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Hydrophobic Ligand Binding by Zn-α_2-glycoprotein, a Soluble Fat-depleting Factor Related to Major Histocompatibility Complex Proteins*

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Zn-α_2-glycoprotein (ZAG) is a member of the major histocompatibility complex (MHC) class I family of proteins and is identical in amino acid sequence to a tumor-derived lipid-mobilizing factor associated with cachexia in cancer patients. ZAG is present in plasma and other body fluids, and its natural function, like leptin's, probably lies in lipid store homeostasis. X-ray crystallography has revealed an open groove between the helices of ZAG's α_1 and α_2 domains, containing an unidentified small ligand in a position similar to that of peptides in MHC proteins (Sanchez, L. M., Chirino, A. J., and Bjorkman, P. J. (1999) *Science* 283, 1914–1919). Here we show, using serum-derived and bacterial recombinant protein, that ZAG binds the fluorophore-tagged fatty acid 11-(dansylamino)undecanoic acid (DAUDA) and, by competition, natural fatty acids such as arachidonic, linolenic, eicosapentaenoic, and docosahexaenoic acids. Other MHC class I-related proteins (FcRn, HFE, HLA-Cw*0702) showed no such evidence of binding. Fluorescence and isothermal calorimetry analysis showed that ZAG binds DAUDA with K_d in the micromolar range, and differential scanning calorimetry showed that ligand binding increases the thermal stability of the protein. Addition of fatty acids to ZAG alters its intrinsic (tryptophan) fluorescence emission spectrum, providing a strong indication that ligand binds in the expected position close to a cluster of exposed tryptophan side chains in the groove. This study therefore shows that ZAG binds small hydrophobic ligands, that the natural ligand may be a polyunsaturated fatty acid, and provides a fluorescence-based method for investigating ZAG-ligand interactions.

Zn-α_2-glycoprotein (ZAG)[1] is a soluble protein present in serum and other body fluids (1, 2). It accumulates in breast cysts, is produced by 40% of breast carcinomas, and is inducible in breast cancer cell lines by glucocorticoids and androgens (2, 3). ZAG is identical in amino acid sequence to lipid-mobilizing factor that is associated with cachexia (4, 5), the wasting syndrome involving depletion of adipose and muscle tissue, such as occurs in many patients with cancer, AIDS, trypanosomiasis, and other life-threatening diseases. Significantly, ZAG is overexpressed in tumors that accompany fat loss, and exogenous ZAG produces cachectic symptoms in experimental animals (4, 5). ZAG, like leptin (6), therefore, participates in lipid store homeostasis, the dysregulation of which has serious implications for survival and the management of cancer and other diseases.

ZAG is a member of a family of proteins typified by the class I MHC proteins (which present peptides to cytotoxic T cells) (7) and includes CD1 (which presents lipidic antigens to T cells) (8, 9), the neonatal Fc receptor (FcRn; involved in transportation of immunoglobulin across epithelia), and HFE (a transferrin-binding protein that regulates iron homeostasis) (10, 11). In contrast to all other MHC-like proteins, ZAG and MIC-A (a divergent member of the MHC family) are not found in association with β_2-microglobulin (β2M) as a light chain (12, 13).

The crystal structure of ZAG reveals an overall fold that is very similar to that of MHC class I molecules (14). The spatial relationship between its three domains is slightly different from that found in class I MHC proteins, which may explain the lack of affinity of ZAG for β2M. As with all members of the family, a prominent feature of the α_1 and α_2 domains is a pair of opposing α-helices that enclose the binding groove for peptides or (glyco)lipids in MHC proteins. In FcRn, HFE, and MIC-A, the groove is closed, and there is no evidence for ligand binding within the region between the helices, although each interacts with other proteins (15–17). In ZAG, however, the x-ray crystal structure shows that the groove is open and contains an additional electron density ascribed to an unidentified ligand (Fig. 1) (14). The presumptive ligand appears curved and nonbranched and lacks the characteristic protrusions of either a peptide or carbohydrate structure. The molecular surface of the central part of the groove where the unresolved density lies is nearly neutral in charge, except for an Arg side chain protrud-

