after isolation by ultracentrifugation, values similar to those for dRER in the absence of polysomes were recorded, thus indicating the absence of polysome binding to the protease-treated membrane.

The isolation of polysomes involves the use of a detergent, DOC. Accordingly, as DOC could be present as a trace contaminant in the polysome preparations and perturb the behavior of the fluorescent fatty acid probe, we checked for the effect of DOC on dRER PDA fluorescence. However, when DOC was added to PDA-labeled dRER in a final concentration of 1% (corresponding to the concentration used during the purification of polysomes), no effect on either monomer or excimer fluorescence was observed (data not shown). The fluidizing effect of ethanol on membranes has been reported in several studies [for a recent review, see Wood and Schroeder (1988)]. The final concentration of ethanol in our experiments was always less than 0.1%. Removing ethanol by pelleting and washing of PDA-labeled dRER membranes with buffer did not alter the fluorescence changes induced by added polysomes.

Binding of TNP-Labeled Polysomes to dRER. In order to study polyribosome-induced changes in RER lipid dynamics in more detail, polysomes were also labeled with TNBS. Due to the extensive overlap of TNP absorption with pyrene monomer emission, TNP can be used as an efficient Perrin–Förster acceptor for pyrene emission (Thuren et al., 1988). TNP-labeled polysomes caused a rapid and concentration-dependent decrease in both the pyrene monomer and excimer emission when added to PDA-containing dRER. The fluorescence changes due to TNP-polysomes are more rapid than those for the nonlabeled polysomes. However, PDA quenching effects observed by TNP-polysomes involve also the inner filter effect of TNP on pyrene fluorescence. Accordingly, the kinetics of these data are limited by the efficiency of mixing of the cuvette contents and cannot be used to analyze the rates of TNP-polysome binding to dRER. On the other hand, when corrected for the inner filter effect (by adding to the measured values the decrease in pyrene fluorescence caused by equimolar concentrations of TNP-Lys which lacks high-affinity binding to dRER), specific quenching due to the dRER association of TNP-polysomes remains (Figure 4). In other words, the I_m and I_e values (corrected for the inner filter effect) measured after the addition of TNP-polysome to PDA-labeled dRER reflect changes in the PDA signal beyond the quenching radii of TNP in polysomes. Binding of TNP-polysomes to PDA-labeled dRER could also be confirmed by ultracentrifugation on sucrose gradients (Borgese et al., 1974; data not shown).

Discussion

Pyrene-labeled lipids are versatile spectroscopic probes and have been extensively used in membrane studies on, for instance, ordering of lipids (Somerharju et al., 1985; Kinnunen et al., 1987; Eklund et al., 1988), membrane binding of a peripheral protein cytochrome *c* (Mustonen et al., 1987), lipid lateral diffusion (Galla & Sackmann, 1975), spontaneous and protein-mediated lipid transfer (Smith & Pownall, 1984), conformation of phospholipids and the alignment of phospholipid acyl chains (Kinnunen & Virtanen, 1982; Thuren et al., 1984; Thuren, 1988), and the action of PLA2 (Thuren et al., 1988) as well as the equilibrium lateral pressure in liposomes (Konttila et al., 1988). A particularly useful feature of pyrene is the ability of the excited monomeric molecule to undergo complex formation with a ground-state pyrene which results in the formation of an excited dimer, excimer (Förster, 1969). Under controlled conditions, the ratio of the excimer and monomer fluorescence intensities (I_e/I_m) for proper pyrene-containing lipid derivatives depends on the ocal concentration of pyrene as well as on the rate of lateral diffusion (Galla & Sackmann, 1975).

The pyrene probes are poorly suited for quantitative energy-transfer measurements, however. There are several reasons for this. First, to obtain efficient quenching, the extent of

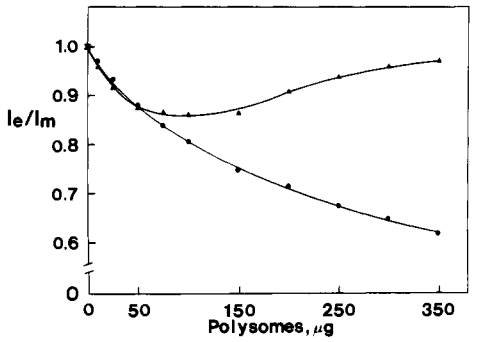

FIGURE 5: Normalized changes in pyrene I_e/I_m due to the binding of polysomes (●) and TNP-polysomes (▲) to PDA-labeled dRER at 25 °C (75 μg of dRER protein in 1.6 mL of TKM). The data for TNP-labeled polysomes have been corrected for the inner filter effect due to the absorbance of the TNP label at the excitation wavelength (343 nm).

