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Deconstructing the DGAT1 enzyme: Binding sites and substrate interactions

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ABSTRACT

Diacylglycerol acyltransferase 1 (DGAT1) is a microsomal membrane enzyme responsible for the final step in the synthesis of triacylglycerides. Although DGATs from a wide range of organisms have nearly identical sequences, there is little structural information available for these enzymes. The substrate binding sites of DGAT1 are predicted to be in its large luminal extramembranous loop and to include common motifs with acyl-CoA cholesterol acyltransferase enzymes and the diacylglycerol binding domain found in protein kinases.
In this study, synthetic peptides corresponding to the predicted binding sites of DGAT1 enzyme were examined using synchrotron radiation circular dichroism spectroscopy, fluorescence emission and adsorption onto lipid monolayers to determine their interactions with substrates associated with triacylglyceride synthesis (oleoyl-CoA and dioleoylglycerol). One of the peptides, Sit1, which includes the FYxDWWN motif common to both DGAT1 and acyl-CoA cholesterol acyltransferase, changes its conformation in the presence of both substrates, suggesting its capability to bind their acyl chains. The other peptide (Sit2), which includes the putative diacylglycerol binding domain HKWCIRHFYKP found in protein kinase C and diacylglycerol kinases, appears to interact with the charged headgroup region of the substrates. Moreover, in an extended-peptide which contains Sit1 and Sit2 sequences separated by a flexible linker, larger conformational changes were induced by both substrates, suggesting that the two binding sites may bring the substrates into close proximity within the membrane, thus catalyzing the formation of the triacylglyceride product.

1. Introduction

The acyl-CoA:diacylglycerolacyltransferase 1 (DGAT1) is a microsomal enzyme located mainly in the endoplasmic reticulum (ER) membrane; it plays an essential role in lipid metabolism by catalyzing the production of triglycerides from acyl-CoA and diacylglycerol substrates [1,2]. Disfunction in this stage of lipid metabolism can lead to serious disorders, such as obesity, which is an important risk factor for cardiovascular diseases, hypertension and diabetes [3,4]. Transgenic DGAT1-deficient mice have been shown to resist induced obesity even when fed on a rich fat-based diet, in addition to showing a remarkable reduction in the accumulation of triacylglycerol in their adipose tissues [5]. This suggests that DGAT1 enzymes could be potential therapeutic targets for the inhibition of obesity [6–9]. In addition, DGAT1 is an important element in food production/agriculture as cattle milk-fat production can be greatly enhanced by a single point mutation in this enzyme [10].

Although DGAT orthologues from a wide variety of mammalian sources have nearly identical sequences (~90%), and even orthologues from distantly related eukaryotes have identities of >40%, there is as yet no crystal structure of any DGAT protein nor of any closely-related protein which might form a good template for modeling of its structure. Bioinformatics analyses indicate that it is an integral membrane protein [11], but that its enzymatic function lies in a large luminal loop in the C-terminal half of the protein. Two peptides in this loop are thought to comprise the binding sites for the two types of substrates: Sit1, which includes the motif FYxDWWN [12] that is highly conserved between the DGAT and acyl-CoA cholesterol acyltransferase (ACAT) enzymes [13,14] (^{356}FGDREFYRDWWNSES^{370}), and Sit2, which includes the putative diacylglycerol binding motif HKWCIRHFYKP found in protein kinase C and diacylglycerol kinases (^{379}NIPVHKWSIRHFY^{391}). In this study, we have examined substrate interactions involving synthetic peptides corresponding to these two motifs, as well as with a third

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peptide, consisting of the central regions of Sit1 and Sit2 peptides joined by a short flexible linker. The Sit1 and Sit2 sequences are located in the same extramembranous loop as the proposed catalytic residue, His^{416}, albeit at some distance from it in the linear sequence. It has been suggested that the two proposed binding sites may be important for directing the substrates into an extended active site, making them available for reaction at the catalytic histidine. Hence, the interactions of the peptides with the oleoyl-CoA and dioleoylglycerol substrates were examined using synchrotron radiation circular dichroism (SRCD) and fluorescence spectroscopies, and adsorption onto lipid monolayers.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-sn-glycerol (DOG) and coenzyme A (CoA) were purchased from Sigma Aldrich; oleoyl coenzyme A (OCoA) and the phospholipid 1-palmitoyl-2-oleoyl-sn-glycerol-3-phospho-rac-(1-glycerol) (POPG) were purchased from Avanti Polar Lipids.

The synthetic peptides were designed based on the primary structure of bovine DGAT1 (UniProt code Q8MK44). Sit1 (its location within the full length protein is noted in Fig. 1) includes the most conserved region present in DGAT1 and the ACAT family, the FYxDWWN segment. Sit2 is the putative diacylglycerol binding domain, and includes the sequence HKWCIRHFYKP, similar to that found in protein kinase C and the diacylglycerol kinases. A third peptide, Sit1&2, links together the most conserved residues of Sit1 and Sit2 with a flexible linker region with the sequence GSG (peptide sequences are in Suppl. Table 1).

