Modification of the Adipocyte Lipid Binding Protein by Sulfhydryl Reagents and Analysis of the Fatty Acid Binding Domain†

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ABSTRACT: The adipocyte lipid binding protein (ALBP) is a member of a multigene family of low molecular weight proteins which stoichiometrically and saturably bind hydrophobic ligands and presumably facilitate intracellular lipid metabolism. To probe the structure–function relationship of the binding domain of ALBP, chemical modification has been employed. Modification of the two cysteinyl residues of ALBP (Cys^1 and Cys^{117}) with a variety of sulfhydryl reagents decreased the apparent affinity for oleic acid in the following order of effectiveness: methyl methanethiosulfonate <<< *p*-(chloromercuri)benzenesulfonic acid < *N*-ethylmaleimide (NEM) = 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Thiol titration of ALBP with DTNB in the presence of bound oleate resulted in the modification of a single cysteinyl residue. The oleate-protected cysteine was identified as Cys^{117} by modification with a combination of reversible (DTNB) and irreversible (NEM) sulfhydryl reagents in the presence or absence of saturating oleic acid. Cys^{117}–NEM ALBP exhibited a large decrease in binding affinity while Cys^1–NEM ALBP exhibited normal binding properties. Neither the modification of ALBP with NEM nor the addition of oleic acid had a significant effect on protein structure, as judged by circular dichroic analysis. These results suggest that Cys^{117} of ALBP resides in the ligand binding domain and that site-specific modification can be utilized to assess the conformational flexibility of the binding cavity.

Low molecular weight, intracellular fatty acid binding proteins have been isolated from a number of lipid-metabolizing tissues. Though the exact physiological function of these proteins is unknown, their tissue-specific expression, lipid binding properties, and cytosolic location indicate that these proteins mediate the uptake, solubilization, and intracellular metabolism of fatty acids. Fatty acid binding proteins (FABPs)[1] have been identified in several tissues to date, including liver (Ockner et al., 1982; Lowe et al., 1985), intestine (Alpers et al., 1984; Lowe et al., 1987), heart (Said & Schulz, 1984; Offner et al., 1986), myelin (Uyemura et al., 1984), and mammary gland (Böhmer et al., 1987). The adipose fatty acid binding protein has been purified and termed adipocyte lipid binding protein (ALBP) (Matarese & Bernlohr, 1988; Baxa et al., 1989).

The crystal structures of two fatty acid binding proteins with their bound ligands have been reported (Jones et al., 1988; Sacchettini et al., 1989a). Despite the fact that the rat I-FABP and bovine P2 proteins share only 27% sequence similarity, their α-carbon backbones are virtually superimposable. This finding leads to the consideration that despite a wide variance in amino acid relatedness all members of the multigene family are likely to adopt a similar multistranded antiparallel β-barrel structure common to many extra- and intracellular hydrophobic ligand binding proteins (Newcomer et al., 1984; Huber et al., 1987; Holden et al., 1987; Matarese et al., 1990). This structure has been referred to as the β-clam (Sacchettini et al., 1989a). The high-resolution structures of rat I-FABP and bovine P2 have revealed residues that line the internal fatty acid binding cavity. Sacchettini et al. (1989a,b) have identified amino acids of I-FABP whose side chains lie within 4.5 Å of the bound palmitate. These include a large number of hydrophobic residues and Arg^{106} which interacts with the fatty acid carboxylate. $Tyrosine^{117}$ was also found to lie near the midregion (C_7–C_{11}) of the acyl chain. Jones et al. (1988) have indicated that in the P2 structure the amino acid side chains of Cys^{117}, Arg^{106}, and Arg^{126} are also oriented internally, toward the bound ligand. The ligand binding site of both proteins is essentially a water-filled cavity lined with hydrophobic amino acid side chains. Buried deeply within the domain is an arginine residue which coordinates the fatty acid carboxylate. This suggests that binding energy is contributed from a combination of hydrophobic and electrostatic interactions. The observation that position 117 of ALBP, like P2, is a cysteinyl residue predicts that sulfhydryl-directed reagents may be utilized as experimental tools to examine the structure and flexibility of the binding domain. The present study was undertaken to determine if Cys^{117} resides within the ligand binding domain of ALBP and, if so, whether modification of this residue could be used to probe the structure of the binding cavity.

