Biochemical and Crystallographic Analyses of a Portal Mutant of the Adipocyte Lipid-binding Protein*

(Received for publication, August 13, 1996, and in revised form, January 23, 1997)

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A number of crystallographic studies of the adipocyte lipid-binding protein have established that the fatty acid-binding site is within an internalized water-filled cavity. The same studies have also suggested the existence of a region physically distinct from the fatty acid-binding site which connects the cavity of the protein with the external solvent, hereafter referred to as the portal. In an attempt to examine the portal region, we have used site-directed mutagenesis to introduce the mutations V32D/F57H into the murine ALBP cDNA. Mutant protein has been isolated, crystallized, and its stability and binding properties studied by biochemical methods. As assessed by guanidine-HCl denaturation, the mutant form exhibited a slight overall destabilization relative to the wild-type protein under both acid and alkaline conditions. Accessibility to the cavity in both the mutant and wild-type proteins was observed by stopped-flow analysis of the modification of a cavity residue, Cys517, by the sulfhydryl reactive agent 5,5'-dithiobis(2-nitrobenzoic acid) at pH 8.5. Cys517 of V32D/F57H ALBP was modified 7-fold faster than the wild-type protein. The ligand binding properties of both the V32D/F57H mutant and wild-type proteins were analyzed using a fluorescent probe at pH 6.0 and 8.0. The apparent dissociation constants for 1-anilinonaphthalene-8-sulfonic acid were approximately 9–10-fold greater than the wild-type protein, independent of pH. In addition, there is a 6-fold increase in the $K_d$ for oleic acid for the portal mutant relative to the wild-type at pH 8.0. To study the effect of pH on the double mutant, it was crystallized and analyzed in two distinct space groups at pH 4.5 and 6.4. While in general the differences in the overall main chain conformations are negligible, changes were observed in the crystallographic structures near the site of the mutations. At both pH values, the mutant side chains are positioned somewhat differently than in wild-type protein. To ensure that the mutations had not altered ionic conditions near the binding site, the crystallographic coordinates were used to monitor the electrostatic potentials from the head group site to the positions near the portal region. The differences in the electrostatic potentials were small in all regions, and did not explain the differences in ligand affinity. We present these results within the context of fatty acid binding and suggest lipid association is more complex than that described within a single equilibrium event.

Lipids are essential molecules that serve a variety of roles in biological systems including formation of the primary structural components of membranes, serving as a source and store of metabolic energy, and as messengers for signal transduction. The intracellular trafficking of these poorly soluble molecules has been hypothesized to be mediated by a family of proteins known as the intracellular lipid-binding proteins (1). These proteins are characterized by their conserved gene structure, similarity of amino acid sequences, and the ability to bind lipophilic molecules such as fatty acids and retinoids (2, 3). The adipose specific member of the gene family is known as the adipocyte lipid-binding protein (ALBP or aP2) (4). This 14.6-kDa protein is found in great abundance in the adipocyte cytosol and has been shown to bind long chain fatty acids and fatty acid analogs with an affinity of 0.1–1.5 μM (4–8).

The crystal structure of ALBP, like all members of the ILBP family, is relatively easy to visualize (1) and a stereodiagram of the main chain is given in Fig. 1. The single polypeptide chain is folded such that the 10 antiparallel β-strands are hydrogen bonded to each other in a barrel-like fashion. Starting from the N-terminal, the strands have been referred to in numerous other publications as βA through βJ. The single exception to the inter-strand H-bonding appears between strands βD and βE. The distance between these two strands is too large to form normal β-sheet hydrogen bonds but the gap is filled with side chains maintaining the integrity of the β-barrel. The ends of the β-barrel are closed off by a helix-turn-helix segment at one end, and the last few residues of the N-terminal and side chains at the other. The helix-turn-helix is located between strands βA and βB.

ALBP and other family members have an unusual structural property. The binding site for fatty acids and hydrophobic ligands is an internalized cavity. This cavity is partially hidden

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* This work was supported in part by National Institutes of Health Grant GM 13925 (to L. B.) and National Science Foundation Grant MCB 9506088 (to D. A. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** Supported by a fellowship from the Arnold H. Johnson Fund.

§§ Supported by National Institutes of Health Training Grant GM 07232.

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from the external milieu in some iLBPs and is nearly totally hidden for ALBP. The internalized cavity is much larger than the ligand and is filled with ordered and presumably disordered water molecules. For most iLBPs, proteins, including ALBP, the ligand-protein stoichiometry is 1:1. The crystal structures of the apo and holo forms of ALBP are essentially the same, which suggests that a conformational change must accompany both the association and dissociation processes (9).

Members of the iLBP family have dissociation constants for their ligands in the micromolar to nanomolar range. The thermodynamics of the association reaction have been determined by titration calorimetry for ALBP binding to oleic and arachidonic acid (10), and by van’t Hoff data for ALBP and a number of other fatty acid-binding proteins (7). These studies reveal that the relatively high affinity of an iLBP for a hydrophobic ligand is influenced by enthalpic factors. This is in agreement with the crystal structures of the apo and holo forms of the protein which suggest that some of the binding energy is derived from the coulombic interactions between the ionized carboxylate of the fatty acid and a binding triad formed by the side chains of two arginines and a tyrosine which are present within the cavity.