Peptides were manually synthesized using Fmoc strategy on Rink Amide MBHA resin. Coupling reactions were performed with two equivalents of Fmoc-protected amino acids, N,N′-diisopropylcarbodimide/N-hydroxybenzotriazole in dimethylformamide (DMF) (1:1, volume ratio) until reaction went to >95% completion, as checked by Kaiser assay [15], and deblockings were done with 20% piperidine in DMF. After the incorporation of all amino acid residues, the final cleavage of the peptide from the resin was performed with a mixture of trifluoroacetic acid (TFA):triisopropylsilane:ethanedithiol:H_2O (94:1:2.5:2.5, v/v) for 4 h. The crude peptides were precipitated with cold diethyl ether, centrifuged and lyophilized. The synthetic peptides were solubilized in 0.1% (v/v) TFA and the purification was carried out with semi-preparative HPLC using a reverse-phase C_{18} column (Vydac, Hesperia, CA) equilibrated with 0.1% (v/v) TFA/water. The peptides were eluted from the column with a linear gradient from 5% to 95% of acetonitrile in water containing 0.1% TFA using a 2 mL/min flow rate, over a 30 min gradient and with absorbance monitored at 220 and 280 nm (Suppl. Fig. 1a). The sequences of the purified peptides were confirmed by mass spectrometry analyses using an ESI-microOTOF-Q (Bruker Daltonics). Peptides were solubilized in formic acid 0.5% and analyzed in the 50–3000 m/z range (Suppl. Fig. 2).

Peptide concentrations were determined by measuring the absorbance at 280 nm, using their calculated molar extinction coefficients [16].

2.2. Prediction of secondary structure propensity and transmembrane segments

The secondary structure propensities of the peptides were calculated based on the sequences as found in the full length bovine DGAT1 enzyme and in the isolated peptides using the GOR [17] method. The prediction of transmembrane segments in DGAT1 used the SACS MEMSAT software [11] to produce Fig. 1A.

2.3. Monolayer surface activity

The surface activities of the Sit1, Sit2 and Sit1&2 peptides were measured in a Kibron trough (Kibron, Finland) by injection of the peptides (10 μM) into the aqueous subphase and recording the surface pressure over 30 min. To measure the kinetics of the adsorption of these peptides onto monolayers formed by 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosglycerol (POPG) and dioleoyl glycerol (DOG), each lipid was solubilized in a mixture of chloroform/methanol (4:1) and spread on the top of the water to achieve surface pressures ranging from 5 to 40 mN/m. After solvent evaporation, each of the DGAT1 peptides (10 μM) was injected in the subphase, and the surface pressure recorded as a function of time.

2.4. Synchrotron radiation circular dichroism spectroscopy in solution

SRCD spectra of each of the peptides in an aqueous solution (0.3 mM) and in the presence of oleoyl-CoA (OCoA) at 1:1, 1:2 and 1:4 peptide:lipid molar ratios were measured on beamline CD1 at the Institute for Storage Ring facilities (Aarhus, Denmark). Triplicate scans of samples and baselines (taken using the same exact condition of the cognate sample, containing all of the components in the sample, except the peptide) were obtained over the wavelength range from 280 to 176 nm in 1 nm increment using a 2 s dwell time in demountable Suprasil quartz cells (Hellma Analytics, UK) with 0.0050 cm pathlength at 25 °C. The sample and baseline scans were each averaged and the averaged baseline subtracted from the averaged sample spectrum, smoothed with a Savitzky–Golay filter, and calibrated with camphorsulfonic acid using CDTool processing software [18]. Replicate measurements were also done at the DISCO beamline of the Soleil Synchrotron (Paris, France) and at the CD12@ANKA beamline of the ANKA Synchrotron (Karlsruhe, Germany). Mean residue weights of 142, 141, and 141, respectively, were used to scale the spectra of the Sit1, Sit2 and Sit1&2 peptides to delta epsilon values.

Additionally, conventional CD spectroscopy was used to investigate the binding of the DGAT1 peptides (25 μM) to CoA at a 1:10 molar ratio. Measurements were obtained over the wavelength from 185 to 280 nm in 1 nm intervals using a Jasco J-715 spectropolarimeter as an average of 6 scans using a 0.1 cm pathlength quartz cuvette, at 25 °C. Baseline subtraction and data processing were performed using CDTool software.

2.5. SRCD spectroscopy of films

Either DOG or POPG (the latter is not a substrate but examined as a control for specificity of the interactions) in chloroform/methanol (4:1) was incubated with each of the DGAT1 peptides at peptide:lipid molar ratios of 1:50. A film of the mixture was prepared by depositing 10 μl of the peptide/lipid mixture onto a circular Suprasil quartz plate (Hellma Analytics, UK), then allowing the evaporation of the solvent; the film was further dried in a desiccator under vacuum for 3 h, and then re-hydrated for 24 h in a specially designed sample chamber [19] at 97% relative humidity. SRCD measurements were obtained at 25 °C, over the wavelength range from 280 to 170 nm with 1 nm step size and 2 s dwell time. Measurements were made at four different rotational positions of the plate separated by 90° in the direction perpendicular to the beam in order to detect any signal due to linear dichroism. These four rotational repeats were averaged to produce the final spectrum. Baseline measurements (obtained with an empty cell) were subtracted from the sample spectra using CDTool software.

Fig. 1. Transmembrane (TM) and secondary structure predictions for DGAT1 enzyme. A) Predicted membrane disposition of bovine DGAT1 and B) secondary structure predictions. The TM region is indicated in orange. In both cases the locations of the Sit1 and Sit2 peptides are in green and cyan overlay, respectively, and the putative catalytic histidine is in red. The secondary structure code is c, coil; e extended; and h, helix.