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[1] The abbreviations used are: ZAG, Zn-α_2-glycoprotein; ANS, 8-anilino-1-naphthalenesulfonic acid; DACA, dansyl-D,L-α-amino-octanoic acid; DAUDA, 11-(dansylamino)undecanoic acid; DHA, docosahexaenoic acid; DSC, differential scanning calorimetry; FcRn, neonatal Fc receptor; HFE, hereditary hematochromatosis protein; ITC, isothermal titration calorimetry; PUFA, polyunsaturated fatty acid; retinol, all-*trans*-retinol; PBS, phosphate-buffered saline; MHC, major histocompatibility complex; β_2M, β_2-microglobulin; PCR, polymerase chain reaction; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

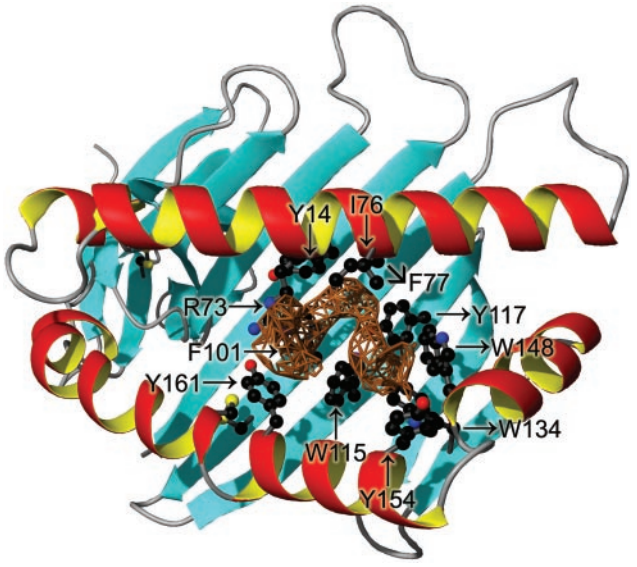

FIG. 1. **The presumptive ligand binding site on ZAG.** A view of the α_1 and α_2 domains of ZAG showing the position of the unresolved density found in the crystal structure (*brown net*) (14) is shown. The amino acid side chains that may interact with the ligand are represented as *balls-and-sticks*. Note the position of the cluster of tryptophans (*W115*, *W134*, *W148*) close to one end of the density, and the prominent arginine side chain (*R73*) at the opposite end.

ing from one side of the groove. The other residues surrounding the presumptive ligand are hydrophobic (three Trp, four Tyr, two Phe, and an Ile). Like CD1, therefore, ZAG may bind a hydrophobic ligand, although the groove in ZAG is not as deep as that in CD1 and could not accommodate lipids of the size and complexity of those bound by this protein.

The nature of the ZAG ligand cannot be determined unambiguously from the crystal structure, because the electron density corresponding to the ligand may arise from a heterogeneous mixture of molecules or represent a loosely bound or large ligand only partially inserted into the groove. The nature of the ligand or ligands is clearly of potential importance to the biological function of ZAG. Moreover, if (like MHC class I) ZAG can bind different ligands of a biochemically similar class, then it is possible that the biological activity of ZAG varies with the precise nature of the bound compound. The nature of the ligand would be of relevance to homeostasis of body lipid stores and the management of disease conditions in which ZAG activity is detrimental to outcome.

Given the chemical characteristics of the groove, and assuming that the unresolved electron density is a useful guide to the nature of the ligand, it is conceivable that the ligand is a fatty acid. Using fluorescent methods and microcalorimetry, we have found that ZAG can bind fatty acids and that one particular fluorescent fatty acid analogue may be particularly useful for screening ZAG ligands.