Structural studies of ALBP in the presence and absence of various fatty acid ligands have also lead to the identification of a distinct region of the protein surface known as the portal, as first described by Xu et al. (9). The portal is a region physically distinct from the fatty acid binding cavity and is formed by helix αII and turns between βC-βD and βE-βF. One of the characteristics of the portal region is the relatively mobile side chain of Phe^57. The position of the side chain of Phe^57 is significantly different in the apo- and holo-protein. The side chain is near the hydrocarbon tail of the fatty acid and points toward the interior of the molecule in the holo-protein. In the apoprotein, the aromatic ring swings back and appears to block access to the cavity. These observations suggest the region around Phe^57 could be indicative of a favored connection between the internal binding site and the environment.

To address the functional role of Phe^57 and the portal region, we have used site-directed mutagenesis to introduce the mutations V32D/F57H into the murine ALBP cDNA. The mutant was designed to form a histidine-aspartic acid salt bridge across a region of the ALBP molecule thought to be the preferential pathway for ligand entry into the binding cavity. Furthermore, the region of ALBP mutated is distant from the internalized arginines and therefore would not be expected to interfere with the coulombic binding reaction. Our goal was to test the portal hypothesis by attempting to confer pH dependence to the binding characteristics.

In this report, biochemical characterization of the mutant protein including stability, ligand binding, and kinetic properties is described. To understand the conformational implications of the mutations, the crystal structure of the mutant form of ALBP is compared at two different pH values. Last, in an attempt to further understand how the mutation could affect the cavity, the conformational implications to the binding characteristics.

Mutagenesis—The V32D/F57H mutant was used in a sequential manner. PCR-mediated site-specific mutagenesis was used to construct the single V32D mutation in the murine ALBP cDNA. The plasmid pGSTALBP (5) was used as a template for two polymerase chain reactions using either the V32D oligo (5'-ACAAGGAAAGACCAGCCATG-3') and an oligo complimentary to the 3' end of the DNA (5'-TGGAT-GGATCTTCCATCCAGG-3') or an internal ALBP oligo (5'-GGTGGTTTTTATGAGTACTCT-3') and an oligo complimentary to the 5' end of the DNA (5'-AGGACGATCTCTCTCGAGG-3'). Two products were obtained, a 350-bp V32D product which corresponds to nucleotides 131–476 of the DNA and a 208-bp fragment corresponding to nucleotides 12–227. The PCR products were mixed, denatured at 95 °C for 10 min, and subsequently allowed to cool to room temperature over a 60-min period. The heteroduplex DNA was then used as the template for a second round of PCR utilizing the 5'- and 3'-oligonucleotides (above) that bracket the ALBP cDNA and which contain BamHI restriction sites to facilitate cloning. The 450-bp PCR product was subcloned into BamHI digested, dephosphorylated, vector 3Zf(9). Ligation products were transformed into Escherichia coli strain JM109 and recombinant plasmids were selected on the basis of blue/white color selection. Plasmids were recovered and V32D mutants were verified by double-stranded DNA sequencing.

The V32D/F57H double mutant was created by single-stranded mutagenesis (11). Plasmids were isolated and screened via restriction digestion with the enzyme DraI. Successfully mutagenized plasmids were then verified via DNA sequencing. By analogy with procedures, the individual F57H mutation was generated using single-stranded DNA prepared from wild-type cDNA.

To obtain large quantities of recombinant mutant proteins, the individual cDNAs were subcloned into the prokaryotic expression vector pJM100A. PCR was used to engineer novel XbaI restriction sites into cDNA flanking regions to facilitate subcloning. A 27-bp 5'-oligonucleotide (5'-GGTGTACACCATGGTGATCTTCTGTT-3') and a 25-bp 3'-oligonucleotide (5'-ATTCCCTCTAGATCCTCCATCCAGG-3') (Genosys Biotechnologies Inc.) were used to produce a 447-bp PCR product that could be directly subcloned. The PCR product was subcloned into XbaI-digested pJM100A. Recombinant plasmids were identified via diagnostic restriction enzyme analysis with HindIII, and sequenced to verify the integrity of the nucleotide sequence.

During the molecular manipulation of the various cDNAs, it became apparent that the use of the NcoI site at the 5' terminus of the cDNA would facilitate the subcloning procedures. The incorporation of the 5' NcoI site resulted in the conservation of the initiating methionine residue but not a subsequent mutation of cysteine 2 to a glycine residue. Previous work in our laboratory (12) had shown that E. coli expressed wild-type ALBP contains a post-translational modification of Cys^9 such that the sulfhydryl group becomes modified and is unreactive to group-specific reagents. As shown by crystallization studies, Cys^9 is found on pA, quite far from the ligand binding site or the portal. Consequently, we anticipated that the C2G mutation would have no effect on ALBP properties. To ensure that the C2G mutation had no adverse effects upon binding of ALBP to intrinsically tryptophan fluorescence was measured and compared with that of wild-type ALBP. The excitation and emission maxima of all forms were essentially identical to wild-type ALBP (results not shown) and concluded that the C2G mutation would have negligible effects upon ALBP structure and function. All protein described within this report contains the C2G mutation.

