Testing of the Portal Hypothesis: Analysis of a V32G, F57G, K58G Mutant of the Fatty Acid Binding Protein of the Murine Adipocyte[†]

Anne E. Jenkins, Jennifer A. Hockenberry, Tan Nguyen, and David A. Bernlohr*

Department of Biochemistry, Molecular Biology and Biophysics, The University of Minnesota, Minneapolis, Minnesota 55455

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ABSTRACT: The portal region of fatty acid binding proteins is hypothesized to function as a dynamic aperture, controlling accessibility of external ligands to the internal fatty acid binding cavity. To test this hypothesis, a triple mutant of the murine FABP4 has been developed (V32G, F57G, K58G, referred to as the portal mutant) that is predicted to constitutively enlarge the opening due to a reduction in the molecular dimensions of the side chains of key portal amino acids. The portal mutant was purified from expressing *Escherichia coli*, its stability was evaluated, and the thermodynamics and kinetics of ligand binding were compared to that of wild-type protein. Introduction of the three amino acid substitutions caused no significant change in the stability of the protein with a free energy of unfolding of 13.7 kJ/mol as compared to 14.0 kJ/mol for the wild-type protein. The portal mutant exhibited a modest decrease (4-fold) in ligand binding affinity using the fluorescent probe 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) as a surrogate ligand. 1,8-ANS displacement assays revealed that the binding affinity for oleate increased from a $K_{0.5}$ of 196 ± 15 nM for the wild-type protein to 165 ± 8 for the portal mutant, while that for arachidonate decreased from the wild type of 186 ± 11 nM to 418 ± 26 nM for the portal mutant. To evaluate cavity accessibility, rate of 1,8-ANS binding was assessed between the portal and wild-type protein. Using equimolar amounts of ligand and protein at 4 degrees, 1,8-ANS bound within the cavity to 95% saturation ($t_{0.95}$) in 750 ms, while the mutant protein was fully modified in less than 1.4 ms. To independently evaluate cavity accessibility, modification of the sole protein cysteine residue, C117 residing within the cavity near C2−C4 of the bound ligand, was monitored using 5,5′-dithio-bis(2-nitrobenzoic acid) modification. The half time for modification ($t_{0.5}$) for the wild-type protein was approximately 20 s, while that for V32G F57G K58G occurred in less than a second. As such, enlargement of the portal region of FABP4 markedly increased the accessibility of ligands to the cavity while having only modest effects on ligand affinity. Taken together, these data provide support for the portal region hypothesis and suggest dynamic fluctuations in this region regulate cavity access, but not ligand affinity or selectivity.

Recent advances in adipose biology have provided support for fatty acids and/or their oxidation products as key regulators of overall energy homeostasis (*1*, *2*). Fatty acids are intimately linked to energy storage through synthesis of triacylglycerol depots, to energy production as substrates for β-oxidation, as regulators of gene transcription as nuclear hormone receptor ligands, and as regulators of signal transduction by association with protein kinases (*3*−*6*). Within the cellular context, fatty acids are not freely diffusing but are bound by specific carrier proteins termed fatty acid binding proteins.

In the adipocyte, two fatty acid binding proteins are expressed, an abundant form, the product of the FABP4[1] gene, and a minor form, the product of the FABP5 gene (*7*, *8*). FABP4 is referred to as the adipocyte fatty acid or lipid-binding protein, and also as aP2. The protein is a member of a multigene family of small, intracellular proteins that have high affinity and selectivity for fatty acids and other hydrophobic ligands (*9*, *10*). The members of the fatty acid binding protein family sequester their ligands from the aqueous external environment within an internalized water-filled cavity of the protein (*11*). Ten antiparallel β-strands organized into two opposing β-sheets form the cavity space. The two sheets form a clam-like structure capped by a helix−turn−helix motif (*12*). For FABP4, binding of a single fatty acid within the cavity occurs in a "head-in" manner as a result of the coordination of the carboxylate of the fatty acid ligand with a tyrosine and two arginine residues buried deeply within the cavity (*13*). The internalized binding cavity is not in obvious contact with the external environment. Entry of the ligand into the cavity is proposed to occur via a region of the protein formed from determinants within the βC−D loop, the βE−F loop, and the N-terminal region of the second α-helix. This region, referred to as the "portal" of the protein, has been studied by numerous investigators by mutational analysis (*9*, *14*−*16*) X-ray crystallography (*14*) and multi-dimensional NMR (*17*, *18*). Portal residues are located on the surface of the protein and are distinct from residues within