Experimental Procedures

Materials. [9,10-^{3}H]Oleic acid (5 Ci/mmol) was obtained from Amersham, and *N*-[*ethyl*-1-^{14}C]maleimide (40 mCi/mmol) was from DuPont–New England Nuclear Corp. Unlabeled oleic acid was purchased from Nu Check Prep, Inc. (Elysian, MN). Unlabeled *N*-ethylmaleimide, methyl methanethiosulfonate, 5,5′-dithiobis(2-nitrobenzoic acid), *p*-(chloromercuri)benzenesulfonic acid, and Sephadex G-75 were obtained from Sigma. Lipidex-1000 [(hydroxyalkoxy-

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[1] Abbreviations: ALBP, adipocyte lipid binding protein; FABP, fatty acid binding protein; I-FABP, intestinal FABP; MMTS, methyl methanethiosulfonate; NEM, *N*-ethylmaleimide; PCMBS, *p*-(chloromercuri)benzenesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, thionitrobenzoate anion; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; TPCK, L-(tosylamino)-2-phenylethyl chloromethyl ketone; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; CD, circular dichroism.

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propyl)dextran] was from Packard, and trypsin [L-(tosylamino)-2-phenylethyl chloromethyl ketone treated] was purchased from Cooper Biomedical. The prepacked Excellulose GF-5 desalting column was from Pierce, and 2,3-butanedione was obtained from Aldrich. All other supplies were reagent grade.

Murine ALBP Preparation. ALBP was purified from cultured murine 3T3-L1 adipocytes according to the method of Matarese and Bernlohr (1988). Protein concentration was determined spectrophotometrically by using a molar extinction coefficient at 280 nm of 1.55×10^4 M^{-1} cm^{-1}.

Oleate Binding. Fatty acid binding was measured by utilizing the liposome delivery assay of Brecher et al. (1984) as previously described (Matarese & Bernlohr, 1988). Binding data were analyzed by calculating a $K_{0.5}$ using $[\text{ligand}]_{\text{free}} = [\text{ligand}]_{\text{total}} - [\text{ligand}]_{\text{bound}}$ as described by Baxa et al. (1989).

Sulfhydryl Modification of ALBP. For sulfhydryl modification, ALBP (2–5 μM) in 50 mM Tris-HCl, pH 8.0, and 100 mM NaCl was reacted with 40 μM DTNB in the presence of 0.1 mM EDTA. Sulfhydryl titration was monitored spectrophotometrically, calculating the extent of modification from the difference in absorbance at 412 nm (ϵ = 13 600; Ellman, 1959) between a protein-containing sample and a buffer blank.

Modification with other sulfhydryl reagents was performed by adding aliquots of 10 mM stock solutions of MMTS, NEM, or PCMBS in 10-fold molar excess to ALBP. After a 45-min incubation at 25 °C, unreacted reagent was removed by dialysis into liposome buffer (10 mM Tris-HCl, pH 7.4, and 100 mM NaCl). All manipulations with PCMBS were done in dim light. In each case, thiol modification occurred to greater than 95% as determined by subsequent spectrophotometric titration with DTNB. To demonstrate that DTNB modification of ALBP was reversible, doubly modified ALBP was denatured with 8 M urea and treated with 10 mM DTT. The reduced protein was dialyzed first against 8 M urea in liposome buffer and then against liposome buffer alone to renature the protein. The recovery of active protein by urea denaturation and renaturation was greater than 80%.

Arginine Modification of ALBP. ALBP (40 μM) in 50 mM Hepes, 50 mM borate, and 100 mM NaCl (final pH 8.3) was reacted with 10 mM 2,3-butanedione at 25 °C. After 2 h, the reaction mixture was applied to a 5-mL Excellulose GF-5 desalting column. Protein-containing fractions were pooled and dialyzed against liposome buffer containing 50 mM borate and assayed for fatty acid binding as above. Native ALBP untreated with butanedione was handled similarly as a control. Arginine modification and all subsequent manipulations with native and modified protein were done in dim light.

Circular Dichroism. The circular dichroic spectra of ALBP samples were determined at 25 °C using a Jasco J-41C spectropolarimeter calibrated with *d*-10-camphorsulfonic acid. Spectra were recorded at a protein concentration of 5 μM in 10 mM Tris-HCl, pH 7.4, and 100 mM NaCl using a cell path length of 5.0 mm. Circular dichroism results were reported in terms of $[\theta]$, the mean residue ellipticity, calculated as described by Adler et al. (1973).