EXPERIMENTAL PROCEDURES

Naturally Produced and Recombinant Proteins—Natural ZAG was purified from human serum using immunoaffinity chromatography as described previously (13). For expression of ZAG in *Escherichia coli*, polymerase chain reaction (PCR) was used to modify the cDNA encoding human ZAG to insert a 5′ *Nde*I site before the codon corresponding to residue 1 of the mature protein and a 3′ *Xho*I site following the stop codon. The PCR product was subcloned into pPCR-Script Amp SK(+) and sequenced, then subcloned into the *Nde*I and *Xho*I sites of the bacterial expression vector pET23a (Novagen). ZAG was expressed in *E. coli* strain BL21(DE)3pLysE, and the protein was renatured from inclusion bodies as described previously (18) and purified on a gel filtration column (HiLoadTM 26/60 Superdex 200 column; Amersham Pharmacia Biotech). The protein migrated as a single peak on the gel filtration column and exhibited a far-UV circular dichroism spectrum similar to natural ZAG isolated from serum (data not shown). Soluble versions of FcRn, HFE, and a class I MHC protein (HLA-Cw*0702) were expressed in CHO cells (FcRn and HFE) or baculovirus-infected insect cells (HLA-Cw*0702) and purified as described previously (16, 19, 20). All the proteins were greater than 95% pure as judged by SDS-polyacrylamide gel electrophoresis stained with Coomassie Blue. The concentrations of the proteins were estimated by absorbance at 280 nm using theoretical extinction coefficients based on their amino acid compositions (21) using the ProtParam program through ExPASy server (expasy.cbr.nrc.ca/tools/).

Ligands—The fluorescent fatty acids 11-((5-dimethylaminonaphthalene-1-sulfonyl)amino)undecanoic acid (DAUDA) and *cis*-parinaric acid were obtained from Molecular Probes (Eugene, OR). Oleic acid, arachidonic acid, docosahexaenoic acid, γ-linolenic acid, *cis*-eicosapentaenoic acid, linoleic acid, palmitic acid, all-*trans*-retinol, and dansyl-α-aminooctanoic acid (DACA) were obtained from Sigma (Poole, Dorset, UK). The dansylated fatty acids were stored as stock solutions of ~3 mg ml^{-1} in ethanol, in the dark at −20 °C, and freshly diluted in phosphate-buffered saline (PBS; 171 mM NaCl, 3.35 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2) to ~1 μM before use in the fluorescence experiments. Competitors of fluorescent fatty acid binding were prepared as stock solutions in ethanol at ~10 mM and diluted in PBS in the case of oleic acid or in ethanol for other competitors. Retinol was dissolved and diluted in ethanol immediately before use and binding to proteins was tested by addition of, typically, 5 μl of this directly to a cuvette containing protein in PBS. The concentrations of both DAUDA and DACA stocks were calculated using a molar extinction coefficient ϵ_{335} of 4800 M^{-1} cm^{-1} in methanol, ϵ_{303} of 76,000 M^{-1} cm^{-1} for *cis*-parinaric acid in ethanol (22), and ϵ_{325} of 52,480 M^{-1} cm^{-1} for retinol in ethanol.

Spectrofluorimetry and Fluorescence-based Ligand Binding—Fluorescence binding emission spectra (uncorrected) were recorded at 20 °C with a SPEX Fluo-Max spectrofluorimeter (Spex Industries, Edison, NJ) using 2-ml samples in a silica cuvette. Raman scattering by solvent water was subtracted where necessary. The excitation wavelengths used for DAUDA, DACA, *cis*-parinaric acid, and retinol were 345, 345, 319, and 350 nm, respectively. Fluorescence data were corrected for dilution where necessary and fitted by standard nonlinear regression techniques (using Microcal ORIGIN software) to a single site binding model to give estimates of the dissociation constant (K_d) and maximal fluorescence intensities (F_{max}).

Isothermal Calorimetry (ITC)—ITC experiments to measure the binding of DAUDA to ZAG were done at 25 °C using a Microcal VP-ITC titration microcalorimeter following standard instrumental procedures (23, 24) with a 250-μl injection syringe and 320 rpm stirring. Proteins were dialyzed against PBS, followed by addition of ethanol to match the ligand mixture (2.5%), and degassed briefly before loading into the calorimeter cell. Ligand (DAUDA) solutions were made up from ethanolic stock solutions by dilution (to 2.5% ethanol) in the same buffer. A typical binding experiment involved 25 × 10-μl injections of ligand solution (450 μM) into the ITC cell (~1.4 ml active volume) containing protein (25 μM). Control experiments were performed under identical conditions by injection of ligand into buffer alone (to correct for heats of ligand dilution) and injection of buffer into the protein mix (to correct for heats of dilution of the protein). Integrated heat effects, after correction for heats of dilution, were analyzed by nonlinear regression in terms of a simple single-site binding model using the standard Microcal ORIGIN software package. For each thermal titration curve this yields estimates of the apparent number of binding sites (n) on the protein, the binding constant (K/M^{-1}) and the enthalpy of binding (ΔH/kcal mol^{-1}). Other thermodynamic quantities were calculated using standard expressions: $\Delta G^0 = -RT\cdot\ln K = \Delta H^0 - T\cdot\Delta S°$.