Expression and Purification of V32D/F57H ALBP—The V32D/F57H ALBP cDNA was transferred into E. coli JM 101 and protein expression induced by the addition of naldixic acid as described previously (12). Proteins were purified as described previously (9) except that cation exchange chromatography was used as the terminal step, replacing immobilized metal affinity chromatography. Concentrated protein solution was dialyzed into 50 mM sodium acetate, pH 5.2, and fractionated utilizing a Mono S HR 10/10 cation exchange column. Recombinant ALBP forms were eluted with a NaCl gradient to 500 mM salt and the single V32D/F57H mutant was verified by double-stranded DNA sequencing. By analogy with procedures, the individual F57H mutation was generated using single-stranded DNA prepared from wild-type cDNA.
dialyzed into either 50 mM MES, pH 5.5, or 50 mM Tris-HCl, pH 8.0, and then diluted to 0.5 μM in the appropriate buffer at 25 °C. The emission spectrum of each protein was recorded in the presence of increasing guanidine HCl with a Perkin-Elmer 650–10S fluorescence spectrophotometer with an excitation wavelength set to 285 nm. The emission was used to determine the concentration of denaturant at the midpoint for each point in the unfolding profile. The Gibbs free energy was determined spectrophotometrically and the amount of thionitrobenzoate released was calculated from the absorbance at 412 nm (ε = 13,600 cm⁻¹ M⁻¹). Protection assays were performed by mixing protein with 50 μM ligand (oleic acid or 1,8-ANS) for 3 min before the addition of DTNB. Stopped-flow analysis was carried out with an Applied Photophysics apparatus and data analyzed with the X suite of kinetic analysis programs. Data were fit to a single exponential equation which used a steady state value to mark the completion of the reaction.

**Sulfhydryl Modification**—Modification of Cys¹¹⁷ was carried out as described previously (13). Briefly, protein was added to a final concentration of 10 μM in 50 mM Tris-HCl, pH 8.5, 100 mM NaCl and reacted with 50 μM DTNB at room temperature. Sulfhydryl titration was monitored spectrophotometrically and the amount of thionitrobenzoate released was calculated from the absorbance at 412 nm (ε = 13,600 cm⁻¹ M⁻¹). Protection assays were performed by mixing protein with 50 μM ligand (oleic acid or 1,8-ANS) for 3 min before the addition of DTNB. Stopped-flow analysis was carried out with an Applied Photophysics apparatus and data analyzed with the X suite of kinetic analysis programs. Data were fit to a single exponential equation which used a steady state value to mark the completion of the reaction.

**Ligand Binding**—Ligand binding to V32D/F57H ALBP and wild-type ALBP was assessed using the fluorescent probe 1,8-ANS (14). The probe was dissolved in absolute ethanol and its concentration was determined spectrophotometrically (ε₂₈₅ = 8000 cm⁻¹ M⁻¹). Final ethanol concentrations were kept below 1% (v/v). Proteins were dialyzed into 50 mM NaPO₄ at either pH 6.0 or 8.0 and added in 1 μM aliquots to 0.5 μM 1,8-ANS. The samples were mixed for 1 min and their fluorescence was measured. The solutions were incubated in a thermostatted (25 °C) Perkin-Elmer 650–10S fluorescence spectrophotometer. All manipulations took place under dimmed lights. Relative fluorescence was plotted versus increasing protein concentration and Scatchard analysis was performed to determine binding parameters.

**Oleate Competition**—Oleic acid was used as a competitor for 1,8-ANS binding to either V32D/F57H ALBP or wild-type ALBP. Subsaturring concentrations of V32D/F57H ALBP (2.3 μM) or wild-type ALBP (0.6 μM) in 50 mM NaPO₄, pH 8.0, were mixed with 0.5 μM 1,8-ANS at 25 °C and the fluorescence signal was measured. Increasing concentrations of oleic acid (from a 10 mM stock in absolute ethanol) were added and the subsequent loss of fluorescence was observed. Relative fluorescence was then plotted versus increasing concentration of oleic acid and a polynomial equation was used to determine the amount of oleate at the midpoint of the competition (50% loss of initial fluorescence) and the apparent Kᵣ (14).

**Crystalization Trials**—The protein was equilibrated in 12.5 mM HEPES buffer, pH 7.5, and concentrated to 10 mg/ml. Crystalization screens were set up using the hanging drop/vapor diffusion method. A pH Phosphate scan was first tried similar to native ALBP crystallization conditions (9). These conditions yielded crystals at 2.4 Å (NH₄)₂SO₄, 50 mM NaPO₄, and 100 mM acetate, pH 4.5.

**X-ray Methods**—X-ray diffraction data was collected on a Siemens multiwire area detector, with CuKα radiation from a Rigaku RU-200 generator implemented with a graphite crystal monochromator. Data was collected with a crystal to detector distance of 12 cm, and a crystal rotation of 0.25° between frames. Data collection statistics are reported in Table I. Neither crystal form was isomorphous with previously reported crystal forms of native ALBP. The pH 4.5 form has the space group P4₃2₁₂, with one molecule per asymmetric unit, and cell dimensions of a = b = 56.4 Å, c = 80.5 Å. The pH 6.4 form belongs to the space group C222₁, also with one molecule per asymmetric unit, and cell dimensions of a = 79.5 Å, b = 97.3 Å, c = 50.7 Å.

**X-ray Refinement**—Phases were obtained using molecular replacement implemented in X-plor (17), with native ALBP used as the search model. The refinement was carried out in X-plor, initially using rigid body and positional refinement, and ending with subsequent rounds of b-factor/positional refinement using a bulk solvent correction. The mutated residues were modeled as alanines through the initial stages of refinement, and were fit to both |Fo| − |Fc| density contoured at 1σ, and |Fo| − |Fc| density contoured at 3σ. Data was added to the refinements in increments of 0.5 Å or less, starting with data from 8.0 Å to 3.5 Å. Waters were added to the model when the resolution of the data reached 2.5 Å in the case of the pH 4.5 form, or during final refinement of the pH 6.4 form. Waters had to meet the following criteria: 1) presence in 2 Fo−|Fc|; 2) spherical shape; and 3) within 2.5 to 3.5 Å of a proper hydrogen bond donor/acceptor. The R⁵⁰/R⁰ was monitored at all times to ensure proper fitting of the model (18). Final refinement statistics are presented in Table I. The coordinates at both pH values of the V32D/F57H are deposited in the Protein Data Bank with the accession codes 1AB0 and 1ACD.