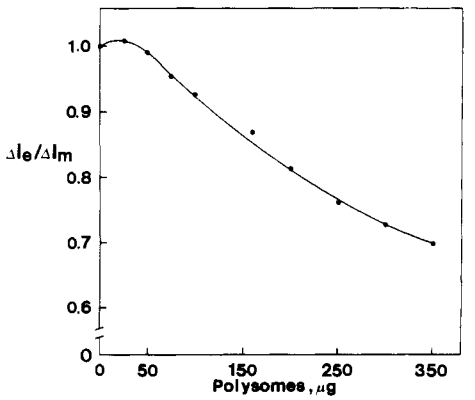

FIGURE 6: Normalized PDA fluorescence changes ($\Delta I_e/\Delta I_m$) in the membrane at the ribosome binding sites as a function polysome concentration. The data were calculated as described under Discussion from the data presented in Figure 4.

labeling was such that each ribosome contained approximately 200 TNP moieties. Assuming the chromophores to be distributed randomly on the surface of a ribosome, this means that their distance between the membrane surface varies between 0 and ≈200 Å. Similarly, the number of PDA molecules within the distance allowing energy transfer to occur is altered as a consequence of polysome binding. Related to the above, the orientation of the donor and acceptor dipoles cannot be defined. Excited monomeric pyrene has two relaxation dipoles at a right angle to each others. Second, the kinetics of pyrene emission are very complex and involve multiple relaxation pathways [e.g., see Lemmetyinen et al. (1989)] and are in addition sensitive to oxygen. The relatively straightforward calculations to characterize the parameters of the energy-transfer process would therefore yield results bearing little if any physical significance. Accordingly, the present data cannot be used to estimate the actual size of the membrane domains involved in polysome binding.

In the present study, changes in the fluorescence of pyrenyldecanoic acid incorporated in dRER membranes caused by the membrane attachment of polysomes were studied. The combined use of both nonlabeled and TNP-labeled ribosomes allows differentiation of changes in PDA fluorescence at the ribosome binding site from those of the surrounding, polysome-free membrane. Accordingly, when changes in I_e/I_m due to nonlabeled polysomes contain PDA signals arising in the whole dRER membrane, those recorded with TNP-ribosomes reflect only the I_e/I_m outside the critical range of Perrin–Förster energy transfer. For reasons summarized above, the data collected in the present study do not allow estimation of the critical range and distance between the fluorescence donor and acceptor. Therefore, only qualitative conclusions can be drawn. Yet, the domain surrounding a membrane-attached TNP-labeled ribosome and depleted of PDA fluorescence should extend <100 Å from the ribosome surface, in line with quantitative data from energy-transfer studies (Stryer, 1978). Quantum mechanical aberrations may additionally be involved (Kinnunen et al., 1987).

As shown in Figure 5, binding of polysomes to PDA-containing dRER results in a monotonous decrease in I_e/I_m, thus indicating an overall decrease in lateral diffusion. However, the corresponding I_e/I_m data collected by using TNP-labeled polysomes reveal more complex, biphasic behavior in the dynamics of PDA in the ribosome-free membrane domain. At low TNP-polysome concentrations, I_e/I_m falls rapidly, in parallel with nonlabeled ribosomes. At higher polysome concentrations, the I_e/I_m values for the ribosome-free membrane increase.

Combining the data obtained with both nonlabeled and TNP-labeled ribosomes allows calculation of PDA fluorescence changes ($\Delta I_e/\Delta I_m$) in the membrane vicinal to the bound ribosomes. For both monomer and excimer fluorescence intensities, the following equation was used to give the fluorescence intensities in the membrane domains quenched by TNP-polysomes:

$$\Delta I = I_0 + (I_B - I_*)$$

where I_0 is the initial fluorescence intensity prior to the addition of polysomes, I_B is the fluorescence intensity after the addition of nonlabeled polysomes, and I_* is the fluorescence measured after the addition of an equivalent amount of TNP-labeled polysomes. The resulting normalized curve for $\Delta I_e/\Delta I_m$ as a function of polysome concentration is shown in Figure 6 and reveals a clear biphasic behavior, a small increase at low polysome concentrations followed by a decrease upon further increase in the amount of polysomes added.