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* Address all correspondence to Dr. David A. Bernlohr, Department of Biochemistry, Molecular Biology and Biophysics, The University of Minnesota, 6-155 Jackson Hall, 321 Church St. SE, Minneapolis, MN 55455, V: 612-624-2712, F: 612-625-2163, E: bernl001@umn.edu.

[1] Abbreviations: FABP, fatty acid binding protein; I-FABP, intestinal FABP; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); 1,8-ANS, 1-anilinonaphthalene-8-sulfonic acid; Gdn-HCl, guanidinium hydrochloride.

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the cavity that form the salt bond or are in van der Waals contact with the bound lipid acyl chain.

Nuclear magnetic resonance studies of the intestinal fatty acid binding protein (I-FABP, FABP2), another member of the FABP family sharing high structural similarity with FABP4, have supported the portal region hypothesis (*17*). High-field NMR experiments have shown that the solution structure of holo FABP2 has little variability in the α-carbon backbone of the protein. In contrast, the backbone of the apo form has extensive variability in the portal region while the remainder of the backbone structure is unaffected, supporting the proposition that the portal is dynamic in nature and undergoes rapid fluctuation.

The apo and holo forms of FABP4 have been studied extensively through X-ray crystallography (*11*−*14*, *19*, *20*). When comparing the apo and holo crystal forms, one of the few noticeable structural changes that occur upon ligand binding is the rotation of F57, a residue in the βC−D loop. In the apo structure, F57 projects outwardly away from the cavity, while after ligand binding it rotates nearly 180° into the cavity (*13*). The inward rotation of F57 may be important for blocking access to the internalized ligand. Single-site substitution mutations of F57 result in proteins with modestly reduced ligand binding affinity, but no change in ligand selectivity (*16*). It should be noted, however, that F57 is not completely conserved in all the nine FABPs implying that general mechanisms for cavity access cannot lie solely on this amino acid but more broadly on the portal region as a functional domain. These studies have led to the hypothesis that the residues of the portal region, including F57, operate as a dynamic aperture controlling access of ligands to the cavity inside but not ligand selectivity (*15*, *17*, *18*, *21*, *22*).

To further evaluate the portal hypothesis, we report here the properties of a mutant of FABP4 in which three portal amino acids, V32, F57, and K58, have been concurrently mutated to glycine residues. The reduced size of the side chains in the portal region is proposed to increase the constitutive area of the portal opening. We have analyzed ligand binding and cavity access in the portal and compared it to wild type FABP4. The results support the model of the portal region functioning to control cavity access while only modestly affecting ligand binding affinity and/or selectivity.

MATERIALS AND METHODS

Materials. Oleate and arachidonate were purchased from Nu Chek Prep (Alysian MN), and 1,8-anilinonaphthalene-8-sulfonic acid (1,8-ANS) was purchased from Molecular Probes (Eugene OR). Muta-Gene in vitro mutagenesis kit was purchased from Bio-Rad (Hercules, CA). All other materials were obtained from Sigma (St. Louis, MO).