**Electrostatics**—All electrostatic calculations were carried out in Delphi, part of the Biosym suite of programs for the study of protein structure. The parameters used in the calculations are listed in Table II. Electrostatic potentials were calculated using the crystal structures of native ALBP, the pH 4.5, and the pH 6.4 mutant forms. Potentials were also calculated using models based on the pH 4.5 form with His⁵⁷ in the three most common rotamer positions to determine the influence of His⁵⁷ position on the electrostatic potential. The models for this calculation were built using O (19). To assess the effect of the mutations on ligand binding, it was decided to measure the electrostatic potential near the arginine-tyrosine-binding site and at ligand atom positions throughout the cavity using crystallographic coordinates previously determined for a ligand. The structure of native ALBP bound to hexadecane sulfonic acid (HDS; Hid in the Protein Data Bank) was used to provide the reference ligand position (20). All structures were superimposed in O, then the coordinates of HDS were used to reference points in the three-dimensional electrostatic potential at each atom position of HDS. Similar calculations were also carried out on hypothetical models of the mutant forms R126L/Y128F and T125Q.

**RESULTS**

The V32D/F57H mutant was designed to test the hypothesis that fatty acids preferentially enter and exit the ALBP cavity via the portal region. Since the mutations involved surface residues, the change from hydrophobic to hydrophilic side...
chains was not expected to notably affect protein stability but would affect accessibility to the cavity. The residues which were mutagenized can be visualized in Fig. 1. Note that they are located on a helix, α-II (V32D), and a turn between β-strands C and D (F57H). The best model called for the creation of the mutations V32D/F57H which based upon distances and geometry, would create a salt bridge between Asp32 and His57 at a pH above the pKₐ of Asp32 and below that of His57.

To purify V32D/F57H ALBP, a combination of pH fractionation, gel filtration, and ion-exchange chromatography was utilized. Protein purity was estimated to be greater than 95% based on SDS-polyacrylamide gel electrophoresis and typical yields of protein were 10–20 mg/liter of ferment. The protein was soluble at a wide range of pH values and ionic strengths and stable when stored at −20 °C. The intrinsic tryptophan fluorescence of V32D/F57H ALBP was assessed and compared with wild-type ALBP as a measure of its folding. The fluorescence excitation and emission maxima for V32D/F57H ALBP were 285 and 332 nm, respectively, at both pH 5.5 and 8.0. These values are virtually identical with those obtained for wild-type ALBP, which indicated that the tryptophan residues found in V32D/F57H ALBP are most likely located in the same environment as the tryptophan residues within ALBP. Integrity of the tertiary structures was confirmed by crystallography as described below.

The stability of V32D/F57H ALBP at pH 5.5 and 8.0 was examined by observing the equilibrium unfolding of the protein in response to increasing concentrations of denaturant. The transition profiles for denaturation of both V32D/F57H ALBP and wild-type ALBP at pH 5.5 had essentially the same sigmoidal shape. The denaturation was reversible; dilution of denatured protein with the appropriate buffer resulted in a return to emission maximum of 334 nm for each protein (results not shown). The concentration of guanidine HCl at the midpoint of the folded/unfolded transition at pH 5.5 was 1.6 ± 0.02 M for native ALBP and 1.1 ± 0.04 M for the V32D/F57H mutant. Similar results were obtained at pH 8.0. Midpoints for native ALBP were 1.5 ± 0.05 and 1.2 ± 0.08 M for the double mutant. At each pH, V32D/F57H ALBP was denatured at a lower concentration of guanidine HCl than wild-type ALBP, indicating that the mutant form is somewhat destabilized relative to wild-type ALBP. Analyzing the denaturation curves suggests that the V32D/F57H mutant is destabilized by approximately 3.0 kcal/mol at pH 5.5 and 0.8 kcal/mol at pH 8.0.

Next, the rate of entry of ligands into the cavity of the portal mutant was compared with the native protein. To do this, stopped-flow kinetic analysis of the modification of Cys117 by DTNB was carried out. Previous work from our laboratory had shown that the side chain of Cys117 is located within the ligand binding cavity of ALBP (22), but nonetheless could be covalently modified by the thiol reactive agent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (13). DTNB is itself large and bulky, and cysteine modification would be expected to be dependent on the accessibility of the reagent to Cys117. Fatty acids protect Cys-117 from modification and conversely, Cys117 modification blocks fatty acid binding (13). Crystal structure analysis has revealed that the side chain of Cys117 resides within 4.5 Å of the second, third, and fourth methylene carbons of the bound acyl chain in the holo-protein (22).

The modification of both mutant and wild-type protein was monitored by the release of the thionitrobenzoate anion as a function of time and the results are shown in Fig. 2. The progress curves were fit to a rate equation which described the reaction as a single exponential function. Rate constants of 0.100 and 0.720 s⁻¹ were obtained for wild-type ALBP and V32D/F57H ALBP, respectively, indicating that the Cys117 site in V32D/F57H ALBP is modified at a rate which is 7-fold faster than the wild-type protein.