In order to estimate to what extent these change in I_e/I_m in the two membrane domains reflect altered lateral diffusion on one hand and on the other hand changes in the distribution of PDA, we defined parameter Q by the equation:

$$Q = aI_m + bI_e$$

where a and b are constants. We then assume (i) that the fluorescence relaxation pathways remain qualitatively unaltered and (ii) that irrespective of the distribution of the emission between the two emitting species the number of radiating fluorophores remains constant. In other words, even when I_e/I_m is changing when corrected for the respective quantum yields of monomer and excimer fluorescence, the total number of quanta emitted by pyrene monomer and excimer species should remain constant.

As only relative values are of interest here, the value for the constant a is assigned to unity. The value for the constant b is then obtained from spectral data by measuring I_m and I_e for the ribosome-free membrane and after the addition of polysomes, for instance. Values for Q for the ribosome-free membrane and the membrane with bound polysomes, Q_1 and Q_2, respectively should be equal, and we may then solve the value for b (=0.33). We can then plot $Q = I_m + 0.33I_e$ as a function of the concentration of polysomes. As expected, Q vs [polyribosome] is essentially constant (Figure 7). In contrast, log Q vs [TNP-polyribosome] gives Q for the polymer-free membrane, which reveals a clear biphasic behavior (Figure 7). We may further calculate relative changes in [PDA] in the membrane underneath the attached polysomes (Figure 8). To conclude, this analysis indicates reciprocal changes in the relative distributions of PDA (a) in the prox-

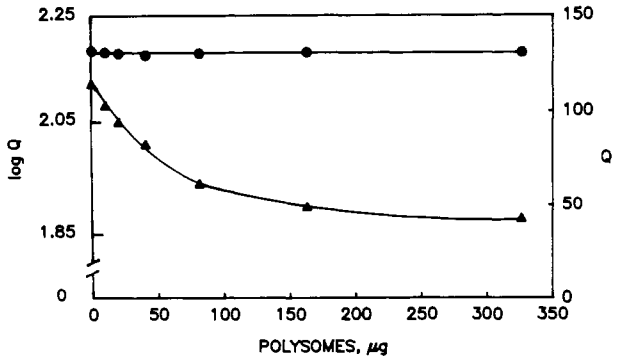

FIGURE 7: Value for parameter Q remained constant when polysomes were added to PDA-labeled dRER (●). Also shown are the relative changes in the lateral distribution of PDA in the polysome-free dRER membrane as a function of TNP-polysomes (▲). For the sake of clarity, the latter curve uses logarithmic values for Q so as to illustrate the biphasic nature of the graph. See Discussion for details.

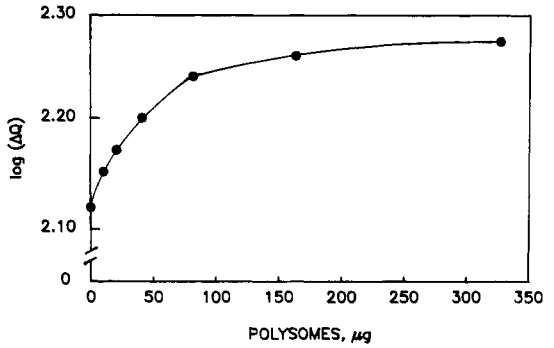

FIGURE 8: Estimated relative changes in the concentration of PDA in the RER membrane vicinal to the polysome binding sites as a function of polysome concentration. To emphasize the biphasic behavior of the parameter Q, its logarithmic values are shown.

imity of the ribosome binding sites in dRER and (b) in the membrane domain beyond the quenching radii of the TNP moieties on polysomes. Accordingly, at low polyribosome concentrations, the decrease of I_e/I_m in the ribosome-free membrane results from a depletion of PDA from this domain with a corresponding relative enrichment of the probe in the membrane attachment sites for ribosomes. At higher ribosome/membrane ratios, the I_e/I_m values for the free membrane again increase. This increase is likely to result from enhanced rates of lateral diffusion of the probe in this domain whereas PDA in the membrane domains participating in ribosome attachment becomes translationally more restricted.

We may conclude our results to indicate that the binding of polyribosomes to dRER results in changes in the distribution of the pyrene-labeled fatty acid, the probe favoring partitioning into the membrane underneath the polyribosomes. The membrane attachment of polysomes appears to result in the rigidification of lipids in the proximity of the binding sites. It is possible that as a consequence of ribosome attachment the distribution of other dRER membrane lipids, acidic phospholipids in particular, would be altered. Accordingly, an enrichment of less fluid lipids underneath ribosomes would result in a corresponding enrichment of fluid lipids in the ribosome-free membrane. This would result in the observed higher rates of lateral diffusion and thus increasing I_e/I_m for PDA in the membrane domain beyond the quenching radii of TNP-labeled polysomes in spite of the relative decrease in the content of PDA in this domain.