Mutagenesis, Expression, and Purification of FABP4 Forms. Single-stranded mutagenesis utilizing the Muta-Gene in vitro mutagenesis kit was used to create a sequential series of FABP4 mutants. All experimentation was carried out on the murine form of the protein, and all mutations were verified by direct DNA sequencing. BL21 DE3 pLysS *Eschericia coli* was transformed with pRSet-FABP4 or pRSET-V32G F57G K58G to produce the wild type or portal mutant protein, respectively. The transformed cells were induced with the addition of 100 μg/mL isopropyl-thio-galactosidase when the cell cultures reached an optical density of 0.8 and harvested after 4 additional hours of culture. Wild type and portal FABP4 were purified as previously described (*11*). Briefly, cells were lysed by sonication, and cell debris was pelleted by centrifugation at 12 000 rpm for 30 min at 4 °C. Protamine sulfate was added to the resulting supernatant to precipitate nucleic acids, which were subsequently pelleted by centrifugation at 8000 rpm for 20 min at 20 °C. The supernatant was acidified to pH 5 with sodium acetate and centrifuged again at 8000 rpm for 20 min at 20 °C. The supernatant was concentrated and centrifuged at 100000*g* for 30 min at room temperature. Finally, the sample was applied to a Sephadex G-75 size exclusion column equilibrated in buffer containing 12.5 mM HEPES, pH 7.5, 250 mM NaCl, and 1 mM DTT. FABP4-containing fractions were detected spectrophotometrically and verified by SDS−PAGE and immunoblotting. Fractions containing FABP4 were concentrated and stored at −70 °C until use.

Chemical Denaturation and Free Energy of Unfolding. The free energy of unfolding of wild type and portal FABP4 was determined by monitoring intrinsic tryptophan fluorescence as a function of denaturant as previously described (*23*). 0.5 μM protein in 25 mM Tris-HCl buffer, pH 7.4, was incubated with increasing concentrations of guanidinium-HCl from 0 to 3 M, and the degree of protein unfolding was assessed by measuring the red-shifting of the emission maximum from 333 to 354 nm. The Pace method of linear extrapolation was used to determine the free energy of unfolding for each protein (*24*).

Binding Studies. The fluorescent probe 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) was used to study the ligand binding characteristics of FABP4 and the mutant as previously described (*25*). Each protein was incubated with 1,8-ANS, and the fluorescence properties of the resulting 1,8-ANS−protein complex was determined. $K_{0.5}$ values for 1,8-ANS binding were determined from Scatchard analysis of the resulting titration curves.

Competition assays for each protein were conducted using two fatty acids, oleate and arachidonate, as representative model ligands. 500 nM 1,8-ANS in 25 mM Tris-HCl buffer pH 7.4 was incubated with protein, and the indicated fatty acid was titrated, and the loss of 1,8-ANS−protein complex was measured by the corresponding loss of fluorescence. Decreasing relative fluorescence was plotted against increasing competitor concentration, and using the equation $K_i = [I]_{0.5}(1 + [1,8\text{-ANS}]_{free}/K_d)$, the competitor constants (K_i) for each fatty acid were determined.

Cavity Accessibility. Sulfhydryl modification of C117 with 5,5′-dithio-bis(2-nitrobenzoic acid) was determined as previously described by Buelt and Bernlohr (*26*). To measure the rate of 1,8-ANS binding to FABP4 forms, association was monitored by recording the increase in fluorescence over a 10-s time period using an Applied Photophysics SX18.MV stopped-flow fluorometer system at 4 °C. Briefly, 10 μM protein and 10 μM 1,8-ANS, both in 100 mM sodium phosphate pH 7.4 buffer, were loaded into separate syringes. After injection, mixing, and excitation at 376 nm, fluorescence emission at 472 nm was measured in increments of 5 ms.

RESULTS

To evaluate the effects of portal size on ligand binding, a V32G, F57G, K58G FABP4 form was created in which three

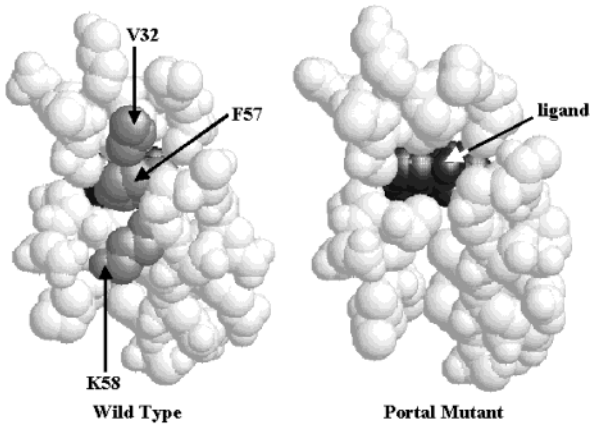

FIGURE 1: Space-filling model of wild-type and portal mutant FABP4. Swiss Model server (*27*−*29*) was used on five existing PDB files (1LIF.pdb, 1LIE.pdb, 1LID.pdb, 2ANS.pdb, 1LIC.pdb) of apo and holo FABP4, and rendered using Ras Mol. Shown at left is the wild-type, and on the right is the portal mutant. 1,8-ANS (ligand) is in black, wild-type V32, F57, and K58 residues are dark gray, and selected surrounding residues are in white.