In addition to examining rate effects, changes in binding affinity were assessed using the fluorescent probe 1,8-ANS. Recently, we have developed a sensitive fatty acid binding assay for lipid-binding proteins using 1,8-ANS fluorescence (14). The assay provides a large signal to noise ratio as the fluorescence enhancement of 1,8-ANS upon binding to ALBP is substantial, approaching values that are greater than 100-fold. Therefore, we used the reagent 1,8-ANS to analyze the effects of pH upon V32D/F57H ALBP’s ligand binding affinities. In addition, we evaluated the effect of the individual mutations, V32D and F57H, on 1,8-ANS binding.

The affinities of both V32D/F57H ALBP or wild-type ALBP for 1,8-ANS at pH 6.0 and pH 8.0 are given in Table III. The results demonstrate that the V32D/F57H mutant protein binds 1,8-ANS with less affinity than the wild-type protein at both pH values. Scatchard analysis of the 1,8-ANS binding iso-
Access to an Internalized Binding Site in ALBP

Ligand fluorescence was characterized by observing the fluorescence spectrum of an equimolar mixture of protein and 1,8-ANS (0.5 μM). Maximum excitation and emission wavelengths are displayed in units of nanometers (nm ± 2) and represent the mean of at least three independent experiments.

| Protein       | pH 6.0 | pH 8.0 |
|---------------|--------|--------|
|               | Excitation | Emission | Excitation | Emission |
| Native ALBP   | 367    | 465    | 367     | 465     |
| V32D          | 369    | 469    | 370     | 468     |
| F57H          | 371    | 470    | 371     | 469     |
| V32D/F57H     | 369    | 472    | 372     | 471     |

**TABLE V**

A comparison of the V32D/F57H crystal structures with native ALBP

Co-RMSD, as calculated by Midas (33).

| Holo-ALBP     | Distance difference in Å |
|---------------|--------------------------|
| V32D/F57H pH 4.5 | 0.34                     |
| V32D/F57H pH 6.4 | 0.62                     |

**TABLE III**

Binding affinities of ALBP forms for 1,8-ANS and oleic acid

1,8-ANS binding assays using 0.5 μM ALBP were performed as described and the apparent $K_i$ determined from Scatchard analysis of the data. The apparent $K_i$ for oleic acid was determined by displacement assay as described (14). All values reported are the means and standard deviations from three to five independent experiments.

| Protein form | pH 6.0 | $K_i$ for 1,8-ANS (μM) | $K_i$ for oleic acid (μM) |
|--------------|--------|-----------------------|--------------------------|
| Native ALBP  | 6.0    | 0.47 ± 0.01           | ND*                      |
|              | 8.0    | 0.74 ± 0.05           | 0.18 ± 0.04              |
| V32D         | 6.0    | 1.27 ± 0.05           | ND*                      |
|              | 8.0    | 1.33 ± 0.05           | 6.14 ± 0.13              |
| F57H         | 6.0    | 1.22 ± 0.01           | ND*                      |
|              | 8.0    | 1.45 ± 0.05           | 0.51 ± 0.03              |
| V32D/F57H    | 6.0    | 4.3 ± 1.3             | ND*                      |
|              | 8.0    | 7.1 ± 0.5             | 1.03 ± 0.1               |

* ND, not determined.
tal lattice contacts were studied for both mutant forms and the native form of ALBP. Symmetry mates of the structures were generated, and the residues around the mutated areas were examined with the program LIGPLOT (24). Native ALBP, in space group C222\(_1\), shows no symmetry related interactions for the region around residue 32, but shows a fairly large hydrogen bond network between the region around residue 57 and a symmetry related molecule. The mutant structure at pH 4.5, in space group P4\(_{1}2_12_1\), shows no symmetry-related interactions at either location, while the mutant structure at pH 6.4, in space group C222\(_1\), shows one hydrogen bond between Asn 59 and a symmetry related Glu54.

Because of the known differences in chemical properties, the refined models were studied carefully to see if the crystal structures could explain the changes. The first question is the overall change in stability. Since the conformations of the mutant are identical except for the new side chains, the temperature factors for the atoms in each structure were examined. They are plotted in Fig. 3. Clear differences between the mutant and wild-type protein are visible but they are mainly in magnitude. As can be seen in Fig. 3, the region containing Val/Asp\(^{32}\) has the highest temperature factors in both the wild-type and mutant structures. In the double mutant at both pH values, the temperature factors in this part of the structure are elevated. The second highest segment of b-factors appears at the loop connecting \(\beta C\) to \(\beta D\). Again they are elevated at both pH values in the V32D/F57H mutant. Although one could justify these increases in many ways, the most straightforward explanation is that the mutations have localized effects on the conformation to produce more disorder. This shows up in the electron density in the regions near Asp\(^{32}\) and His\(^{57}\), which is poor at both pH values.

Despite the uncertainty in the positioning of Asp\(^{32}\) and His\(^{57}\), the final refined positions were examined to check their interactions. Strong ionic interaction via the ion pair, Asp\(^{32}\)-His\(^{57}\), was not observed in the mutant crystal structures at either pH. In fact at pH 4.5, there appears to be a local displacement of the \(C_{\alpha}\) of His\(^{57}\) away from Asp\(^{32}\), pushing the side chain further away from the region thought to be the site of preferred ligand entry. This displacement is approximately 3.5 Å away from the conformation adopted by Phe\(^{57}\) in the native crystal structure. The orientation of the mutated residues at both pH values can be seen more clearly in Fig. 4. In the pH 4.5 structure, the temperature factor for Asp\(^{32}\) is very high and it was assumed that it is pointing into the solvent as is illustrated in Fig. 4.