It is necessary to emphasize that the present study involves the introduction of relatively high amounts of a perturbing probe into the dRER membrane. The asymmetry of lipid composition as well as fluidity has been shown for murine fibroblast and erythrocyte plasma membrane leaflets (Schroeder, 1980; Sweet et al., 1987; Morrot et al., 1986). Obviously, the interpretation of our data would be complicated by such phenomena. However, the possible asymmetry of RER remains to be verified. Additionally, partitioning of the fatty acid probe between the two leaflets of the bilayer may change upon polysome binding. Another potential complication could be introduced due to the storage of the membrane preparations at subzero temperatures which may result in phase separation processes as well as irreversible changes in lipid distribution.

Therefore, more elaborate studies are required before any definite conclusions can be drawn. Yet, the experimental approach described here should be applicable to the characterization of membrane proteins in general, assuming that the labeling procedure used does not alter the properties of the protein under study too seriously. With pyrene-labeled lipids, qualitative changes both in the translational dynamics as well as in the probe distribution can be estimated. The functional significance, if any, of the above findings is currently being investigated in our laboratory.

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Low-Temperature ^{2}H NMR Spectroscopy of Phospholipid Bilayers Containing Docosahexaenoyl (22:6ω3) Chains†

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ABSTRACT: Polyunsaturated fatty acids are widely distributed components of biological membranes and are believed to be involved in many biological functions. However, the mechanisms by which they act on a molecular level are not understood. To further investigate the unique properties of ω3 polyunsaturated phospholipid bilayers, deuterium nuclear magnetic resonance (^{2}H NMR) studies have been made of the liquid–crystalline (L_α) and gel phases of a homologous series of mixed-chain phosphatidylcholines containing docosahexaenoic acid: (per-^{2}H-*n*:0)(22:6)PC, where n = 12, 14, 16, and 18. The moments of the ^{2}H NMR lineshapes have been evaluated, and from these the warming and cooling main phase transition temperatures were determined. The transition temperatures of the mixed-chain series were found to be significantly lower than those of the corresponding lipids in the disaturated series, di(per-^{2}H-*n*:0)PC, with hystereses ranging from 2 to 14 °C. Distinct effects of the docosahexaenoyl chain on bilayer order were found, though these effects varied across the mixed-chain series. In evaluating the moment data, an empirical method for normalizing the moments with respect to differences in temperature was applied, in addition to using the reduced temperature method. For the systems studied here, the method of normalization had no significant effect on the interpretation of the moment data.

Deuterium nuclear magnetic resonance (^{2}H NMR)[1] is uniquely suited to the study of membranes in that it yields information on both time-averaged conformations and molecular motions within lipid bilayers and biological membranes (Seelig, 1977; Davis, 1979; Seelig & Seelig, 1980; Griffin, 1981; Brown, 1982; Davis, 1983). Polyunsaturated ω3 phospholipids in bilayers are of particular interest because of widespread evidence suggesting their participation in many biological functions. Although metabolically costly to synthesize, long-chain polyunsaturated fatty acids are extraordinarily abundant in certain tissues. There is extensive evi-

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[1] Abbreviations: (per-^{2}H-12:0)(22:6)PC, 1-perdeuteriolauroyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; (per-^{2}H-14:0)(22:6)PC, 1-perdeuteriomyristoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; (per-^{2}H-16:0)(22:6)PC, 1-perdeuteriopalmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; (per-^{2}H-18:0)(22:6)PC, 1-perdeuteriostearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; di(per-^{2}H-12:0)PC, 1,2-perdeuteriolauroyl-*sn*-glycero-3-phosphocholine; di(per-^{2}H-14:0)PC, 1,2-perdeuteriomyristoyl-*sn*-glycero-3-phosphocholine; di(per-^{2}H-16:0)PC, 1,2-perdeuteriopalmitoyl-*sn*-glycero-3-phosphocholine; di-(per-^{2}H-18:0)PC, 1,2-perdeuteriostearoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; ^{2}H NMR, deuterium nuclear magnetic resonance; DSC, differential scanning calorimetry; T_m, main (gel–liquid crystalline) phase transition temperature; T_{red}, reduced temperature; T_{rel}, relative temperature.

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