Selective NEM Modification of ALBP. The cysteinyl residues of ALBP were selectively reacted with radiolabeled NEM as follows. To modify both sulfhydryls, ALBP (20 μM) was incubated with a 10-fold molar excess of [^{14}C]NEM (3.5 mCi/mmol) for 45 min at 25 °C. The reaction was stopped by the addition of excess β-mercaptoethanol, and unreacted reagent was removed by dialysis into 50 mM acetate at pH 5. The radiolabeled protein was then divided into two aliquots; one aliquot was dialyzed into liposome buffer for ligand binding analysis, and the second aliquot was dialyzed into 50 mM Tris-HCl, pH 7.4, for enzymatic digestion.

To modify the cysteinyl residue not protected by bound fatty acid, ALBP (20 μM) was reacted with a 10-fold molar excess of [^{14}C]NEM (3.5 mCi/mmol) in the presence of 100 μM [^{3}H]oleate (2.6 mCi/mmol). After 45 min, the reaction was stopped with β-mercaptoethanol and dialyzed into 50 mM acetate, pH 5. The ALBP was delipidated by chromatography on Lipidex-1000 resin (0.5 × 1.5 cm) at 25 °C. Greater than 95% of the oleate was removed as judged by the loss of radioactivity from the protein. The modified sulfhydryl was identified by tryptic peptide analysis, and the protein was analyzed for fatty acid binding as above.

To selectively modify the ligand-protected cysteinyl residue, ALBP (40 μM) was first titrated with 200 μM DTNB in the presence of 200 μM [^{3}H]oleate (3.1 mCi/mmol). The 1-mL reaction mixture was immediately applied to a Sephadex G-75 column (1.0 × 50 cm) to separate modified ALBP from unreacted DTNB and the thionitrobenzoate anion. Bound oleate was removed by subsequent delipidation on Lipidex-1000 resin. The newly accessible thiol was then modified by reaction with a 10-fold molar excess of [^{14}C]NEM (1.8 mCi/mmol) for 45 min at room temperature. After dialysis into 50 mM Tris-HCl, pH 8, and 100 mM NaCl buffer, the TNB adduct was released by reduction with 1 mM DTT. After further dialysis to remove contaminants, the NEM-modified cysteinyl residue was identified as above, and the protein was analyzed for its ligand binding affinity. Unmodified ALBP was treated similarly as a control. ALBP was incubated with 100 μM [^{3}H]oleate, delipidated, and assayed for fatty acid binding.

Peptide Isolation. For digestion with trypsin, TPCK-treated bovine trypsin was added in two aliquots to a final concentration of 2% of the mass of ALBP, and the sample was incubated for 48 h at room temperature in 50 mM Tris-HCl, pH 7.4, and 11.5 mM $CaCl_2$. Tryptic peptides were isolated as described by Matarese and Bernlohr (1988). Peptide sequencing was performed with an Applied Biosystems 477A pulse-liquid-phase protein microsequencer, and amino acid analysis was performed with a Beckman 6300 amino acid analyzer (Microchemical Facility of Human Genetics Institute, University of Minnesota).

Results and Discussion

On the basis of the rat I-FABP and myelin P2 structures, Cys^{117} of ALBP was predicted to reside within the ligand binding cavity. The possibility existed therefore that modification of this cysteinyl residue may serve as a probe of the structure and dynamics of the binding domain. To test this, ALBP was treated with DTNB to modify the two reduced cysteinyl residues and assayed for its fatty acid binding activity using the liposome assay of Brecher et al. (1984). Native unmodified ALBP exhibited saturable, stoichiometric association with oleic acid, with 1 mol of oleate bound per mole of ALBP. Half-maximal saturation occurred at approximately 75 μM oleate. ALBP modified with DTNB showed a significant decrease in the apparent binding affinity, with an estimated $K_{0.5}$ of 440 μM (Figure 1). Because of the instability of liposomes prepared with very high levels of fatty acid (greater than 6.5 mM), saturable binding could not be demonstrated, and so the true $K_{0.5}$ value for DTNB-modified ALBP may be much higher. In fact, the binding observed in DTNB-modified ALBP may represent partial association with the ligand binding site or weak interaction with other sites on the protein such that the true effects of DTNB may be quite large. However, it is clear that DTNB modification of ALBP

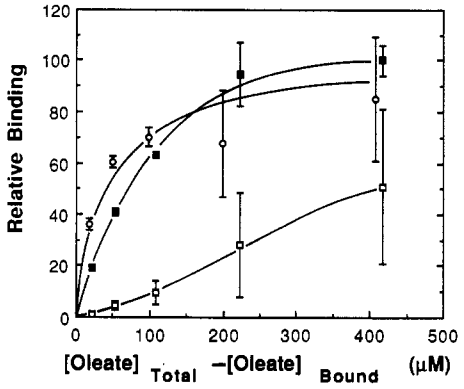
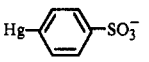
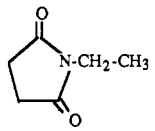
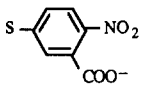