Differential Scanning Calorimetry (DSC)—DSC to examine the thermal stability of ZAG and ZAG·DAUDA complexes was done using a Microcal VP-DSC, normally scanning from 10 to 100 °C with a scan rate of 60 °C h^{-1} (25). Scans were repeated after cooling with the sample *in situ* to check for reversibility of the thermal unfolding transition. Protein samples (12.5 μM) were prepared as above for ITC, and both sample and reference (buffer) solutions were degassed prior to loading. In experiments with added ligand, the samples also contained ethanol (0.5–5%, depending on ligand addition), and separate control experiments were performed to confirm the absence of any significant ethanol effect on protein stability under these conditions. Normalized DSC data, corrected for instrumental baseline, were fitted to standard non-two-state models using Microcal ORIGIN software.

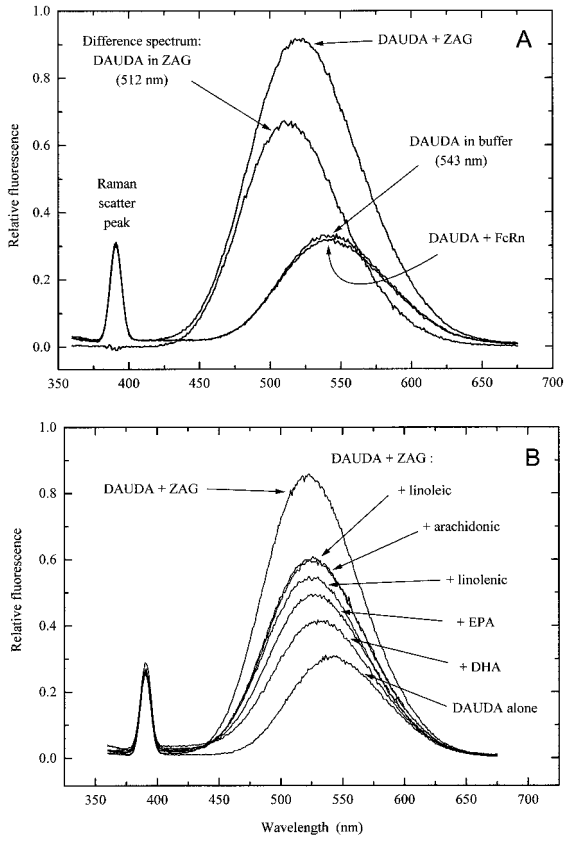

FIG. 2. **Fluorescence analysis of ligand binding by ZAG.** *A,* fluorescence emission spectra (λ_{Exc} = 345 nm) of 2 μM DAUDA alone in buffer or upon addition of 0.39 μM ZAG. The difference spectrum indicates the wavelength of peak emission by DAUDA when bound to ZAG. Other proteins structurally related to ZAG (FcRn, HFE, and the class I MHC molecule HLA-Cw*0702) produced no change in the fluorescence emission spectrum of DAUDA, and the effect of FcRn is given for example. *B,* the reversal of ZAG-induced changes in DAUDA emission by competition with 20 μM arachidonic, linoleic, linolenic, eicosapentaenoic (*EPA*), or docosahexaenoic (*DHA*) acids added to a preformed complex of ZAG·DAUDA complex. The concentrations of DAUDA and ZAG were 2 and 0.39 μM, respectively.

RESULTS AND DISCUSSION

Human serum-derived and recombinant ZAG, and control proteins of the MHC family, were obtained as detailed under "Experimental Procedures." The naturally produced and recombinant ZAG behaved similarly in preliminary ligand binding and intrinsic fluorescence experiments, and only experiments using the natural protein are described unless differences were noted.

