Mutants of Rat Intestinal Fatty Acid-binding Protein Illustrate the Critical Role Played by Enthalpy-Entropy Compensation in Ligand Binding*

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Site-specific variants of rat intestinal fatty acid-binding protein were constructed to identify the molecular interactions that are important for binding to fatty acids (FAs). Several variants displayed affinities that appeared incompatible with the crystal structure of the protein-FA complex. Thermodynamic measurements provided an explanation for these apparent inconsistencies and revealed that binding affinities often inaccurately reported changes in protein-FA interactions because changes in the binding entropy and enthalpy were usually compensatory. These results demonstrate that understanding the effects of amino acid replacements on ligand binding requires measurements of enthalpy and entropy, in addition to affinity.

Fatty acid-binding proteins (FABPs) are approximately 15-kDa cytosolic proteins that may play important roles in fatty acid (FA) trafficking (1–3). X-ray crystallography reveals that the FA binding site is an internal cavity in the protein (4–8). Crystal structures of rat intestine FABP (I-FABP) and its complex with FA show that the hydrocarbon chain of the FA interacts directly with about 19 amino acid residues and several bound waters within this cavity (2, 4, 9). Binding of FA to I-FABP involves desolvation of the FA followed by insertion into the binding cavity. The net free energy for these steps for wild type I-FABP is approximately −10 kcal/mol (10, 11) and is predominately enthalpic (10–12).

To understand how the amino acid residue-FA interactions revealed by the crystal structure contribute to the energy of binding we have used site-specific mutagenesis to alter amino acid residues within the binding cavity. Most mutagenesis studies aimed at understanding ligand binding interactions have relied on changes in affinity to determine which residues play important roles in the active site (13, 14). However, the affinity is related to the free energy change ($K_d = \frac{c_c}{c_p} \cdot (\Delta G^o/RT)$) and, through $\Delta G^o = \Delta H^o - T\Delta S^o$, to the enthalpy and entropy changes of the binding reaction. Because mutations alter both enthalpic and entropic contributions to ligand binding, the changes in the underlying molecular interactions may not be correlated with changes in affinity (15, 16). In this present study, therefore, we have determined the free energy, enthalpy, and entropy changes of binding for each mutant interacting with long chain FAs.

EXPERIMENTAL PROCEDURES

Fatty Acid-binding Proteins—Mutants were constructed by extension of overlapping oligonucleotides, which together spanned restriction endonuclease sites in I-FABP, and insertion of the resulting double-stranded DNA as described (17). Mutant and WT proteins were expressed in the pET/BL21 system as described (18). Protein for all mutants except E51A and F93A were isolated from cell lysates. E51A and F93A were expressed as inclusion bodies and were solubilized by denaturation in 4 M GdnHCl followed by renaturation by dialysis against a buffer consisting of 10 mM HEPES, 150 mM NaCl, 5 mM KCl, and 1 mM NaHPO4, at pH 7.4. This buffer was also used in all the binding measurements. Protein purification and delipidation for all proteins was done as described (18). ADIFAB was prepared from acrylodan-derivatized I-FABP as described (19) and is available from Molecular Probes, Eugene, OR.

Fatty Acid Binding to FABP—Measurements of the binding of FA to FABP were done by using ADIFAB fluorescence to monitor the binding of the sodium salts of the FA to each FABP at temperatures between 10 and 45 °C as described (10). For each combination of FA and FABP, a binding isotherm was measured for each temperature and in all cases showed a stoichiometry of 1 FA binding per FABP monomer. The temperature dependence of the $K_d$ values for each FA and FABP exhibited linear van’t Hoff behavior, and $\Delta H^o$ values were determined from the slopes of each of the van’t Hoff plots. For the results shown in Figs. 2 and 3, $\Delta G^o$ values were evaluated from the $K_d$ values measured at 25 °C, and $-T\Delta S^o$ was calculated as $\Delta G^o - \Delta H^o$.

RESULTS AND DISCUSSION

To obtain information about the nature of the FA-FABP interaction we constructed 24 mutants of I-FABP and measured their binding to FA. We substituted Ala for 16 of the 19 residues interacting with the FA in the cavity (Fig. 1A), as well as Gln at residue 106. We also investigated Ala substitutions of the more distal amino acid residues shown in Fig. 1B, FABP binding to each mutant was measured as a function of temperature using the fluorescent probe ADIFAB (10, 19). Dissociation constants ($K_d$) determined at 37 °C for binding of 6 long chain FAs to the wild type protein and to each of the 24 mutants were found to range between 0.5 and 4500 nM (Table I and Fig. 3). These results show that substitution of single residues in I-FABP can result in proteins that bind with affinities ranging from 30-fold higher (Leu-72, Arg-106) to almost 30-fold lower (Met-18, Phe-68) than the wild type (WT) protein.