At pH 6.4, regions near the mutational sites have somewhat lower temperature factors. As is also shown in Fig. 4, His\(^{57}\) refines to a position somewhat closer to Asp\(^{32}\). However, both mutated residues would be closer to the methyl end of the hydrophobic chain belonging to a bound fatty acid. This then could be one of several explanations for the observed reduction in binding affinity. The mutant conformation puts two hydrophilic residues at the portal and because of the positions they have adopted, they would be relatively close to the methylene end of a bound fatty acid. The crystal structures show no evidence that a strong polar interaction forms at either pH between the mutated residues as was originally designed.

Two other structural features related to ligand affinity were then examined in both the pH 4.5 and 6.4 crystal structures. The first feature is the bound water molecules within the cavity; the second was the electrostatic potential in the cavity at the normal positions of ligand atoms. The latter was done by homologous positioning of hexadecanesulfonic acid at the binding site in the V32D/F57H mutant structure. The position of water molecules is done by comparing those refined positions from the crystallographic studies of the mutant protein. Previously, a network of 10 crystallographically conserved waters had been identified within the ALBP binding cavity (20). Comparison of these waters with the higher resolution mutant form, pH 4.5, shows that most appear to be conserved. Seven of the core cavity waters previously reported are again observed in the electron density maps, as well as other surface waters in the \(\beta D-\beta E\) turn which appear necessary for proper folding. Cautious comparisons of the water sites is necessary since the crystal structures were not all obtained with x-ray data to the same resolution. The native ALBP crystal structure was solved to 1.6-Å resolution, while the pH 4.5 form of the V32D/F57H was obtained at 1.9 Å.

At pH 6.4, only 2.7 Å data was used. The water molecule which is shown to assist in ligand binding by bridging Arg\(^{106}\) and the ligand is not seen in either apo-mutant structure, having presumably displaced into bulk solvent. Nonetheless, the seven waters were placed in homologous sites in the mutant structures. At least with the present structural data on the V32D/F57H mutant, major perturbations of the water structure within the binding cavity seems not to have occurred.

With no obvious differences in the structure of the binding cavity or the bound waters, another possible explanation for the reduction in ligand binding affinity was that it was the result of local charge changes. To test whether or not a ligand would sense the charge differences between the V32D/F57H mutant and wild-type protein, the electrostatic potential was
monitored at positions in the binding cavity where ligand atoms are normally located. To make these calculations, the crystallographic coordinates of native ALBP and the V32D/F57H mutant structures and modeled coordinates of T125Q and R126L/Y128F were used. Positions marking the location of atoms belonging to a fatty acid ligand correspond to those of hexadecanesulfonic acid. The R126L/Y128F mutant of ALBP is known to have a marked decrease in affinity for fatty acids (20), and the T125Q mutant has been shown to have a slightly increased ligand affinity.

The results of the electrostatic calculations are shown in Fig. 5. Note that the potential at X0 on the curve marked “native” in Fig. 5 corresponds to that normally experienced by the carboxylate of bound fatty acid in the wild-type or T125Q ALBP. Not surprisingly, the mutant with known decreased affinity for fatty acid, R126L/Y128F, showed a marked decrease in positive electrostatic potential near the carboxylate group. When the electrostatic potential was calculated for the V32D/F57H form using the coordinates from both pH studies, the electrostatic potential was slightly higher than native ALBP near the carboxyl binding position, and lower near the proposed entrance. To assure the higher potential was not an effect of the rotamer selection of His57, potentials were calculated with His57 in the three most favored rotamer positions for histidine. While the potentials differed slightly (data not shown), they were all still above that of native. Therefore, the electrostatic calculations suggest that the decrease in binding affinity of V32D/F57H ALBP for fatty acids is not due to any general electrostatic effects near the carboxyl binding position.

**DISCUSSION**

The earlier analysis of the crystal structure of apo-ALBP has revealed that the interior ligand binding site is poorly accessible to the external milieu. Close examination of the oleate-ALBP crystal structure suggested that a small opening might exist in a region bounded by helix α-II and the turns connecting strands βC-βD and βE-βF as defined in Figs. 1 and 4. We and others have called this region the ligand binding portal. Important to this location is the residue Phe57, which is disordered in some crystal structures and may undergo a conformational change upon binding fatty acid. To examine the portal region as a site for ligand entry/exit into the cavity, we designed the site-specific mutant V32D/F57H which was predicted to form a pH-dependent, steric barrier at this site. The attractiveness of such a system was that the barrier would be formed by the electrostatic interactions between Asp32 and His57 at low pH, but would be less effective at higher pH.

Examination of the crystal structures of V32D/F57H ALBP at two pH values indicates that unexpected changes occurred. Ion pair formation between Asp32 and His57 appears to be only weakly present in the pH 6.4 crystal structure and unlikely at all at pH 4.5. At both pH values, electron density near the mutational sites is not definitive, and the refinement indicated a high degree of thermal motion in this region of the protein. While electron density is poor in this region, the main chain can be traced fairly effectively in both forms, and indicates that the conformation of the mutant is still very similar to that of the wild-type protein. The Cα model of V32D/F57H ALBP was shown in stereo in Fig. 4 and the close agreement between the crystallographic model at two pH values is clearly visible. Within the usual limitations of crystal/solution conformations, the V32D/F57H mutation had little or no effect on the conformation that forms the cavity binding site. As shown in Fig. 5, within the limitations of electrostatic calculations, the double mutation did not have a major effect on the electrostatic potential at the position normally occupied by the carboxylate of a bound fatty acid.