Fatty Acid Binding—Synthetic fatty acid analogues bearing environment-sensitive fluorophores were used to investigate fatty acid binding by ZAG. These compounds alter their fluorescence emission intensities and wavelengths of peak emission upon entry into protein binding sites. The most useful proved to be DAUDA, which is a saturated fatty acid with a dansyl fluorophore attached at its ω-methyl terminus, and has been used extensively in the study of fatty acid-binding proteins, from which it can be displaced by specific fatty acids (26, 27). DAUDA also binds to serum albumins, although it appears in this case to bind in the hydrophobic binding site for bilirubin (28). Addition of ZAG to a solution of DAUDA was accompanied by a substantial increase in DAUDA fluorescence intensity and a shift in the fluorescence emission maximum wavelength from 543 to 512 nm (Fig. 2*A*). Control experiments with FcRn, HFE, or HLA-Cw*0702 under similar conditions gave no alteration in the fluorescence emission by DAUDA.

The blue shift in fluorescence emission of the dansyl fluorophore indicates transfer from solvent water to an apolar protein binding site. A blue shift to 512 nm with DAUDA is substantial, but is less than that observed upon DAUDA binding to liver or intestinal fatty acid binding proteins (496 and 492 nm, respectively) (29, 30), tear lipocalin (490 nm) (31); the most extreme blue shifts in DAUDA emission have been recorded upon interaction with unusual lipid-binding proteins from nematode worms (475 and 485 nm) (32–34). The relatively small blue shift when DAUDA interacts with ZAG is perhaps indicative of a binding site that is more solvent-exposed than in lipid transporter proteins, such as would be expected for an open-sided binding groove. ZAG failed to produce any detectable change in the fluorescence emission of a fatty acid analogue in which the dansyl group is attached at the α-carbon (dansyl-D,L-α-amino-octanoic acid) or in the fluorophore alone (in the form of dansylamide). Consequently, dansylation close to the carboxylate of a fatty acid interferes with binding, and the dansyl group itself probably does not contribute to the binding. Fluorescence titration experiments in which ZAG was added incrementally to a solution of DAUDA provided an estimate of the DAUDA·ZAG dissociation constant (K_d = 4.2×10^{-7} M; data not shown), which was similar to that obtained from calorimetry (see below).

Addition of natural fatty acids to a ZAG·DAUDA complex resulted in a reversal of the fluorescence effect, presumably by competitive displacement of DAUDA into solvent (Fig. 2*B*). Different fatty acids varied in the efficiency with which they did this on a molar basis, the ranking being docosahexaenoic > eicosapentaenoic > linolenic ≈ arachidonic > linoleic > oleic acid, but no changes were detected upon addition of cholesterol, deoxycholic acid, or arterenol (noradrenaline). ZAG was found to increase the fluorescence of ANS, which is generally regarded as a probe for exposed hydrophobic surfaces on proteins, and this change was also reversed upon addition of fatty acids (data not shown). ZAG failed to alter the emission of the intrinsically fluorescent steroid dehydroergosterol (which binds to liver fatty acid-binding protein (35)), all-*trans*-retinol, 12-(9-anthroxyl)stearic acid (which binds to several different fatty acid-binding proteins (36, 37)), or *cis*-parinaric acid. The latter result is surprising given that *cis*-parinaric acid is known to bind to other fatty acid-binding proteins, including those that do not bind DAUDA (38). This may, however, be due to the fact that this fatty acid is highly conjugated and may lack the conformational freedom to adapt to the ZAG binding site. Eicosanoids such as arachidonic acid, in contrast, are known to be more flexible and bind to proteins in different conformations. For instance, arachidonic acid binds in a hairpin conformation in adipocyte lipid-binding protein (39), but in a more extended conformation in prostaglandin synthase, as does leukotriene A_4 in leukotriene A_4 hydrolase (40, 41).

Soluble forms of proteins belonging to the MHC class I family, such as FcRn, HFE, and an MHC molecule (HLA-Cw*0702) all failed to bind DAUDA and ANS in control experiments. Thus, ZAG appears to specifically bind hydrophobic ligands, with an apparent preference for fatty acids, although many more types of natural ligand need to be tested for its true specificity to be understood. DAUDA may or may not bind to ZAG in a fashion analagous to its natural ligand(s), but it is likely to be useful in screening for natural ligands by competitive displacement. The characteristics of its interaction with ZAG were therefore investigated further.