From the temperature dependence of the $K_d$ values we determined the differences in enthalpy ($\Delta H^o$), entropy ($\Delta S^o$), and free energy ($\Delta G^o$) of FA binding between each mutant and the WT protein. These results, arranged so that the $\Delta G^o$ values for linoleate increase monotonically, are shown in Fig. 2 and demonstrate that changes in affinity, equivalent to $\Delta G^o$,
Enthalpy-Entropy Compensation in Site-specific Mutagenesis

Values are not correlated with $\Delta \Delta H^0$ and $T \Delta \Delta S^0$. This lack of correlation results because $\Delta \Delta H^0$ and $T \Delta \Delta S^0$ tend to compensate in these binding reactions. As a consequence, the mutation-induced changes in binding enthalpy and entropy are almost always larger than $\Delta \Delta G^0$, so that relatively small mismatches in the enthalpy/entropy compensation can result in highly significant changes in binding affinity. A striking feature of these results is that the changes in affinity are not related uniquely to the changes in enthalpy and entropy. For example, although Ala substitutions for Leu-72, Arg-106, and Tyr-117 all result in substantial increases in affinity, the molecular interactions that generate these increases are different in each case. The increase in affinity for L72A is caused by an increase in $|\Delta H^0|$ with a smaller decrease in entropy, that for R106A is caused by a decrease in $|\Delta H^0|$ with a larger increase in entropy, and that for Y117A by both of these kinds of changes, but generally with smaller magnitudes. At the other end of the scale, M18A and F68A have substantially lower affinities than WT protein. This is achieved by quite large (>6 kcal/mol) decreases in $|\Delta H^0|$ with smaller increases in entropy for M18A but quite modest (<2 kcal/mol) changes in entropy and enthalpy, that are not quite compensatory, for Phe-68, which has the smallest affinity of any mutant. In the cases of Tyr-14, Leu-78, and Asn-11, Ala substitutions result in virtually no change in affinity and the appearance of no interaction between the FA and the WT residue. However, for Y14A and L78A, the lack of change in affinity results from quite large but virtually exactly compensating changes in enthalpy and entropy, although of opposite signs for the two sites. Only for N11A is the lack of a change in affinity consistent with virtually no change in the underlying molecular interactions.

An especially clear example of the value of measuring binding enthalpy and entropy is provided by the results for Arg-106. The crystal structure of I-FABP suggests that electrostatic interactions between the carboxylate oxygens of the FA and the NH groups of Arg-106 contributes significantly to FA binding (2, 4, 12). Surprisingly, elimination of these interactions by the R106A substitution results in an up to 28-fold increase in binding affinity (Table I and Fig. 3). Also shown in Fig. 3 are the results of a Gln substitution for Arg-106. This mutant, for which Gln-106 would be expected to have a reduced level of attractive electrostatic interactions relative to Arg-106, reveals at most a modest reduction in FA binding, with the exception of palmitate. Examination of Fig. 3, however, reveals that although $\Delta \Delta G^0$ is decreased, increased, or unchanged by the Ala and Gln mutations, both these mutations result in large reductions (average of about 5 kcal/mol) in the enthalpy of binding. At the same time, an increase in entropy either compensates for (R106Q) or is significantly greater than (R106A) the loss in enthalpy in enthaplly. These results indicate that using site-directed mutagenesis to assess the role of individual residues in ligand binding requires an accurate understanding of how the $\Delta \Delta H^0$ and $T \Delta \Delta S^0$ values are generated at the structural level.

These results indicating significant increases in binding entropy for the Arg-106 mutants are consistent with the crystal structures of the WT and R106Q mutant complexed with oleate (9). These structures reveal that for the C-5 to C-18 portion of the FA hydrocarbon chain, the conformation and degree of order are similar in the WT and R106Q mutant. However, C-4 to C-1 of oleate exhibits considerably greater disorder in R106Q than in the WT protein. This suggests that the loss of an attractive electrostatic interaction in the mutant, as reflected by the decrease in enthalpy of binding, is counteracted by an increase in entropy derived, at least in part, from the increase in disorder of C-4–C-1 of the FA.