FIGURE 1: Oleate binding of DTNB-treated ALBP. Liposomes containing varying concentrations of [^{3}H]oleate were incubated with unmodified ALBP (■), DTNB-treated ALBP (□), or DTNB-treated ALBP reduced with DTT (○) for 60 min at 25 °C and then pelleted by centrifugation at 400000*g*. Data were normalized to account for variability in stoichiometries of ligand binding between protein preparations which ranges from 0.7 to 1.1 mol of oleate bound per mole of ALBP. Error bars represent the sum of one standard deviation from the averages of radioactivity in the protein-containing samples plus that from buffer blanks.

Table I: Effect of Sulfhydryl Modification on Fatty Acid Binding

Sulfhydryl Reagent	ALBP-S-R, where R =	$K_{0.5}$ (μM)
----	H	75
MMTS	S-CH_3	80
PCMBS		180
NEM		400
DTNB		440

greatly decreased the binding affinity for oleic acid. Reduction of DTNB-modified ALBP with DTT to liberate the TNB adduct restored normal binding of oleic acid, demonstrating the reversibility of the process.

In native ALBP, both Cys^1 and Cys^{117} are reduced and react readily with DTNB within 3–4 min. To determine whether the effect of DTNB on ligand binding was due to disruption of an electrostatic or hydrogen-bonding interaction or was the result of steric hindrance, ALBP was reacted with a variety of thiol-directed reagents. The sulfhydryl reagents used to modify ALBP ranged from those that incorporated small, neutral molecules to those that introduced bulky charged adducts into the protein; results are shown in Table I. Modification of ALBP with the bulky uncharged NEM to form a thiol ether hindered ligand binding in a fashion similar to that following modification with DTNB. However, when the thiols were coupled with the small, non-hydrogen-bonding methanethiol group from MMTS, the protein exhibited normal binding with a $K_{0.5}$ of 80 μM. Formation of a mercaptide with the organomercurical PCMBS appeared to inhibit ligand binding, but to a lesser extent than reaction with either NEM or DTNB. The normal binding properties of ALBP modified

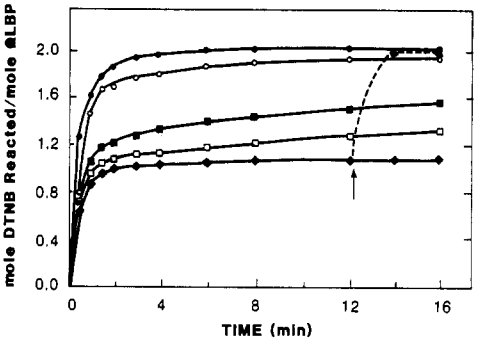


FIGURE 2: Effect of oleate concentration on sulfhydryl accessibility. ALBP (5 μM) in 50 mM Tris-HCl, pH 8, 100 mM NaCl, and 0.1 mM EDTA was incubated with varying concentrations of oleate and subsequently treated with 40 μM DTNB. Concentrations were 0 (●), 1 (○), 5 (■), 10 (□), and 50 μM (♦) oleate. Arrow: Addition of SDS to 1% final concentration to the 50 μM sample.

with MMTS indicated that cysteinyl residues were not involved in electrostatic or hydrogen-bonding interactions. Moreover, only small adducts can be readily accommodated in the ligand binding cavity, as the incorporation of larger reagents had dramatic inhibitory effects on binding affinity.

Oleate Protection of a Single Cysteinyl Residue. Modification of sulfhydryl groups with bulky adducts specifically inhibited ligand binding. That being so, the presence of a bound fatty acid would be expected to protect the amino acids from chemical reaction with bulky thiol reagents. When ALBP was titrated with DTNB in the absence of bound oleate, both sulfhydryls readily reacted within 3–4 min (Figure 2). In contrast, titration of ALBP with DTNB in the presence of saturating oleate yielded only a single cysteinyl residue available for modification. Upon denaturation of ALBP with SDS (to 1%) to liberate the bound ligand, the second cysteine became accessible. The availability of the protected group increased with decreasing concentrations of oleate. Protection of a single cysteinyl residue by oleic acid occurred in a concentration-dependent fashion, indicating that one cysteine resides within the ligand binding domain. The concentration of oleic acid necessary for half-maximal protection (5 μM) was identical with the concentration of oleic acid necessary for half-maximal binding when assayed by the decrease in intrinsic tryptophan fluorescence (Armstrong et al., 1990). In fluorescence assays, the ligand was delivered as an aqueous dispersion or from an alcoholic stock solution, and a half-maximal value of 2–4 μM was observed. However, when the liposome assay (Brecher et al., 1984) was utilized, a half-maximal value of 75 μM was obtained (Figure 1). Dissociation constants are independent of the method utilized, unlike the half-maximal ($K_{0.5}$) values, which are assay dependent. In the oleate protection experiments, the lipid was added from methanol, such that one would expect the concentration of ligand necessary to prevent thiol modification would be similar to the $K_{0.5}$ for binding using the fluorescence titration assay.

