Monoacylglycerol binding to human serum albumin: Evidence that monooleoylglycerol binds at the dansylsarcosine site

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Abstract **The binding of monoacylglycerides of long-chain fatty acids to human serum albumin has been examined using monooleoylglycerol as the ligand. Binding was investigated using changes in tryptophan fluorescence and also the displacement of a variety of well-studied fluorescent ligands from human serum albumin (HSA). Monooleoylglycerol caused a decrease in fluorescence from tryptophan-214 when measured at 350 nm while oleic acid had no effect on fluorescence at this wavelength and did not compete with monooleoylglycerol. In contrast, oleic acid caused an increase in fluorescence at 330 nm whereas monooleoylglycerol did not affect fluorescence intensity at this wavelength. These results suggest that these two ligands do not bind to the same site on HSA. From competition studies using dansylglycine, dansylsarcosine, 11-(dansylamino)-undecanoic acid and 1-anilino-8-naphthalenesulfonic acid it was proposed that monooleoylglycerol binds at the dansylsarcosine site (site II) of HSA. Monooleoylglycerol was a competitive inhibitor of dansylsarcosine binding with a K_d of about 2.5 μM whereas oleic acid was not competitive with dansylsarcosine binding.—**Thumser, A. E. A., A. G. Buckland, and D. C. Wilton. **Monoacylglycerol binding to human serum albumin: evidence that monooleoylglycerol binds at the dansylsarcosine site.** ***J. Lipid Res. 1998.*** 39: **1033–1038.**

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A major function of human serum albumin (HSA) is to transport fatty acids that bind with high affinity to the protein (1–3). In addition, HSA binds a wide variety of other non-polar compounds including physiological ligands such as bilirubin and lysophospholipids (1, 4). However, the binding of monoglycerides such as monooleoylglycerol has received minimal attention (5) despite monoglycerides and fatty acids being the primary products of serum triglyceride hydrolysis by lipoprotein lipase. It is not clear whether albumin has a role in removal of 2-monoglyceride as lipoprotein hydrolysis proceeds or whether there is further hydrolysis by a plasma or cellular monoglyceride lipase. In order to understand the possible role of albumin in lipoprotein hydrolysis it is important to determine the nature of binding of monoglyceride to HSA in terms of stoichiometry, affinity and the location of the binding site(s) within the albumin molecule.

A number of separate ligand binding sites have been identified within the albumin molecule (1), however many ligands can bind to more than one type of site while protein conformational changes on binding of a ligand to one site can affect the binding characteristics of other sites (1, 2). A major binding feature is that of long chain fatty acid sites and up to three separate high affinity sites have been proposed with a total capacity for up to 6 molecules of fatty acid being seen under extreme in vivo conditions. A high affinity bilirubin site separate from the highest affinity long chain fatty acid sites has been demonstrated. In addition further functional sites, site I and site II, have been proposed that bind a wide range of non-polar ligands (1).

HSA contains a single tryptophan, at position 214, that has been used extensively as a fluorescent reporter group for ligand binding and conformational studies (1). In addition, fluorescent probes, particularly those containing a polarity-sensitive dansyl-type fluorophore, have been used to obtain information about these ligand binding sites. We have previously demonstrated that the primary binding of a fluorescent fatty acid probe (DAUDA) is at a site discrete from the long chain fatty acid sites and at a site apparently shared with medium chain fatty acids and bilirubin (6). As a result, displacement from this site by oleic acid only starts at HSA : oleic acid ratios of approximately 1:3 (4, 6).

The binding of fluorophore, 1,8-ANS, and several other dansylated ligands to HSA have been well characterized (6–11). The binding of 1,8-ANS occurs at one high-affinity

Abbreviations: HSA, human serum albumin; DAUDA, 11-(dansylamino)-undecanoic acid; 1,8-ANS, 1-anilino-8-naphthalenesulfonic acid.

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site while at least three sites of lower affinity have been reported (8). In contrast, dansylsarcosine and dansylglycine bind to HSA at a single site with affinities in the μm range (8) and the use of these compounds in displacement studies makes interpretation of experimental data simpler. The exact site of dansylglycine binding is controversial as Sudlow, Birkett and Wade (8, 9) have defined dansylglycine as a site I probe whereas other groups disagree with this location (11). In contrast, dansylsarcosine has been identified as the marker ligand for site II, a site known to have a preference for non-polar ligands either without charge or with an anionic charge located more peripherally on the molecule (1).