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The somewhat ambiguous role of Arg-106 in FA binding raises the question of what other interactions are responsible for binding within the cavity. Analysis of the crystal structure suggests that FA binding is due to a “series of feeble forces”
Fig. 2. Mutant-wild type changes in thermodynamic parameters. Values for $\Delta G^0$, $\Delta H^0$, and $T\Delta S^0$ were calculated as $\Delta G^0 = \Delta G_{WT}^0 - \Delta G_{mut}^0$, $\Delta H^0 = \Delta H_{WT}^0 - \Delta H_{mut}^0$, and $T\Delta S^0 = T\Delta S_{WT}^0 - T\Delta S_{mut}^0$, respectively. Values for $\Delta G^0$, $\Delta H^0$, and $T\Delta S^0$ were determined at 25 °C for each of the 4 FAs. Thermodynamic parameters for the WT protein at 25 °C were determined previously (10) and for PA, OA, LA, and AA are (in kcal/mol), respectively: $\Delta G^0$, 11.6, 9.5, 12, 11, 11, 11, and $T\Delta S^0$, 0, 1, 2.2. The results in this figure have been arbitrarily arranged so that $\Delta G^0$ values for linoleate increase monotonically. The scale for $\Delta G^0$ is amplified to 5- to 4-fold relative to those for $\Delta H^0$ and $T\Delta S^0$. Negative values for all 3 parameters indicate more favorable binding. Standard deviations (in kcal/mol) are approximately 0.1-0.2 and 1-2 for $\Delta G^0$ and $\Delta H^0$ and $T\Delta S^0$, respectively.

resulting from protein-FA interactions along the length of the FA (2, 9). Our results (Fig. 2 and Table II) are consistent with this notion because in all cases $|\Delta \Delta G^0| \leq 2$ kcal/mol. At the same time, however, the variation of $\Delta \Delta H^0$ and $T\Delta \Delta S^0$ for these mutants suggests that at the molecular level a wide spectrum of interactions contribute to FA binding, with many being quite strong ($|\Delta \Delta H^0| > 5$ kcal/mol). Several of the mutants illustrate that large enthalpy changes of either sign can occur even in the absence of apparent electrostatic interactions. For instance, an Ala substitution of Leu-102 results in an average 4.7 kcal/mol more favorable enthalpy while the enthalpy of the M18A mutant has a less favorable enthalpy by an average of about 6.5 kcal/mol. In contrast to both of these examples in which large changes in the underlying molecular interactions are reflected in large changes in binding, the Y14A mutant reveals almost no change in binding, but up to 6 kcal/mol of almost exactly compensating $\Delta \Delta H^0$ and $T\Delta \Delta S^0$.

If the thermodynamic parameter differences for each Ala substitution indicate the individual interaction energies between the FA and the amino acid residues for the WT protein, then the sum of the individual interactions should be comparable with the total binding energies for the WT protein. The energies of Fig. 2 and Table II reflect interactions only within the cavity, while the measured $\Delta G^0$ values are for binding including interactions involved in the desolvation step. A significant portion of the desolvation free energy is entropic (10), and the last row of Table II shows the thermodynamic parameters with this contribution removed. As Table II indicates, the sums of the thermodynamic contributions for all of the mutants are significantly different from the measured $\Delta G^0$, $\Delta H^0$, and $T\Delta S^0$ values. That these sums do not equal the actual binding energies is not surprising because the $\Delta \Delta G^0$, $\Delta \Delta H^0$, and $T\Delta \Delta S^0$ values are generally not independent (16) and the values for the mutants may not accurately reflect the interactions in the WT protein (15). Studies of the gene V protein of bacteriophage f1 suggest that additivity might be more accurate for well separated mutations (20). An improvement is obtained by restricting the summation for the I-FABP mutants to 7 well-separated residues out of the total 15 cavity residues (the asterisk row of Table II), but significant differences remain. In particular, the sums of the cavity enthalpies differ systematically with double bond number, although the measured binding enthalpy is virtually identical (−11 kcal/mol) for each of the 4 FA (Table II). This variation may reflect interactions internal to the FA that differ in the bound and free state, and this difference may be different for each of the FA (21) and would be consistent with a compensating entropy as discussed previously (10). Thus accurate estimates of the FA’s internal energy differences in the solvent and I-FABP bound states would help to determine whether summation over mutation-induced changes in thermodynamic parameters provides an accurate estimate of the total binding energetics within the cavity.

Some of the substituted I-FABPs involved residues that are more distal to the FA binding site (Fig. 1B). Perhaps the most interesting of these involves Arg-126, which appears to play a direct role in FA binding to all FABPs except intestine where it is more than 6.5 Å from the FA (3). Nevertheless, as Fig. 2 shows, R126A has a substantial effect on binding due to large $\Delta \Delta H^0$ and $T\Delta \Delta S^0$. Because very similar changes are produced by the D94A mutation (Fig. 2), and because Arg-126 forms an electrostatic interaction with Asp-34 (2, 3, 9), the large effect on FA binding by both of these mutations suggests that this electrostatic bond is important, perhaps for the conformation of the binding cavity. Relatively small $\Delta \Delta H^0$ and $T\Delta \Delta S^0$ at distal locations can also profoundly affect ligand binding as illustrated by Ala mutations of Phe-47 and Phe-68, which together with Phe-62 form a barrier between the interior and exterior of the protein and are ≥4.5 Å from the FA at the carboxylate end (2). These mutations result in substantial reductions in affinity.