The inability to modify a sulfhydryl group in the presence of saturating oleate suggested that the binding domain is sufficiently occupied and inflexible as to prevent diffusion of DTNB into the cavity. To examine if less bulky thiol reagents could modify holo-ALBP, MMTS was employed. When ALBP was modified with the small uncharged reagent MMTS in the presence of saturating oleate, approximately 1.5 mol of MMTS reacted per mole of ALBP (data not shown). This would indicate that although the binding cavity is inaccessible to bulky reagents when a lipid is bound, there is enough flexibility to allow small sulfhydryl reagents to diffuse into the binding domain.

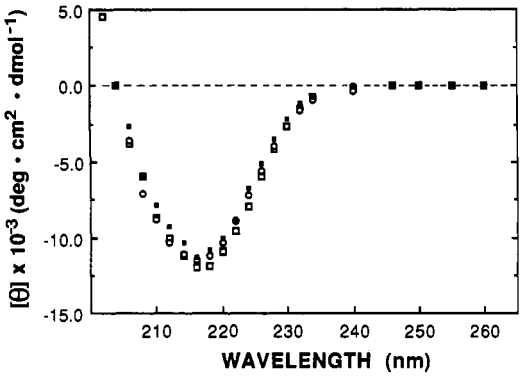


FIGURE 3: Circular dichroic spectra of native and modified ALBP. CD spectra of native ALBP (□), NEM-modified ALBP (○), and ALBP in the presence of saturating oleate (■) were taken at 25 °C. Protein concentration was 5 μM in 10 mM Tris-HCl, pH 7.4, and 100 mM NaCl.

An alternate possibility for our results is that modification of ALBP with sulfhydryl reagents induced a structural change in ALBP which hindered ligand binding and, reciprocally, ligand binding altered ALBP sufficiently to eliminate the modification of a single cysteinyl residue. To address these possibilities, we conducted circular dichroic analysis of ALBP. The CD spectrum of ALBP is shown in Figure 3. Consistent with the predicted β-barrel of ALBP based upon its similarity to I-FABP and P2 proteins, ALBP exhibited a large negative ellipticity from 235 to 204 nm with a minimum at 217 nm indicative of a high degree of β-structure. From the data in Figure 3, we calculate ALBP possesses 42–58% β-structure, dependent upon the algorithm utilized (Greenfield & Fasman, 1969; Chen et al., 1972). This result is in good agreement with the reported values from the CD spectra of other family members (Fournier et al., 1983; Offner et al., 1986) but is somewhat lower than that obtained from the crystal structure (Jones et al., 1988). The addition of saturating oleic acid to ALBP did not significantly alter the CD spectrum. Similarly, the NEM-treated ALBP CD spectrum was indistinguishable from that of native ALBP. Taken together, these results indicate that at the resolution of circular dichroism we do not detect significant structural changes in ALBP upon either ligand association or sulfhydryl modification. However, we cannot rule out the possibility that subtle changes unseen by circular dichroism occur as a consequence of these treatments. Given the proximity of position 117 of I-FABP and P2 to the bound ligand, we consider the simplest explanation for our results is that cysteine modification sterically hinders ligand association.

Identification of a Cysteinyl Residue in the Binding Domain through Selective NEM Modification. The experiments described above indicated a single cysteinyl group was associated with the fatty acid binding domain. Our assumption, based upon the P2 crystal structure, was that Cys^{117} was the residue of interest. To examine this issue, the sulfhydryl groups of ALBP were selectively modified utilizing a combination of irreversible (NEM) and reversible (DTNB) reagents in the presence or absence of a bound fatty acid.