Binding Affinity and Stoichiometry—ITC experiments showed that binding of DAUDA to ZAG is exothermic at 25 °C, giving thermal titration curves consistent with simple 1:1 complex formation (Fig. 3). Mean thermodynamic parameters for

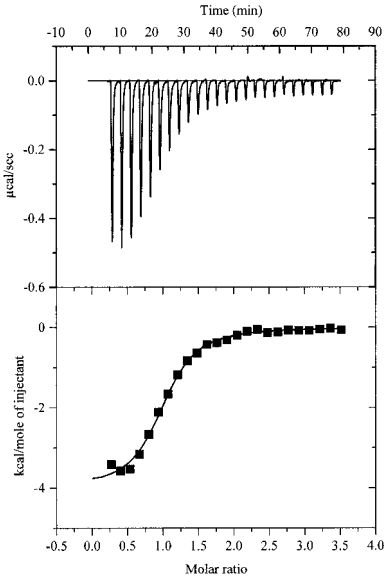

FIG. 3. **ITC analysis of ZAG-ligand interaction.** Typical ITC thermograms for sequential 10-μl additions of DAUDA (450 μM) to ZAG (25 μM). The *upper panel* shows raw data, with negative heat pulses indicating exothermic binding, decreasing to base-line levels at higher ligand concentrations. The *lower panel* shows integrated heats, giving a differential binding curve. The *line* shows the best fit to these data using a single-site binding model with $n = 0.99$, $K = 6.7 \times 10^5$ M (K_d = 1.5 μM), $\Delta H = -3.99$ kcal mol^{-1}, $\Delta S^0 = +13.3$ cal K^{-1} mol^{-1}.

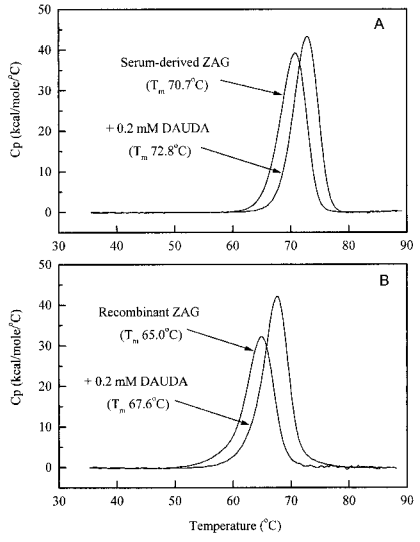

FIG. 4. **Increased thermal stability of ZAG upon ligand binding.** Normalized DSC data, corrected for buffer base line showing cooperative endothermic unfolding of wild-type (*A*) and recombinant ZAG (*B*) in the presence and absence of 0.2 mM DAUDA.

binding of DAUDA to ZAG, determined from a series of calorimetric experiments, are: $K_d = 1.3$ ($\pm$0.4) μM, $\Delta H^0 = -5.2$ ($\pm$1.0) kcal mol^{-1}, $\Delta S° = +9.7$ ($\pm$3.8) cal K^{-1} mol^{-1} (1 cal = 4.184 J). These data are consistent with the micromolar dissociation constants determined from the DAUDA fluorescence titrations and confirm that fluorescence methods, although intrinsically indirect and less precise than direct calorimetric methods, are sufficiently robust to be used for routine ligand binding studies in this system. The positive ΔS^0 for binding is consistent with an increase in solvent (water) entropy anticipated due to disruption of the hydration layer around the hydrophobic ligand.

ITC experiments using the natural ligands were not feasible under the conditions used because of the poor solubility of the ligands at the concentrations required in the injection syringe.

The serum-derived ZAG used here for binding studies was purified using similar procedures to those used for the original crystallographic work (though lacking the additives used for protein crystallization) and may be similarly loaded with competing natural (endogenous) ligand. Bacterial recombinant ZAG may also be loaded with the same or a related compound derived from the synthesizing cells. Although we have no direct evidence for such endogenous ligand(s) in our preparations, any competition for the binding site would serve only to reduce the apparent binding affinities for exogenous ligands that we have observed. The good fit of fluorescence and ITC titration data to simple binding models, with $n = \sim 1$ stoichiometry, shows that endogenous ligand (if present) is exchangeable with DAUDA or natural fatty acids and would act simply as a competitive inhibitor in binding affinity studies. Consequently, the apparent ligand dissociation constants (K_d) determined here represent an upper limit for the true K_d, and the binding strength of ZAG for the natural ligand may be substantially stronger. Given that the K_d values obtained for ZAG·DAUDA were of a similar order of magnitude (micromolar) to those measured for interactions between lipids and lipid transport proteins (26, 27), the binding may be sufficiently strong for the ZAG·ligand complex to remain intact during its passage from the synthesizing cell through blood and tissue spaces to the target cells.