Fig. 3. Thermodynamic parameter differences for Arg-106. Mutant – WT differences at 25 °C are shown for Gln and Ala substitutions at position 106. These measurements were done for the 4 FA used in Fig. 2 plus linolenate (18:3). The disparity between previous calorimetry measurements showing a 20-fold reduction in oleate binding to the R106Q mutant (12), as compared with the 3-fold reduction observed in the present study, is likely the result of the inability of calorimetry to measure accurately the binding affinities of FA-FABP complexes (11, 23).
and indeed the F68A mutation produced the largest $\Delta G^0$ (~1.9 kcal/mol) of all 24 mutants. These alterations of $\Delta G^0$ are, however, achieved by relatively small (generally less than 2 kcal/mol) non-compensating changes in $\Delta H^0$ and $\Delta S^0$ (F68A is the only mutant not exhibiting enthalpy/entropy compensation). The quite small effect for the relatively neutral residue Asn-11, which is predicted to be involved in the initial binding step (2) but is >10 Å from the FA, demonstrates that not all residues affect FA binding.

The ability of FABPs to discriminate among different FA is an important issue in FA metabolism. In general FA metabolism exhibits a high degree of FA specificity, distinguishing clearly among FA on the basis of chain length and saturation (for example, Ref. 22). Presumably, FA recognition by proteins occurs at various steps in FA metabolism. Although FA binding to FABPs does not reveal the kind of selectivity observed in cellular metabolism (18, 19), FABPs might provide a good model of how selectivity can be built into FA recognition. Unfortunately, most of the mutants generated little change in binding specificity, and much of the specificity apparent in Table I (for example, $K_d$ values for all mutants are in the order SA<OA<LA<LN) is a reflection of FA solubility differences (10, 18, 19). However, a few of the mutants such as R106A, W82A, Y117A, Y14A, F93A, and M21A have significant effects on binding specificity. In the case of Trp-82, for example, the Ala mutation results in a monotonic increase in binding with double bond number for the 18-carbon series of FA, producing a 10-fold increase in binding of linolenate (18:3) relative to stearate (18:0). The molecular details of how these alterations are achieved are unclear, although the predominance of aromatic residues within the I-FABP binding cavity and the frequent deviations of arachidonate’s (20:4) thermodynamic parameters relative to the other FA raise the possibility that the aromatic double bond interaction may play a role in modulating FA binding specificity.

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### Table II

Thermodynamic parameter differences for site-specific alanine mutants of amino acid residues in direct contact with bound FA and the WT I-FABP

| Mutant | PA | OA | LA | AA |
|-------|----|----|----|----|
| F68A  | 2.1| 1.9| 1.7| 1.4|
| M18A* | 1.7| 1.8| 1.2| 1.4|
| F55A  | 1.3| 1.3| 1.1| 1.1|
| I23A  | 1.3| 1.1| 0.8| 0.3|
| F62A* | 1.2| 0.8| 0.7| 1.1|
| F17A  | 1  | 0.7| 0.5| 0.2|
| V60A  | 0.8| 0.4| 0.3| 0.4|
| L78A  | 0.4| 0.3| 0.3| 0.3|
| F93A* | 0.2| 0.3| 0.1| 0.1|
| Y70A* | 0.4| 0.1| 0.2| 0.2|
| W82A* | 0.8| 0.5| 0.1| 0.5|
| Y14A  | 0.2| 0.1| 0.3| 0.2|
| Y117A*| 0.5| 0.2| 0.1| 0.1|
| L102A | 0.7| 0.5| 0.4| 0.4|
| R106A*| 0.6| 0.7| 2  | 2  |
| L72A* | 1.5| 1.6| 2  | 2  |

$\Delta G^0$, $\Delta H^0$, and $\Delta S^0$ are calculated at 25°C.

$a$ Values in this row are estimates of the WT thermodynamic parameters ($\Delta G^0$, $\Delta H^0$, and $\Delta S^0$) calculated as: $\Delta G^0 = \Sigma \Delta H^0 - \Sigma \Delta S^0$, $\Delta S^0$ relative, respectively, where $i$ represents each of the mutants in column 1.

$b$ Values in this row are sums of a subset of mutants indicated by $i$. These mutants were chosen using the crystal structure (4) to pick residues that are at well-separated locations and therefore may reflect greater independence.

$c$ WT values are the measured $\Delta G^0$, $\Delta H^0$, and $\Delta S^0$ reported previously (10).

$d$ Values in this row are WT – the contributions estimated previously for the desolvation step ($\Delta G_{\text{desol}}$) in binding (10).