To identify which cysteine residue was chemically modified by each procedure, tryptic mapping was employed in conjunction with sequence and compositional analysis. [^{14}C]-NEM-modified ALBP samples were digested with trypsin and fractionated by reverse-phase HPLC on an Altex C-18 column. Elution profiles are shown in Figure 4. As expected, two major radioactive peaks were detected from the peptide map of [^{14}C]NEM-modified ALBP (Figure 4A). Peak 1, which eluted at 83 min, was determined by direct sequencing to be

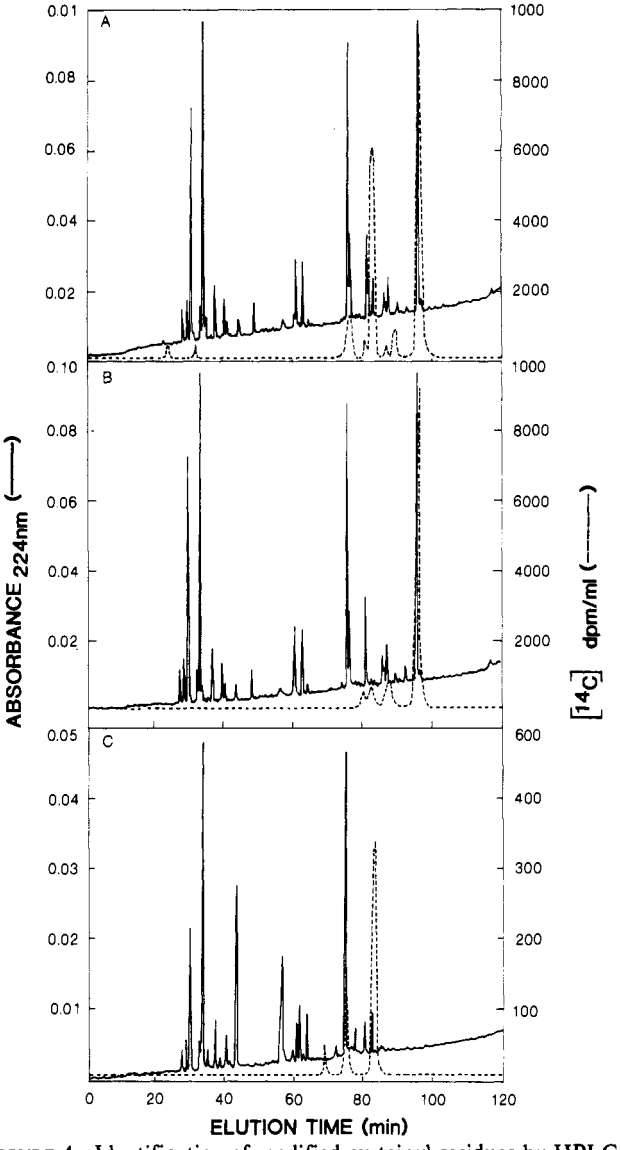


FIGURE 4: Identification of modified cysteinyl residues by HPLC. ALBP forms modified with [^{14}C]NEM were digested with trypsin for 48 h. Peptides were separated on a C-18 Altex reverse-phase column using a linear gradient of 0–71% solvent B (70% acetonitrile in 0.01 M TFA) over 150 min. Peaks 1 and 2 correspond to those peptides eluting at 83 and 95 min, respectively. (Panel A) ALBP modified on both cysteinyl residues; (panel B) ALBP modified on Cys^{1}; (panel C) ALBP modified on Cys^{117}.

a single peptide with the sequence Leu-Val-Val-Glu-CysX-Val-Met-Lys (Table II). This sequence corresponded exactly to that encompassing Cys^{117}. Peak 2, eluting at 95 min, was blocked to sequencing and therefore was identified by compositional analysis. Fast atom bombardment mass spectrometry of the amino-terminal tryptic peptide of ALBP had demonstrated previously that the initiating methionyl residue had been removed and that the cysteinyl amino terminus of the mature protein was N^{α}-acetylated (V. Matarese and D. A. Bernlohr, unpublished results). The composition of peak 2 showed equivalent molar amounts of alanine, aspartic acid, glycine, lysine, phenylalanine, threonine, valine, and a single modified amino acid identified as PTH-*S*-succinylcysteine (Table II). This composition corresponded to the amino-terminal tryptic peptide (Matarese & Bernlohr, 1988), tryptophan having been destroyed upon acid hydrolysis. The recovery of radioactivity in peaks 1 and 2 was greater than 70%.