ZAG Stability and Ligand Binding—DSC experiments (Fig. 4) showed that ZAG proteins undergo a typical endothermic cooperative reversible unfolding transition in solution with a transition midpoint (T_m) at around 65–70 °C under physiological conditions. Recombinant ZAG samples had the slightly lower thermal stability (T_m 65 °C) compared with serum-derived material (T_m 70 °C) under the same conditions, possibly because the recombinant proteins lack the ligand or *N*-linked carbohydrates (14). The crystal structure of ZAG shows nine ordered carbohydrate residues arranged in a biantennary arrangement, attached to Asn^{239} on a loop in the α_3 domain directly beneath the platform formed by the α_1-α_2 superdomain, and other sites with *N*-linked carbohydrates are also evident (14). The reduced stability of the bacterial recombinant ZAG is therefore probably due to the absence of these glycosylations, although the DSC data and the intrinsic fluorescence spectra (see below) indicate that the recombinant protein backbone is properly folded. Addition of increasing concentrations of DAUDA raised the protein T_m progressively for both the natural and recombinant ZAG by up to 5 °C with 200 μM added ligand. This is typical of the enhancement in protein folding stability generally observed in the presence of ligands that bind specifically to the native state (25). It is important to emphasize here that the enhanced stability of the protein-ligand complex does not necessarily imply any conformational change in the protein (as is sometimes assumed), rather it represents simply the additional free energy required to dissociate the native protein-ligand complex before the protein can unfold.

Intrinsic Fluorescence and the Position of the Fatty Acid Binding Site—The results detailed above strongly indicate that DAUDA binds to ZAG at a hydrophobic binding site, from which it is displaceable by fatty acids. The simplest hypothesis is that the binding site is in the groove lying between the α_1 and α_2 domains, analogous to the peptide or lipid ligand binding sites of class I MHC molecules. The binding data, however, provide no direct evidence that this is the binding site in ZAG, and it is conceivable that DAUDA and fatty acids bind elsewhere on the protein. We therefore exploited intrinsic tryptophan fluorescence in an attempt to localize the binding site. ZAG possesses eight tryptophans, three in the α_3 domain, two at the extreme end of the groove and distant from the region

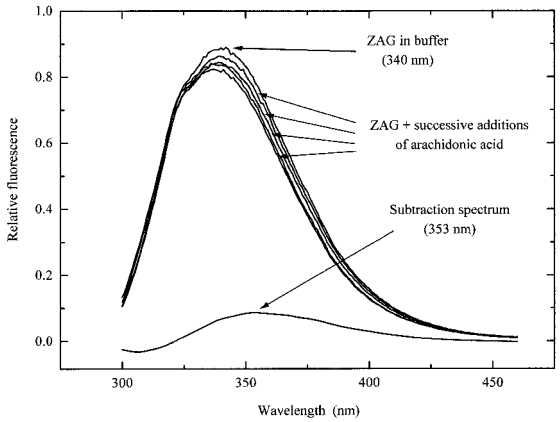

FIG. 5. **Change in intrinsic fluorescence emission of ZAG upon addition of ligand.** Fluorescence emission spectra of ZAG in the presence and absence of incremental additions of arachidonic acid. λ_{Exc} = 290 nm. The spectra were corrected for light scatter produced by the addition of arachidonic acid in control experiments in which arachidonic acid was added to PBS in the absence of ZAG. The addition of arachidonic acid produced a quenching and blue shifting of the fluorescence emission by the protein, and the subtraction spectrum is the difference between the initial emission spectrum of ZAG alone in buffer and at the final addition of arachidonic acid. The peak in the subtraction spectrum (353 nm) is comparable with the emission spectrum of tryptophan fully exposed to solvent water.