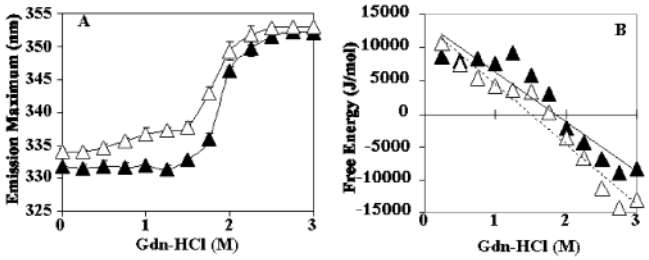

FIGURE 2: Effect of guanidine denaturation on intrinsic tryptophan fluorescence of wild type and portal mutant FABP4. 0.5 μM protein was incubated with 0 to 3 M Gdn-HCl, and tryptophan emission fluorescence was measured (λ_{ex} = 278 nm). Each curve represents three independent experiments. Wild type is shown as a black triangle, and portal mutant protein is shown as a white triangle.

residues were mutated forming a protein referred to as the portal mutant. The reduction in side chain size is predicted to effectively increase the dimension of the portal and/or flexibility within this region. Molecular modeling of the portal mutant and wild-type protein is shown in Figure 1 (*27*−*29*). In the model of the portal mutant, the decrease in size of the side chains is predicted to increase the size of the static portal. Our working hypothesis is that the reduction in side chain volume increases portal opening size.

The portal mutant was compared to wild-type protein for macromolecular stability by measuring the extent of protein denaturation in the presence of increasing concentrations of Gdn-HCl. As shown in Figure 2, the C_m for the wild type (1.55 M) did not vary appreciably from the portal (1.5 M), and free energies of unfolding were similarly unaffected, 14.0 and 13.7 kJ/mol, respectively. We conclude that mutations in the portal region did not appreciably change the stability of the portal protein as compared to wild type FABP4 and that the postulated enlargement of the portal region did not adversely affect overall conformational stability.

To address the functional characteristics of the portal FABP4 mutant, ligand binding was analyzed using 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS). 1,8-ANS is an environment-sensitive probe that fluoresces when bound to fatty acid binding proteins but is relatively nonfluorescent in solution (*30*−*32*). Figure 3 shows the binding isotherms

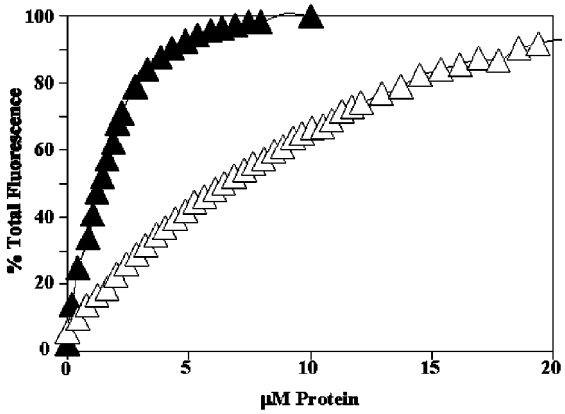

FIGURE 3: 1,8-ANS binding to wild-type and portal mutant protein. 500 nM 1,8-ANS in 25 mM Tris-HCl pH 7.4 buffer was incubated with increasing concentrations of protein, and the fluorescence of the 1,8-ANS−protein complex was measured. Fluorescence measurements (λ_{ex} = 376 nm, λ_{em} = 472 nm) are reported as a percent of the total fluorescence change. Wild type is shown as a black triangle, and portal mutant protein is shown as a white triangle.