To determine which sulfhydryl group was accessible to [^{14}C]NEM in the presence of bound ligand, the tryptic map

Table II: Sequence Data of Tryptic Peptides

(A) Automated Edman Degradation of Peak 1		
cycle	amino acid	yield (pmol)
1	Leu	156
2	Val	157
3	Val	157
4	Glu	72
5	Cys-X[a]	33
6	Val	166
7	Met	133
8	Lys	40

(B) Amino Acid Composition of Peak 2		
amino acid	yield (pmol)	residues[c]
Cys	377[b]	0.82
Asp	397	0.86
Thr	326	0.71
Gly	578	1.26
Ala	468	1.01
Val	460	1.00
Phe	354	0.77
Lys	378	0.82

[a] Identified as PTH-Cys-NEM adduct eluting at 29.2 min and quantitated by assuming an extinction equivalent to that of methionine. [b] Quantitated as sum of PTH-cysteic acid and PTH-*S*-succinylcysteine. [c] Values refer to number of residues normalized to yield of valine.

shown in Figure 4B was obtained. A single radioactive peptide corresponding to peak 2 was obtained which eluted at 95 min. The yield of radioactivity was approximately 60%. Lost from the elution profile was the peak at 83 min corresponding to a peptide containing a reduced thiol. In our hands, peptides with unmodified cysteines were recovered in poor yield from the reverse-phase column. No new peaks indicative of a peptide containing oxidized sulfhydryl groups were observed. These results indicated that not only was peak 2 (containing Cys^1 modified by [^{14}C]NEM) retained, but peak 1 (containing a reduced Cys^{117} protected by the ligand) was lost.

To positively identify which cysteine was protected by oleic acid, ALBP incubated with oleate, modified with DTNB, delipidated, and subsequently treated with [^{14}C]NEM and DTT was subjected to tryptic fractionation. In this case, a single radioactive peptide eluted at 83 min. The sequence of this peptide was identical with that surrounding Cys^{117} (Table II). The recovery of radioactivity in peak 1 was approximately 70%. Again, the peptide corresponding to peak 2 which contains the amino-terminal cysteine as a free thiol was lost from the chromatogram, indicating that this was the residue originally modified with DTNB and then reduced to free the TNB adduct. These results demonstrate that ALBP had been prepared with specific modification at either Cys^1 and/or Cys^{117}. Moreover, oleic acid selectively protected Cys^{117} from modification.

Having demonstrated that oleic acid specifically blocked modification of Cys^{117}, we wanted to establish that modification of this amino acid alone would yield a fatty acid binding protein with markedly reduced affinity for oleic acid. When the four permutations of ALBP were assayed for their ability to bind lipid, unequivocal results were obtained (Figure 5). The Cys^1–SH Cys^{117}–SH and the Cys^1–NEM Cys^{117}–SH forms of ALBP exhibited normal oleic acid binding properties. The $K_{0.5}$ value for both proteins was about 65 μM. In contrast, the Cys^1–SH Cys^{117}–NEM and the Cys^1–NEM Cys^{117}–NEM forms showed significantly reduced binding affinity with $K_{0.5}$ values estimated to be about 430 μM. Modification of Cys^{117} solely, therefore, was sufficient to reduce the affinity of ALBP for fatty acids.

Our conclusion from these results is that Cys^{117} of ALBP resides in the fatty acid binding domain and that the addition

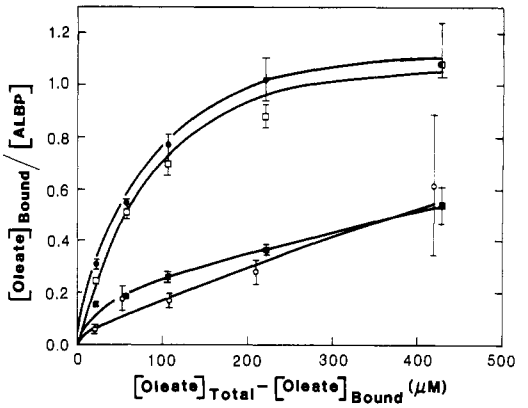


FIGURE 5: Oleate binding of ALBP modified with NEM. ALBP preparations Cys^1-SH Cys^{117}-SH (□), Cys^1–NEM Cys^{117}–NEM (■), Cys^1–NEM Cys^{117}–SH (●), and Cys^1–SH Cys^{117}–NEM (○) were incubated with liposomes containing various concentrations of [^{3}H]oleate for 60 min at 25 °C. The liposomes were then pelleted, and bound fatty acid was determined from the radioactivity in the supernatant of a protein-containing sample relative to a buffer blank.

of a bulky substituent on the thiol group sterically blocks fatty acid binding. This might suggest that the ligand binding cavity does not possess enough flexibility to allow for the binding of a fatty acid if the region around Cys^{117} is occupied. However, Storch et al. (1989) reported that the binding of anthroyloxy-substituted fatty acids to rat liver FABP and murine ALBP (Wootan et al., 1990) occurred with approximately equivalent affinity as do unmodified fatty acids. These results imply that the binding cavity does have enough flexibility to allow for the presence of bulky substituents on the lipid.