Here we show that monooleoylglycerol binds to HSA with a decrease in tryptophan fluorescence (at 350 nm) while, in contrast, oleic acid causes an increase in fluorescence (at 330 nm). In addition, monooleoylglycerol binds competitively with the site II probe dansylsarcosine to HSA providing a K_d of about 2.5 μM.

MATERIALS AND METHODS

Materials

DAUDA was purchased from Molecular Probes, Eugene, OR, HSA was the highest purity essentially fatty acid free material from Sigma. All other chemicals were obtained from Sigma.

Fluorescence assays

The fluorescence assays (1 ml) were performed at 20°C in 50 mm HEPES·NaOH, pH 7.5, on a Hitachi F-4500 fluorimeter. HSA was dissolved in water to give a 0.1 mm stock solution. Monooleoylglycerol, oleic acid and fluorescent ligands were added to assays as 1 mm solutions in methanol.

For binding studies using tryptophan fluorescence, the fluorescence was measured using an excitation wavelength of 295 nm (slit 5 nm) with emission at 330 and 350 nm (slit 10 nm). After each addition of ligand, the solution was left for 30 sec and then the emission spectrum was scanned three times between 300 nm and 400 nm to ensure that equilibrium had been achieved.

For displacement assays using fluorescent ligands, the excitation and emission wavelengths were 335 nm/500 nm (DAUDA), 335 nm/490 nm (dansylglycine), 335 nm/480 nm (dansylsarcosine) and 370 nm/480 nm (1,8-ANS) with 10 nm slit widths (12). The fluorescence emission was monitored with time to achieve a constant fluorescence reading in order to ensure that equilibrium had been attained, normally less than 1 min. The % initial fluorescence due to the fluorescent probe was calculated as: (fluorescence in the presence of added ligand) ÷ (fluorescence in the absence of added ligand) × 100. No corrections were required for fluorescence inner filter effects and the addition of monooleoylglycerol to fluorophores caused no significant change in fluorescence in the absence of protein (data not shown).

Calculation of dissociation constants (K_D) using fluorescence displacement

The fluorescence values were corrected for fluorescence values in the absence of protein and the data were fitted to a hyperbolic equation: $F = (B_{max} \times [S]) / (K_D + [S])$, where F is measured fluorescence and [S] is the concentration of fluorophore (dansylsarcosine). B_{max} is the calculated maximum fluorescence value and K_D the apparent dissociation constant. The inhibition constant (K_i) which will be equivalent to K_d was calculated from a replot of K_D/B_{max} versus monooleoylglycerol concentration (13).

RESULTS

Effect of added oleic acid and monooleoylglycerol on tryptophan fluorescence

HSA contains a single tryptophan at residue 214 and changes in tryptophan fluorescence have been used to determine HSA affinity for a number of ligands (1) including non-anionic ligands such as gossypol (14). Moreover, fluorescence energy transfer experiments have previously predicted that this tryptophan is nearer to site II than site I ligands (15, 16) making it a useful reporter group for ligand binding studies.

A decrease in tryptophan fluorescence at 350 nm was observed on addition of monooleoylglycerol (**Fig. 1a**), but no effect was observed with oleic acid (Fig. 1a), suggesting that these two ligands do not bind to the same site(s). Because addition of oleic acid to HSA had no effect on tryptophan fluorescence at 350 nm, the fluorescence decrease observed with monooleoylglycerol was also measured in the presence of increasing amounts of oleic acid (Fig. 1b). It can be seen that although the oleic acid is added up to a molar ratio of 5:1 with respect to albumin, there is an immediate decrease in fluorescence on adding monooleoylglycerol. This result suggests that the monooleoylglycerol is binding at a site distinct from the primary long-chain fatty acid binding sites. However, the decrease in tryptophan fluorescence was smaller with increasing oleic acid than that seen in the absence of oleic acid (Fig. 1a) and suggests that the binding of oleic acid had an effect on monooleoylglycerol binding. This observation is consistent with the ability of fatty acids to induce a change in albumin conformation (1, 2) which may subsequently decrease the effect of monooleoylglycerol binding on tryptophan fluorescence.