Table 1: Apparent Binding Constants for Wild Type and Portal FABP4

	binding affinity (K_d apparent)		
protein	1,8-ANS (μM)	arachidonate (nM)	oleate (nM)
wild-type FABP4	1.68 ± 0.6	186 ± 11	196 ± 15
V32G, F57G, K58G	6.67 ± 2.9	418 ± 26	165 ± 8

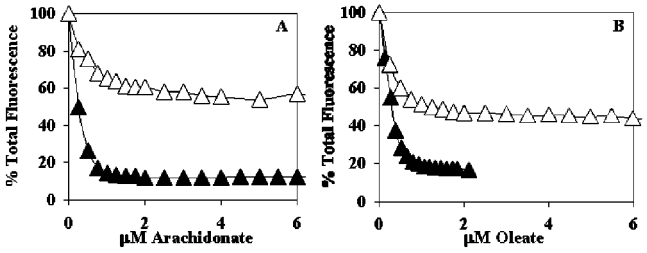

FIGURE 4: Displacement of 1,8-ANS from wild-type and portal mutant protein by arachidonate and oleate. Preformed 1,8-ANS−protein complexes were subjected to competition assays using increasing ligand concentration. Panel A shows displacement with arachidonate, while panel B shows oleate. Black triangles represent wild-type protein; white triangles represent portal mutant protein.

for wild type and portal FABP4. The binding affinity of the portal FABP4 for 1,8-ANS was reduced as a consequence of the mutation, reducing the affinity 4-fold from 1.68 ± 0.6 μM in the wild type to 6.67 ± 2.9 μM for the mutant (Table 1).

To characterize the fatty acid binding properties of the portal mutant, we evaluated the concentration dependence of 1,8-ANS displacement from the binding cavity by oleate and arachidonate (*9*). Figure 4 shows competition curves obtained by adding increasing concentrations of fatty acid to the preformed 1,8-ANS−FABP4 complexes. Table 1 presents the binding affinities of each protein for oleate and arachidonate. The binding affinity of V32G F57G K58G FABP4 for oleate increased slightly relative to the wild type, while the binding affinity for arachidonate decreased slightly. Overall, the differences in fatty acid binding were modest and indicated that the affinity of fatty acids for fatty acid binding protein was not appreciably influenced by alterations in the geometry of the portal region.

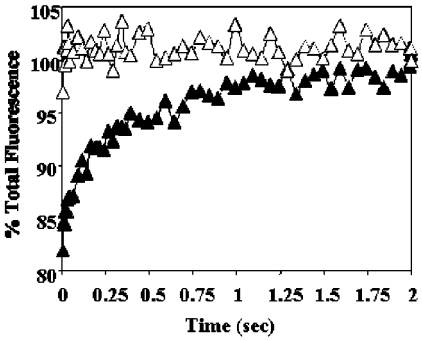

FIGURE 5: Time course of 1,8-ANS binding to wild type and portal FABP4. Equimolar protein and 1,8-ANS (10 μM) were mixed at 4 °C using an Applied Photophysics SX18.MV rapid mixing fluorometer and emission at 472 nm measured every 5 ms for a period of 10 s after excitation at 376 nm. Plotted is a single curve representative of three separate experiments.

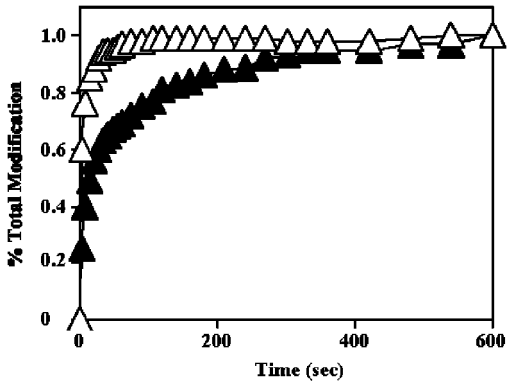

FIGURE 6: Rate of DTNB modification of cysteine 117 of wild-type and portal mutant protein. Equimolar protein and DTNB (1 μM) were mixed, and the release of the thionitrobenzoate ion was followed spectrophotometrically at 412 nm. Black triangles represent wild-type protein; white triangles are portal mutant protein. Plotted is a single curve representative of three separate experiments.