To reconcile these observations, we consider a model whereby the primary forces that contribute to overall binding energy are electrostatic. Sacchettini et al. (1989a,b) have suggested that a lipid enters the binding domain carboxylate first through a "portal" bounded by helix I and β-strands D and E. The fatty acid would migrate into the cavity, directed by ionic interactions with its carboxylate, until coordinated with Arg^{106} in a solvated complex described as a "fatty acid quintet". The lipid takes on a bent conformation with a slight left-handed helical twist. In this model, as applied to ALBP, the lipid would diffuse past a region in proximity to Cys^{117} and be coordinated with either Arg^{106} or Arg^{126}. If Cys^{117} is modified with a bulky group, the primary stabilizing forces, i.e., the formation of ionic interactions with Arg^{106}/Arg^{126}, cannot be formed effectively and hence would lead to weaker binding. In fact, studies on anthroyloxy-substituted fatty acid binding to ALBP indicated that the highest degree of steric constraint occurred in the middle region of the lipid, where Cys^{117} is predicted to reside (Wootan et al., 1990). In the case of anthroyloxy-labeled lipids, the carboxylate–Arg^{106}/Arg^{126} interactions are permitted, and, as such, the primary contributions to energetic stabilization are retained. Consistent with this hypothesis, Brecher et al. (1984) have reported that hepatic fatty acid binding protein has little affinity for fatty acid methyl esters, indicating that carboxylate interactions are of primary importance. Furthermore, ALBP has previously been shown to bind retinoic acid, but not retinol, with high affinity (Matarese & Bernlohr, 1988). This model predicts that arginine modification would disrupt the fatty acid binding characteristics of ALBP. To test this, apo-ALBP was modified with 2,3-butanedione and subsequently assayed for binding activity. Butanedione-modified ALBP exhibited a marked decrease in binding affinity for fatty acids (Figure 6). The weak binding of fatty acids was reminiscent of that observed with DTNB-modified ALBP, suggesting that nonspecific or

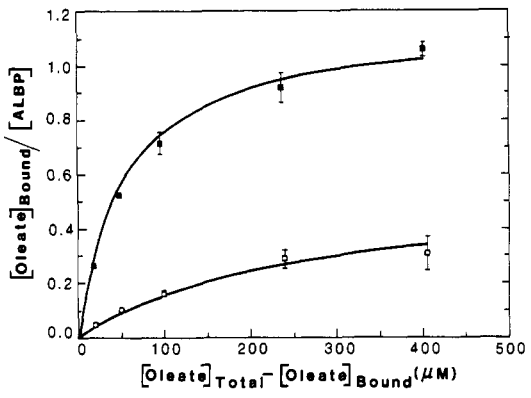


FIGURE 6: Oleate binding of 2,3-butanedione-treated ALBP. ALBP (40 μM) was treated with (□) or without (■) 10 mM 2,3-butanedione under dim light for 2 h and subsequently assayed for binding activity by using the liposome delivery assay.

purely hydrophobic association may be taking place. This inhibition of binding does not appear to be due to an alteration in the net charge of the protein, since complete modification of lysine residues of ALBP with acetic anhydride does not alter binding affinity (V. Matarese and D. A. Bernlohr, unpublished observations). Moreover, butanedione-modified ALBP exhibited a CD spectrum comparable to native ALBP treated under similar conditions (results not shown).

Our results indicate that the binding cavities of lipid binding proteins are flexible with the potential to accommodate a variety of ligands. However, in order to attain high-affinity binding, the ionic interactions with basic amino acids (i.e., Arg^{106}/Arg^{126}) must be established. Prevention of such interactions by sterically blocking the accessibility to these amino acids or by chemically modifying arginine residues leads to a drastic decrease in binding affinity. This result implies that the coordination of a basic residue within the hydrophobic cavity is the primary factor which influences binding. The hydrophobic contributions from side chain amino acids are of secondary importance to the electrostatic interaction of the fatty acid carboxylate with arginine residues. Further, our results suggest that the synthesis of fatty acid analogues with sulfhydryl-reactive groups positioned from C_7 to C_{11} could act as "active site" directed reagents with the potential to serve in situ as experimental tools to evaluate the metabolic function of ALBP.

Registry No. L-Cys, 52-90-4; L-Arg, 74-79-3; oleic acid, 112-80-1.

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