occupied by the unresolved density, and three more that are closely grouped together in the groove immediately adjacent to the density. The intrinsic fluorescence emission spectrum of ZAG (λ_{Exc} = 290 nm) peaked at 340 nm, and there was a gradual quenching and blue shift in λ_{max} with successive additions of arachidonic acid (Fig. 5); a similar spectral shift was found upon addition of docosahexaenoic acid (not shown). Subtraction of the spectrum of ZAG in the absence of added fatty acid from that of a ZAG/arachidonic acid mixture provided a spectrum peaking at 353 nm. This is indicative of the quenching of tryptophans upon ligand binding and that the tryptophans concerned emit at 353 nm in the absence of ligand. Emission at this wavelength is indicative of a tryptophan side chain fully exposed to solvent water (42, 43), such as would be observed in tryptophan in solution or the tryptophans in a fully unfolded protein. Such an effect would occur if the ligand were to alter the charge environment of a tryptophan side chain or if binding were to induce a conformational change in the protein. In either case, the results strongly indicate ligand binding at a site close to, or congruent with, the position of the unresolved density.

These data demonstrate that ZAG can bind hydrophobic compounds and that the natural ligand may be a fatty acid, possibly a polyunsaturated fatty acid (PUFA). These findings are mainly based on the binding by ZAG of a synthetic fluorescent fatty acid, DAUDA. The characteristics of this interaction, as quantified here by fluorescence and microcalorimetry, provide a basis for the screening of natural compounds for binding to ZAG by competitive displacement of DAUDA from the ZAG binding site. Use of this approach showed that ZAG can bind several different types of fatty acids, all of which are abundant in human tissues. Any preparation of ZAG will probably therefore contain a heterogeneous mixture of fatty acids, which would thereby provide an explanation for the failure to resolve the density found in crystals of ZAG. Even if DAUDA were not of the same generic class as ZAG's true ligand(s), and fatty acids are not relevant *in vivo*, DAUDA may nevertheless be valuable for the screening of hydrophobic compounds, natural or synthetic, for binding to ZAG.

The shape and chemical environment of the unresolved electron density in the crystal structure of ZAG show characteristics commensurate with a fatty acid; it is a curved, nonbranched tube, which is embedded in a groove near a cluster of hydrophobic amino acids. Moreover, the only prominent charged amino acid side chain projecting into the groove is an arginine (Arg^{73}), which is positioned ≤4.5 Å from one end of the density. Args and Tyrs are commonly involved in anchoring fatty acids in binding proteins. For example, the carboxylate of arachidonic acid is anchored by interactions with a pair of Arg side chains and a Tyr in adipocyte lipid-binding protein (39) and in prostaglandin synthase by a salt bridge to an Arg and a hydrogen bond to the OH group of a nearby Tyr (40), arrangements that are also possible in ZAG.

If, like class I MHC molecules and CD1, ZAG binds a variety of ligands *in vivo,* albeit of a single generic class (peptides for class I, large, complex lipids for CD1), then it is possible that the particular fatty acid bound to ZAG may determine its biological effect. PUFAs in particular are notable for their pharmacological effects, and it is perhaps worthy of note that the fatty acid found to bind best to ZAG was DHA, which has recently been found to be an activation ligand for the retinoid X receptor in mouse brain (44). It may also be pertinent that eicosapentaenoic acid (which also binds well to ZAG) in conjunction with tumor-derived lipid mobilizing factor induces lipolysis in cultured adipocytes, whereas DHA has no such activity (45, 46). It may therefore be possible to manipulate the biological effects of endogenous ZAG in clinical situations (such as cachexia) by direct administration of an appropriate ZAG-binding ligand or of an appropriate ZAG·ligand complex.

All of the class I-type proteins require binding to a specific receptor for their biological activity to be fulfilled. No receptor has yet been described for ZAG, but it is conceivable that interaction with a receptor can be modified (favored or inhibited) by the presence of ligand and that the particular ligand so delivered then specifies the biologically activity of the recipient cell. An intriguing possibility, however, is that there is more than one receptor for ZAG and that different receptors discriminate different ZAG·ligand combinations, in an analagous fashion to MHC class I·peptide and CD1·lipid combinations interacting with their specific T cell receptor. The particular receptor activated may then determine the biological effect. Whatever the case, it is clearly important to establish the range of ligands that can bind to ZAG, those important *in vivo,* and how the biological effects of ZAG can be manipulated.

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