In terms of their crystal structures, is there any difference between the mutant and wild-type proteins? Two α-helices cover the opening of the barrel-like structure. In the orientation shown in Fig. 1, the lid of the barrel would be hinged to the right of the molecule and the helix, αII, is close to the βC-βD and βE-βF turn. The contact region between the lid and the β-barrel is crudely represented by the dotted band. The mutations V32D and F57H were in the fourth residue in from the COOH-terminal end of αI and on the very tip of the βC-βD turn, respectively. Because of the high crystallographic b-factors in the vicinity of the changes, one could argue that the cavity is more accessible in the V32D/F57H mutant. This would coincide well with chemical modification studies which showed that Cys117 on the interior of the cavity is modified 7-fold faster in V32D/F57H than in native ALBP.

Could the poor electron density and high crystallographic b-factors be a result of crystal lattice contacts, and not the mutations? To address this, the crystal lattice contacts around the portal region were examined in both mutant forms and native ALBP. Native ALBP did show more crystallographic contacts around the portal region than the mutant at either pH. However, the structure of another mutant of ALBP has...
obtained from the crystallographic studies described in the text for using modelled coordinates. The curves labeled pH 4.5 and pH 6.4 were superimposable. Similarly, the curve for R126L/Y128F was calculated type (native) and the modelled mutant T125Q. These were essentially structural reference. Values for the potential were calculated for wild-type and 0.8 kcal/mol at the high pH. At these two pH values, the increase in free energy between native and denatured for the polar head group, X16 recently been solved in our laboratory, E72K, which is isomorphous with the pH 6.4 form, and thereby has the same crystal contacts. This structure exhibits low crystallographic b-factors and good electron density around the portal region, implying that extensive crystal lattice contacts are not needed for good electron density and low b-factors. While the best evidence that the local instability and flexibility is induced by the mutations would be to crystalize the native form in a space group isomorphous to either mutant, this has not been accomplished to date.

To attempt to explain why the structural changes produce a change in fatty acid affinity we first considered if the electrostatic potential near the R126/Y128 binding site was altered. Ligand interaction with these internalized polar groups provides a major part of the binding energy. However, based on the crystal coordinates and the calculated electrostatic potentials, V32DF57H ALBP should actually bind fatty acids better because the potential is higher in the carboxylate region of a bound ligand. In fact, ligand binding affinity is actually reduced. Insofar as the electrostatics using crystal coordinates apply to the solution studies, the mutations should have had a small opposite effect to what was observed in the binding of ANS.

In the absence of explanations related to electrostatics of the region near the R126/Y128 binding site, three hypotheses are reasonable to consider. The first is a relatively simple idea which suggests that stability of ALBP or mutant forms is coupled to affinity. That is, mutant forms which stabilize the apo-protein is not coupled to ligand affinity. Consistent with this interpretation, Herr et al. (8) have recently demonstrated with chemically modified ALBP that when all surface lysine residues are acetylated, the stability of the protein is affected (destabilized) while the affinity is unaltered. Similarly, in studies of cellular retinoic acid-binding protein 1 (25), substitution mutations of critical arginine residues rendered the protein unable to bind retinoic acid but stabilized the molecule to thermal denaturation. Again, the interpretation is that in general, affinity of a lipid-binding protein for hydrophobic ligands is not formally coupled to protein stability.

Second, while the changes in binding affinity did not appear to correlate with alterations in electrostatic potentials near the triad, one cannot rule out some effects introduced in the portal region. Referring to Fig. 5, the reader will notice that the electrostatic potentials near the portal region (X15 and X16), are lower than native ALBP, and are even lower than the R126L/Y128F mutant. If this is the preferred site of ligand entry, and assuming the fatty acid is presented to ALBP with a negatively charged head group, the negative electrostatic potential introduced by V32D could serve as a negative factor in the binding energy. As mentioned earlier, some C18 fatty acid ligands have been observed to make van der Waals contacts with Phe77 in the bound state. The introduction of these two hydrophilic residues for formerly hydrophobic residues would eliminate any such favorable interaction.

A third reasonable hypothesis is that the lipid binding proteins exist in “open” and “closed” conformations as described by Cistola et al. (26). A simple conformational difference of this sort would mean there are really four forms of the protein since one would expect open/closed types of both the apo- and holo-proteins. A diagram of the proposed states is shown in Fig. 6.

The crystal structures reported here would be in the so-called closed conformation. In the iLBP family, so far only crystal structures of the apo closed and holo closed forms have been observed except in the case of cellular retinoic acid-binding protein I where both an open and closed form of the apoprotein appears to be present (27). The cellular retinoic acid-binding protein contained a conformation where the tip of the βC-βD loop moved away from helix αII making the cavity more accessible. It is this loop which is the most destabilized in terms of the crystallographic b-factors as was shown in Fig. 3. In this model, the open and closed forms would be in rapid equilibrium. However, the rather limited accessibility of a fatty acid through the portal in the apoprotein suggests that the preferred ligand binding pathway may be from apo-closed to apo-open. Under both acidic and basic conditions, V32DF57H ALBP reached the midpoint of denaturation at lower concentrations of guanidine hydrochloride than the wild-type protein. The mutant protein was destabilized by 3.0 kcal/mol at the low pH and 0.8 kcal/mol at the high pH. At these two pH values, the affinity for 1,8-ANS was reduced identically. Therefore, while the change in free energy between native and denatured for wild-type and mutant was affected by pH, there was no evidence for a pH-dependent change in binding affinity. These results suggest that in general, stability of the apo- or holo-protein is not coupled to ligand affinity.