Although oleic acid has no significant effect on tryptophan fluorescence when measured at 350 nm, this fatty acid does produce an increase in fluorescence when measured at 330 nm (Fig. 1a) whereas monooleoylglycerol does not (Fig. 1a), again suggesting different sites for binding of the two ligands. Monooleoylglycerol at up to 20 μm did not prevent the increase in tryptophan fluorescence on subsequent addition of oleic acid (Fig. 1c).

Displacement studies using fluorescent ligands

Attempts were made to measure the displacement by monooleoylglycerol of four different fluorophores that are known to bind to HSA; these were DAUDA, dansylglycine, dansylsarcosine and 1,8-ANS (6–11). No significant change in fluorescence characteristics was observed on addition of monooleoylglycerol to solutions containing HSA and DAUDA or 1,8-ANS (**Fig. 2a**, **c**), while a small loss in fluorescence was observed with dansylglycine but only at higher concentrations of monooleoylglycerol (Fig. 2b). A lack of apparent fluorescence change was not due to the probe partitioning into aggregates of monooleoyl-

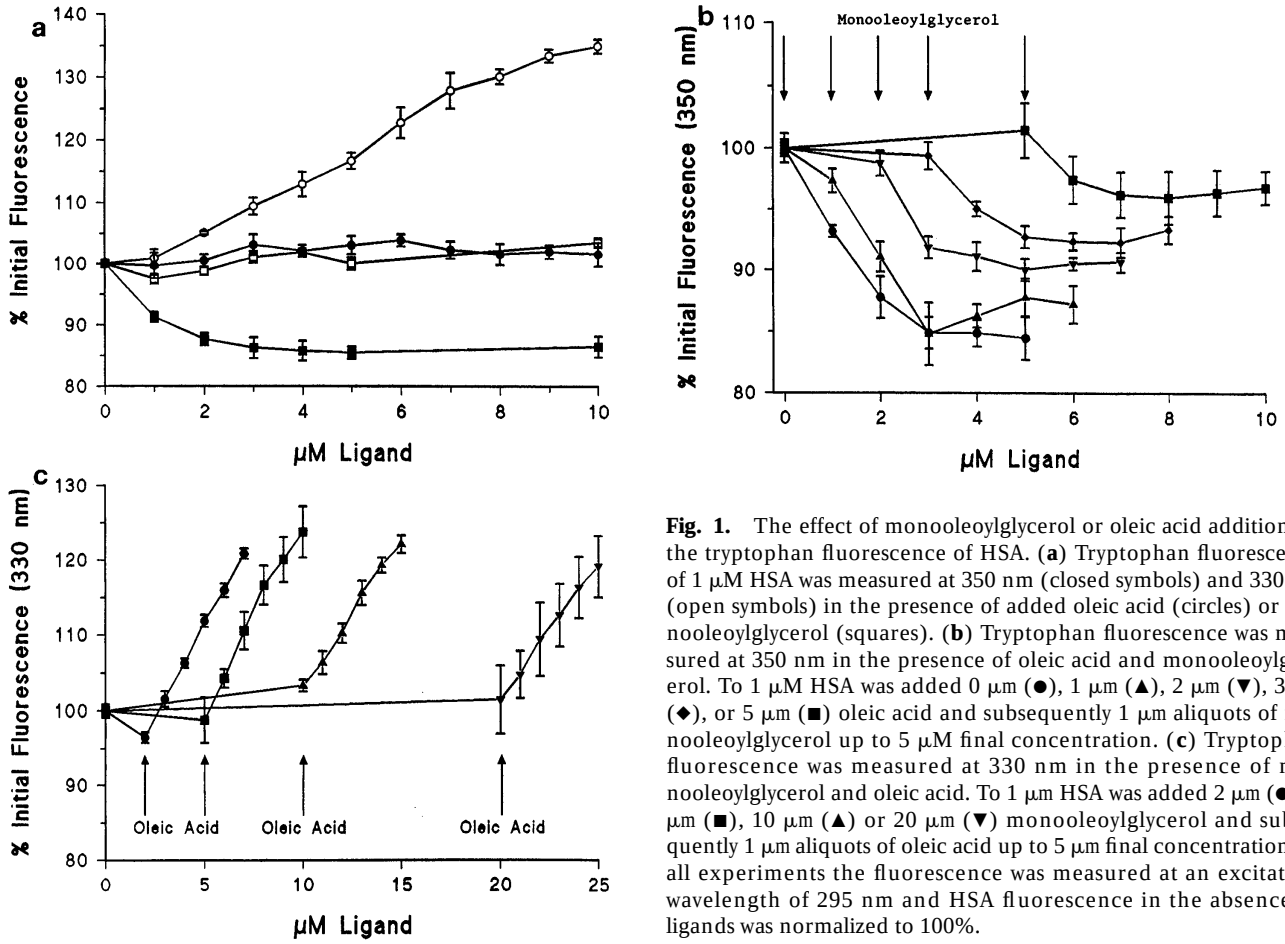

Fig. 1. The effect of monooleoylglycerol or oleic acid addition on the tryptophan fluorescence of HSA. (**a**) Tryptophan fluorescence of 1 μM HSA was measured at 350 nm (closed symbols) and 330 nm (open symbols) in the presence of added oleic acid (circles) or monooleoylglycerol (squares). (**b**) Tryptophan fluorescence was measured at 350 nm in the presence of oleic acid and monooleoylglycerol. To 1 μM HSA was added 0 μm (●), 1 μm (▲), 2 μm (▼), 3 μm (◆), or 5 μm (■) oleic acid and subsequently 1 μm aliquots of monooleoylglycerol up to 5 μM final concentration. (**c**) Tryptophan fluorescence was measured at 330 nm in the presence of monooleoylglycerol and oleic acid. To 1 μm HSA was added 2 μm (●), 5 μm (■), 10 μm (▲) or 20 μm (▼) monooleoylglycerol and subsequently 1 μm aliquots of oleic acid up to 5 μm final concentration. In all experiments the fluorescence was measured at an excitation wavelength of 295 nm and HSA fluorescence in the absence of ligands was normalized to 100%.