We hypothesized that the portal region controlled access to the binding cavity. To evaluate this, we examined the rate of 1,8-ANS binding in the wild type protein and compared it to the portal mutant. In preliminary studies, we noted that the rate of complex formation was too rapid to measure quantitatively using the conditions typically utilized for binding assays. As a consequence, we reduced the temperature to 4 °C and used equimolar amounts of ligand and protein to slow the reaction. As shown in Figure 5, the fluorescence increase due to the formation of the 1,8-ANS−FABP4 complex was monitored over 10 s. The wild-type protein reached 95% saturation within 750 ms, while the mutant protein reached saturation in less than 1.4 ms, equivalent to the minimum detection time for the rapid mixing fluorometer. Minimally, the results indicate that the rate of complex formation is more rapid in the portal mutant by at least 2 orders of magnitude.

Since the kinetic analysis of 1,8-ANS binding suggested that access to the binding cavity was markedly increased in the portal mutant, we used 5,5′-dithio-bis (2-nitrobenzoic acid) modification of C117 as an independent method to assess this point. Cysteine117 is the sole thiol of FABP4 (C2 was mutated to alanine as a consequence of cDNA cloning) and whose side chain extends into the binding cavity. The side chain of C117 resides adjacent to the C2−C4 position of the bound fatty acid (*19*, *20*). As such, fatty acid binding occludes C117 modification by DTNB and conversely, C117 modification by DTNB sterically hinders fatty acid binding. Modification of C117 was monitored kinetically by the release of the thionitrobenzoate anion, and Figure 6 shows that the rate of product release was markedly increased in the portal mutant relative to wild type protein. For wild type protein, 50% modification was reached in approximately 20 s with 95% modification taking nearly 600 s. In contrast, 50% modification of V32G, F57G, K58G was achieved in less than two seconds. Since modification requires not only entry into the cavity and binding, but also catalysis, to produce the colored thionitrobenzoate anion, the process is more complex than simple entry and binding as required by 1,8-ANS. Even so, as with 1,8-ANS, the rate of DTNB modification of C117 was markedly increased in the portal mutant.

DISCUSSION

The fatty acid binding proteins have been the subjects of numerous structural studies by both X-ray crystallography and nuclear magnetic resonance (*12*−*14*, *17*, *18*). The solved crystal and NMR structures of many members of the family have provided detailed information concerning the location and coordination of the bound ligand, the conformation of the β-barrel backbone and side chains, and dynamic motion of the polypeptide. From this, the proposition that the region surrounding the βC−D loop, the βE−F loop, and the N-terminal region of the second α-helix serves as a dynamic entry/exit port for fatty acid ligands has gained acceptance. However, experimental evidence for the portal hypothesis and evaluation of the role of the portal residues in terms of ligand selectivity, ligand affinity, and cavity accessibility have been less well defined. In a previous report, mutagenesis of FABP4 F57 to a variety of single amino acid substitution forms was described, and it was concluded that the portal region did not control selectivity but had modest effects on ligand affinity depending upon the substitution (*16*). However, because each mutant had a differing stability relative to wild type, it could not be concluded if differences in binding were the result of protein structure or the amino acid substitution per se. Cavity accessibility in terms of portal structure/size has not been previously addressed experimentally as a component of the portal hypothesis. This study was undertaken to build upon single amino acid substitution studies previously reported by generating a triple mutation in the portal region that based on model building was proposed to significantly increase the size of the portal, thereby allowing access of ligands to the cavity.

The residues mutated in these studies were selected for several reasons. Crystal structure analysis of FABP4 forms has shown that there are very few differences in the conformation of the apo and holo protein (*11*−*13*, *19*, *20*). Most significantly differing is the positioning of F57 within the βC−D loop. In the apo structure, the phenylalanine is rotated over the portal, whereas in the holo structure the side chain rotates away from the portal opening (*13*). This change suggests that F57 might play a role in cavity accessibility and/or ligand binding and selectivity. Valine 32 resides within the helix αII region, and its side chain protrudes into the "top" of the portal area. Depending upon the angle of ligand entry into the cavity, the side chain of V32 may sterically block access to the cavity. The side chain of lysine

58 similarly lines the portal, and its side chain forms the "base" of the portal domain. As such, restructuring of the portal to eliminate the base may also affect cavity accessibility. In sum, the V32G, F57G, K58G triple mutant was developed to take into consideration maximal access for ligand entry.