Consistent with this interpretation, Herr et al. (8) have recently demonstrated with chemically modified ALBP that when all surface lysine residues are acetylated, the stability of the protein is affected (destabilized) while the affinity is unaltered. Similarly, in studies of cellular retinoic acid-binding protein 1 (25), substitution mutations of critical arginine residues rendered the protein unable to bind retinoic acid but stabilized the molecule to thermal denaturation. Again, the interpretation is that in general, affinity of a lipid-binding protein for hydrophobic ligands is not formally coupled to protein stability. Second, while the changes in binding affinity did not appear to correlate with alterations in electrostatic potentials near the triad, one cannot rule out some effects introduced in the portal region. Referring to Fig. 5, the reader will notice that the electrostatic potentials near the portal region (X15 and X16), are lower than native ALBP, and are even lower than the R126L/Y128F mutant. If this is the preferred site of ligand entry, and assuming the fatty acid is presented to ALBP with a negatively charged head group, the negative electrostatic potential introduced by V32D could serve as a negative factor in the binding energy. As mentioned earlier, some C18 fatty acid ligands have been observed to make van der Waals contacts with Phe77 in the bound state. The introduction of these two hydrophilic residues for formerly hydrophobic residues would eliminate any such favorable interaction. A third reasonable hypothesis is that the lipid binding proteins exist in “open” and “closed” conformations as described by Cistola et al. (26). A simple conformational difference of this sort would mean there are really four forms of the protein since one would expect open/closed types of both the apo- and holo-proteins. A diagram of the proposed states is shown in Fig. 6.

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open followed by lipid association to yield holo-open. Mutations that stabilized the open form would be expected to reduce the apparent affinity of the protein for fatty acids. However, as described earlier, mutants that are unstable are not necessarily in the open conformation because stability does not appear to be linked to affinity. The solution structure of the holo-open form of a member of the iLBP family would be an invaluable tool in the assessment of this hypothesis.

It is clear that the process of ligand binding requires the internalized ligand form of the ionic association with the reactive triad of Arg\(^{106}/\text{Arg}^{126}/\text{Tyr}^{128}\). In addition, van der Waals contacts between the lipid acyl chain and cavity side chains are established as a consequence of ligand binding. The cavity of the protein is roughly 3–4 times the size of the ligand suggesting that a ligand may be internalized without association with Arg\(^{126}\) or Tyr\(^{128}\). This has been shown most clearly for the binding of 12-(9-anthroyloxy)oleic acid to the R126L/Y128F mutant of ALBP where binding occurred within the cavity without triad association (21). These results imply that although the model describes the binding events in simple terms, multiple equilibria contribute to the binding energy and differentiate between ligand internalization and triad association.

Recent observations by other groups are consistent with the model for a multiple equilibria binding process. For example, in the intestinal fatty acid-binding protein 2 (IFABP 2) from the Pima Indian population, a polymorphism at position 54 results in an alanine to threonine substitution. This polymorphism increases the in vitro binding affinity 2-fold, and correlates with greater in vivo lipid oxidation and insulin resistance (28). Position 54 in this protein is found at the IFABP 2 portal region and most likely affects the opening or closing equilibria controlling ligand access into the cavity. Similarly, Prinsen and Veerkamp (29) report on several mutations in human muscle FABP (e.g. T40E) which decrease binding affinity by affecting either the electrostatic triad or the van der Waals association. Kleinfeld and colleagues (30) have examined the kinetics of fatty acid binding to ALBP, heart FABP, and intestinal FABP and concluded that the rate-limiting step in the binding process is the entry/exit of ligand through the portal into the cavity. This is consistent with our interpretation that the opening and closing of the portal are central to the binding event and that equilibria other than those related to formation of the ionic triad are critical for high affinity fatty acid association. Consistent with this, Cistola, Frieden and co-workers (26, 31) have developed a mutant of intestinal FABP lacking the two helices which connect \(\beta\) to \(\beta\) and form some of the components of the portal region. The mutant IFABP, termed \(\Delta^{17-7}\)-SG, is essentially an all \(\beta\)-barrel protein and binds palmitate with an affinity 20–100-fold lower than wild-type. The affinity of \(\Delta^{17-7}\)-SG is virtually identical to that for the R106T IFABP mutant. As evaluated by heteronuclear two-dimensional NMR, the interaction of the bound palmitate with protein side chains is essentially identical for \(\Delta^{17-7}\)-SG and wild-type protein. However, there are dramatic differences in the kinetics of ligand association and dissociation. Since \(\Delta^{17-7}\)-SG lacks helix \(\alpha II\), which contributes to formation of the portal, it is reasonable to conclude that the equilibrium of opening and closing are affected, biasing the mutant toward the open conformation. The IFABP studies, like our own, indicate that mutant proteins with altered ligand binding affinity can arise via effects on any of several equilibrium events.

In summary, our biochemical and structural results describe a portal mutant of ALBP and confirm the hypothesis that changing the region bounded by helix \(\alpha II\) and turns between \(\beta C-\beta D\), and \(\beta E-\beta F\) affects fatty acid affinity. In addition, we present a description of fatty acid binding equilibria that suggests ligand association is more complex than that described within a single equilibrium event. The thermodynamic contributions of the various binding steps to the overall binding free energy remains to be determined.
Biochemical and Crystallographic Analyses of a Portal Mutant of the Adipocyte Lipid-binding Protein
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J. Biol. Chem. 1997, 272:9793-9801.
doi: 10.1074/jbc.272.15.9793

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