glycerol as determined by control titrations for 1,8-ANS and DAUDA in the absence of HSA (data not shown). These fluorophores were displaced by oleic acid although only a relatively small change in fluorescence was observed until a HSA: oleic acid molar ratio of 1:3 was reached (Fig. 2a, b, c). This lag in displacement is indicative of oleic acid first binding to high-affinity binding sites for long-chain fatty acids (4, 6). The inability of monooleoylglycerol to displace DAUDA (Fig. 2a) suggests that these two ligands do not share the same site, while previously we have suggested that DAUDA binds to the high affinity bilirubin site (6). The only fluorescent ligand that was substantially displaced by both monooleoylglycerol and oleic acid was dansylsarcosine (Fig. 2d) and it is noteworthy that no lag was observed for the displacement of this ligand by oleic acid. The ability of monooleoylglycerol to reduce dansylsarcosine fluorescence as a result of the monooleoylglycerol binding to HSA was investigated in detail.

Determination of an inhibition constant (K_i)

The binding of dansylsarcosine to HSA gave a hyperbolic saturation curve and the K_d obtained for dansylsarcosine, assuming a single binding site, was 1.8 μm, which is similar to the values of between 2–6 μm previously published (8, 11). Monooleoylglycerol displaces dansylsarcosine from HSA, as is apparent from a loss of dansylsarcosine fluorescence (Fig. 2d), and therefore monooleoylglycerol could be treated as an inhibitor of dansylsarcosine binding to HSA. The fluorescence values obtained in the presence of monooleoylglycerol were fitted to a hyperbolic equation and the replotted parameters showed competitive inhibition with a calculated inhibition constant (K_i) of 2.5 μm (**Fig. 3a**, **b**).

In contrast, although added oleic acid also resulted in a loss of dansylsarcosine fluorescence (Fig. 2c), a replot of the data indicated that the inhibition was more mixed in nature consistent with oleic acid inducing a conformational change that reduced the binding of dansylsarcosine to HSA.