The portal amino acids mutated in this study are conserved to varying degrees among the fatty acid binding family members (*10*). Position 32 (FABP4 numbering) is conserved in 7 of the 9 family members as an aliphatic amino acid with only FABP6 (ileal) and FABP1 (liver) expressing positively charged residues. That region of helix αII (amino acids 30−32) is interesting for there appears to be a general charge/polarity reversal between the FABP1/6 pair and other members of the FABP family. Phenylalanine 57 is conserved among six of the nine family members. Again, as with position 32, the major outliers being FABP6, FABP1, and FABP9 (testis) have small glycine or cysteine residues in the corresponding positions. Lysine 58 is the most highly conserved portal amino acid, with every member of the family having a positively charged histidine, lysine, or arginine residue.

Ligand entry through the portal region in the wild-type protein is restricted by the physical constraints of size. Molecular visualization modeling (*27*−*29*) (Figure 1) and atom to atom calculations suggest a portal diameter of roughly 9 Å, while the ligand 1,8-ANS is measured as 10 Å across at its widest portion. When van der Waals radii of both the portal and ligand are taken into account, ligand entry and exit are essentially completely restricted without a major dynamic movement of the portal region. In comparison, the V32G F57G K58G FABP4 mutant has a portal diameter closer to 15 Å, conceivably large enough for 1,8-ANS access to the cavity without major structural redistribution. Using the CASTp server (*33*), Connolly's surfaces can be determined. This measurement, based on electron density fields, determines the molecular surface of the portal region into the cavity pocket. The value determined for the wild type protein was 7.92 Connolly units, and based on estimations from the atom to atom distances, the portal mutant would have a value of 11.8 units. The increase in Connolly surface represents a significant change in molecular architecture between the two portals, and therefore the possibility of a significant change in ligand entry.

The portal mutant exhibited a markedly enhanced rate of cavity association, as measured by direct binding (1,8-ANS fluorescence) and indirectly by C117 mixed disulfide formation (DTNB modification). This has been interpreted as increased access and accessibility to the cavity via the portal region. The results presented here should be examined within the context of a kinetic study of 31 single site mutations of intestinal FABP (FABP2) completed by Richieri et al. (*31*). From those studies and others, the K_{on} for fatty acid binding was determined to be slower than diffusion, suggesting that the portal serves as a physical barrier to cavity access. Moreover, several mutations were shown to reciprocally affect both the on and off rate of fatty acid binding, strongly supporting the portal of I-FABP as the site of both ligand entry and exit. In I-FABP, the positively charged side chain of R56, analogous to K58 in FABP4, was suggested to be involved in an initial surface binding step with the carboxylate of the fatty acid in a transient, electrostatically driven "docking" process. Mutation of R56 of I-FABP to alanine resulted in decreased affinity, a lower K_{on}, and higher K_{off}. Since our mutation of FABP4 at K58 was carried out within the context of a V32G, F57G, K58G triple mutation, we did not directly address if K58 serves in an initial docking process. However, our studies are consistent with those from I-FABP and support the proposition of the portal as a barrier to ligand accessibility.

Our results indicate that creating three mutations in the portal of a FABP4 significantly increases cavity access by reducing the barrier to accessibility. While we cannot calculate a K_{on} of fatty acid binding because of the rapid association of 1.8-ANS to the triple mutant, qualitatively we find an increase of at least 2 orders of magnitude in fatty acid binding. It is reasonable to conclude that the residues of the portal provide a significant barrier to fatty acid binding, and reducing the size of portal side chains by introduction of multiple glycine residues increase both the K_{on} and K_{off}. On the basis of the abundance of fatty acid binding protein, the concentration of free fatty acids within the cell is predicted to be in the low nanomolar range. As this is significantly below the apparent K_d, control of accessibility of fatty acids to the binding cavity may play a role in the rate of intracellular fatty acid trafficking.

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