DISCUSSION

Albumin is the major serum transport protein and the ligand binding properties of this protein have been studied in great detail, especially with regard to drugs and long-chain fatty acids (1, 2). The products of triglyceride metabolism by lipases are fatty acids, which bind to HSA at multiple sites with varying affinities (1–3), and 2-monoacylglycerides, for which there is minimal binding information (5). The multiple binding sites on albumin and the non-polar natures of the ligands make it difficult to perform definitive binding studies. We have investigated the binding of mono-

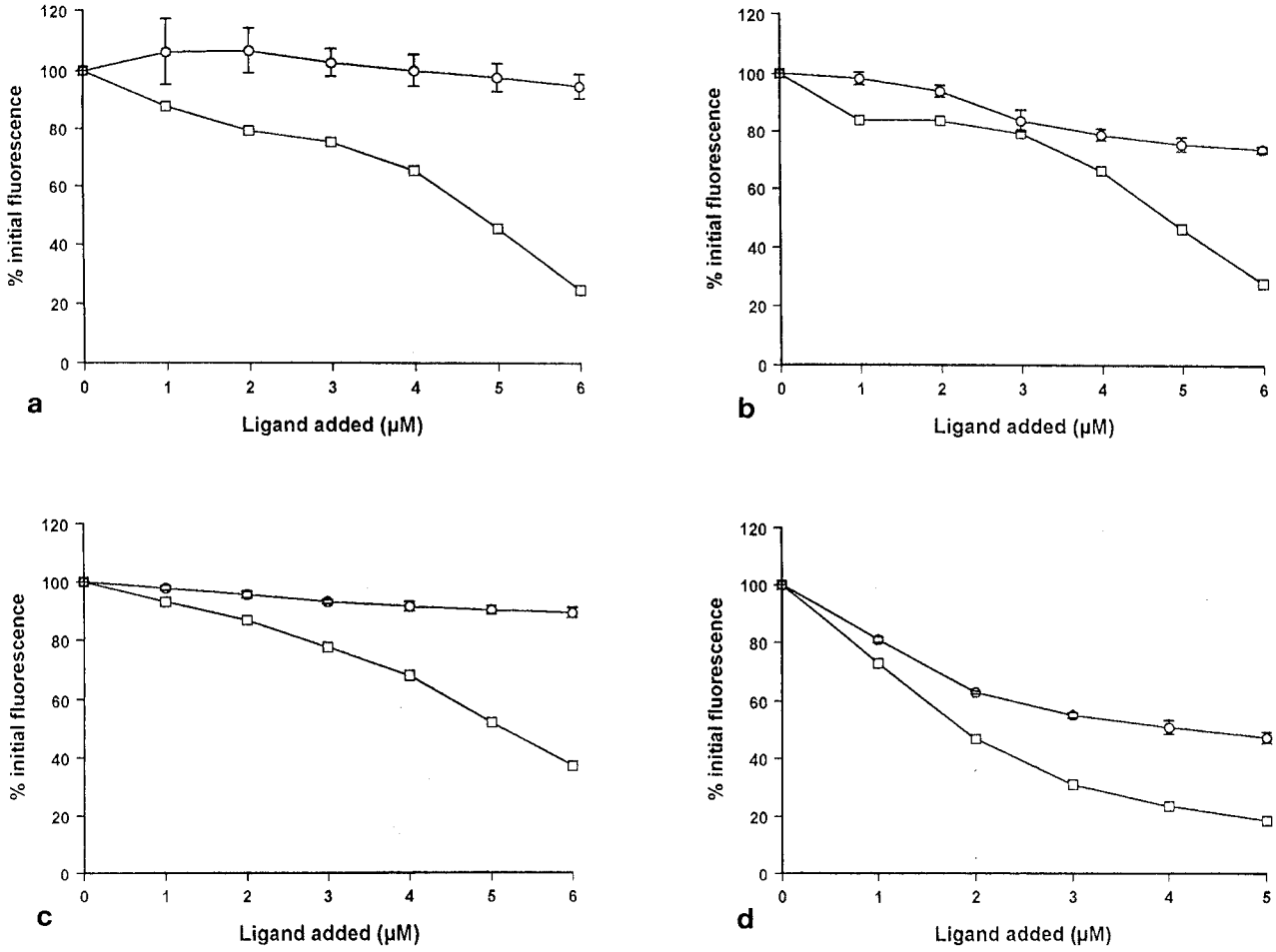

Fig. 2. Monooleoylglycerol and oleic acid displacement of various fluorophores from HSA. Samples contained 1 μm HSA and 1 μm fluorophore to which were added aliquots of monooleoylglycerol (○) or oleic acid (□). The displaced ligands were (**a**) DAUDA; (**b**) dansylglycine; (**c**) 1,8-ANS; (**d**) dansylsarcosine.

oleoylglycerol to HSA by measuring HSA tryptophan fluorescence and using competitive fluorescence displacement assays involving fluorescent ligands for HSA.

HSA contains a single tryptophan at position 214 (1) and the fluorescence emission of this tryptophan at 350 nm was decreased on binding of monooleoylglycerol, but not oleic acid. In contrast, oleic acid produced an increase in fluorescence intensity at 330 nm not seen with monooleoylglycerol while prior addition of monooleoylglycerol did not prevent this increase in fluorescence, suggesting that different sites were involved for the two ligands.

Using a similar protocol, prior addition of oleic acid to HSA did not prevent the decrease in tryptophan fluorescence at 350 nm obtained when monooleoylglycerol was added, suggesting again that monooleoylglycerol and oleic acid are binding at different sites. However, the decrease in fluorescence on addition of monooleoylglycerol was attenuated by prior addition of oleic acid and we propose that the binding of oleic acid induces a conformational change in HSA that affects the binding of monooleoylglycerol. Similar conclusions have been drawn concerning the effects of fatty acids on the binding of other ligands to albumin (1, 2, 17).

The displacement of fluorescent ligands by oleic acid and monooleoylglycerol (Fig. 2) produced significant differences, which suggested that these ligands were binding to different sites on the protein. Dansylsarcosine is a well-characterized fluorescent ligand that binds to site II of HSA (8, 9), now identified as sub-domain IIIA (1, 18). This ligand was displaced from HSA by monooleoylglycerol in a competitive manner with a calculated K_d of approximately 2.5 μm (Fig. 3a, b), which suggests that dansylsarcosine and monooleoylglycerol bind to the same site. In contrast, oleic acid appeared to be more non-competitive in terms of its effect on dansylsarcosine binding (Fig. 3c).

The displacement of dansylsarcosine by oleic acid shows a significant difference from the displacement curves for the other dansyl-related ligands. Displacement of these other ligands shows a clear lag indicating that about 3 mols of fatty acid is required to first saturate the high affinity sites for long chain fatty acids. In contrast, no such lag is seen for the displacement of dansylsarcosine. We have already shown that oleic acid reduces the change in tryptophan fluorescence when monooleoylglycerol binds and we suggested that such fatty acid binding affects the monooleoylglycerol binding site as a result of conforma-

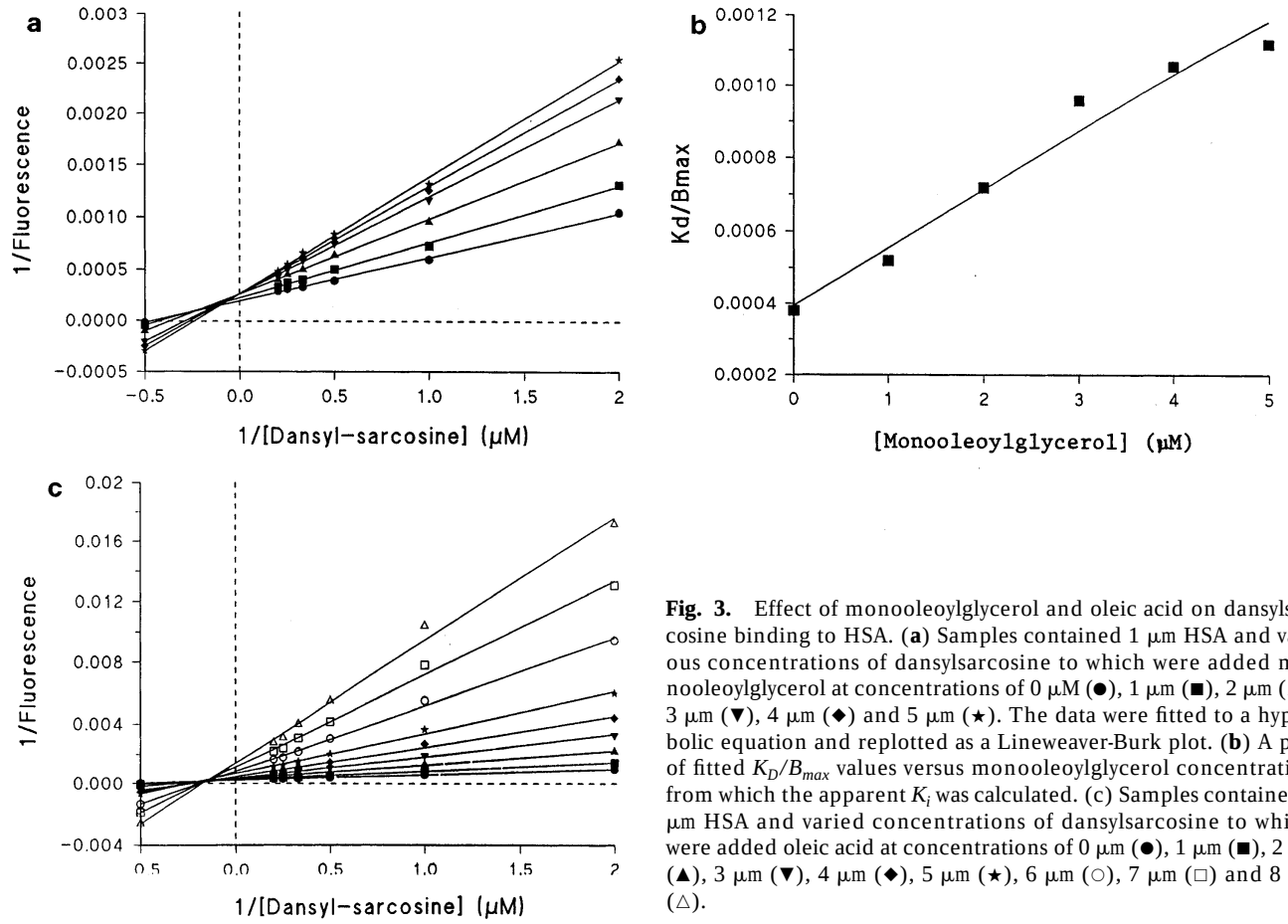

Fig. 3. Effect of monooleoylglycerol and oleic acid on dansylsarcosine binding to HSA. (**a**) Samples contained 1 μm HSA and various concentrations of dansylsarcosine to which were added monooleoylglycerol at concentrations of 0 μM (●), 1 μm (■), 2 μm (▲), 3 μm (▼), 4 μm (◆) and 5 μm (★). The data were fitted to a hyperbolic equation and replotted as a Lineweaver-Burk plot. (**b**) A plot of fitted K_D/B_{max} values versus monooleoylglycerol concentration from which the apparent K_i was calculated. (c) Samples contained 1 μm HSA and varied concentrations of dansylsarcosine to which were added oleic acid at concentrations of 0 μm (●), 1 μm (■), 2 μm (▲), 3 μm (▼), 4 μm (◆), 5 μm (★), 6 μm (○), 7 μm (□) and 8 μm (△).

tional changes in HSA. It is possible that binding of oleic acid at its primary sites can also affect the binding of dansylsarcosine by a similar mechanism and this results in an immediate loss of fluorescence as oleic acid is added (Fig. 2d).

The characterization of dansylglycine as a site I probe is contentious (8, 11), but the results shown here demonstrate that dansylglycine and dansylsarcosine do not show the same displacement characteristics with monooleoylglycerol or oleic acid (Fig. 2b, d), probably indicating different locations on HSA. It is surprising that 1,8-ANS is not displaced by monooleoylglycerol (Fig. 2c) as this compound has also been defined as a site II probe (8). However, there are multiple sites for 1,8-ANS on albumin (8) while in the present study only the highest affinity site will be monitored as a result of using a 1:1 molar stoichiometry of ligand to protein. The studies with DAUDA (Fig. 2a) indicate that monooleoylglycerol does not bind to the bilirubin-binding sites on HSA (6).

In conclusion, these results suggest that monooleoylglycerol binds primarily to HSA site II causing a conformational change that results in a change in tryptophan fluorescence. The binding site for monooleoylglycerol is distinct from the primary sites for long-chain fatty acids and bilirubin. The lower affinity of HSA for monoacylglycerol (monooleoylglycerol) as compared with fatty acid would suggest that albumin may not have a major role in the further metabolism of lipoprotein lipase-generated monoacylglycerol, particularly as long chain fatty acids (oleic acid) may affect the capacity of this site. However, the high concentration of albumin present within both the intravascular and extravascular spaces should normally overcome actual problems of affinity and capacity at the molecular level if binding of monoacylglycerol is required.

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