Thermodynamics of Ligand Binding to Acyl-Coenzyme A Binding Protein Studied by Titration Calorimetry†

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ABSTRACT: Ligand binding to recombinant bovine acyl-CoA binding protein (ACBP) was examined using isothermal microcalorimetry. Microcalorimetric measurements confirm that the binding affinity of acyl-CoA esters for ACBP is strongly dependent on the length of the acyl chain with a clear preference for acyl-CoA esters containing more than eight carbon atoms and that the 3′-phosphate of the ribose accounts for almost half of the binding energy. Binding of acyl-CoA esters, with increasing chain length, to ACBP was clearly enthalpically driven with a slightly unfavorable entropic contribution. Accessible surface areas derived from the measured enthalpies were compared to those calculated from sets of three-dimensional solution structures and showed reasonable correlation, confirming the enthalphically driven binding. Binding of dodecanoyl-CoA to ACBP was studied at various temperatures and was characterized by a weak temperature dependence on $\Delta G°$ and a strong enthalpy−entropy compensation. This was a direct consequence of a large heat capacity ΔC_p caused by the presence of strong hydrophobic interactions. Furthermore, the binding of dodecanoyl-CoA was studied at various pH values and ionic strengths. The data presented here state that ACBP binds long-chain acyl-CoA esters with very high affinity and suggest that ACBP acts as a housekeeping protein with no pronounced built-in specificity.

Acyl-CoA binding protein (ACBP)[1] is a 10 kDa cytosolic protein which is able to bind long-chain acyl-CoA esters with high affinity. We have previously determined the association constant to be 2.2×10^{13} M^{-1} (Rasmussen *et al*., 1994). ACBP is involved in intracellular acyl-CoA metabolism, and experimental evidence supports this assumption: (1) ACBP specifically binds acyl-CoA esters with high affinity but shows no affinity toward nonesterified fatty acids and free CoA (Mikkelsen *et al*., 1987; Mikkelsen & Knudsen, 1987; Rasmussen *et al*., 1990; Rosendal *et al*., 1993); (2) ACBP is able to mediate transport of acyl-CoA esters to mitochondrial β-oxidation and microsomal glycerolipid synthesis *in vitro* (Rasmussen *et al*., 1994); (3) overexpression of ACBP in yeast dramatically increases the pool of acyl-CoA esters showing that ACBP binds and forms a pool of acyl-CoA esters *in vivo* (Mandrup *et al*., 1993; Knudsen *et al*., 1994). However, other functions have been suggested for this protein.

Guidotti *et al*. (1983) isolated a peptide on its ability to displace diazepam from the γ-aminobutyric acid (GABA) receptor which led to the hypothesis that this peptide is an endogenous allosteric modulator of the $GABA_A$ receptor (Costa, 1991; Costa & Guidotti, 1991). By amino acid comparison, it was shown that this peptide, DBI, was identical to ACBP (Knudsen *et al*., 1989). ACBP has also been postulated to be involved in regulation of steroid hormone synthesis (Yanagibashi *et al*., 1988; Besman *et al*., 1989; Boujrad *et al*., 1993) and to be involved in regulation of glucose-induced insulin secretion (Chen *et al*., 1988; Östenson *et al*., 1990; Borboni *et al*., 1991).

ACBP specifically binds acyl-CoA esters, it shows no or only low binding affinity toward nonesterified fatty acids and free CoA, and does not bind acylcarnitines, cholesterol, and a number of nucleotides, indicating that both hydrophilic and hydrophobic interactions are involved in ligand binding (Rosendal *et al*., 1993). EPR binding studies show that the binding of acyl-CoA esters to ACBP is strongly chain-length dependent with a clear preference for acyl-CoA esters with 16−22 carbon atoms in the acyl chain (Rosendal *et al*., 1993). The binding stochiometry is 1 mol of acyl-CoA bound/mol of ACBP (Knudsen *et al*., 1989; Rasmussen *et al*., 1990). By using a photoreactive acyl-CoA analogue, the hydrophobic binding site was localized to include residues 23−38 (Hach *et al*., 1990), which was later assigned to be part of the second of the four α-helices of ACBP (Andersen *et al*., 1991). The apoprotein is a four-helix bundle protein, and this structure is maintained in the holoprotein. The four α-helices are A1 of residues 3−15, A2 from residue 21 to 36, A3 from residue 51 to 62, and A4 from residue 65 to 84. Determination of the three-dimensional structure of recombinant bovine ACBP complexed with hexadecanoyl-CoA using nuclear magnetic resonance has revealed the nature of specific interactions between the ligand and the

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[1] Abbreviations: ACBP, acyl-CoA binding protein; ASA, accessible surface area; CoA, coenzyme A; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance spectroscopy.

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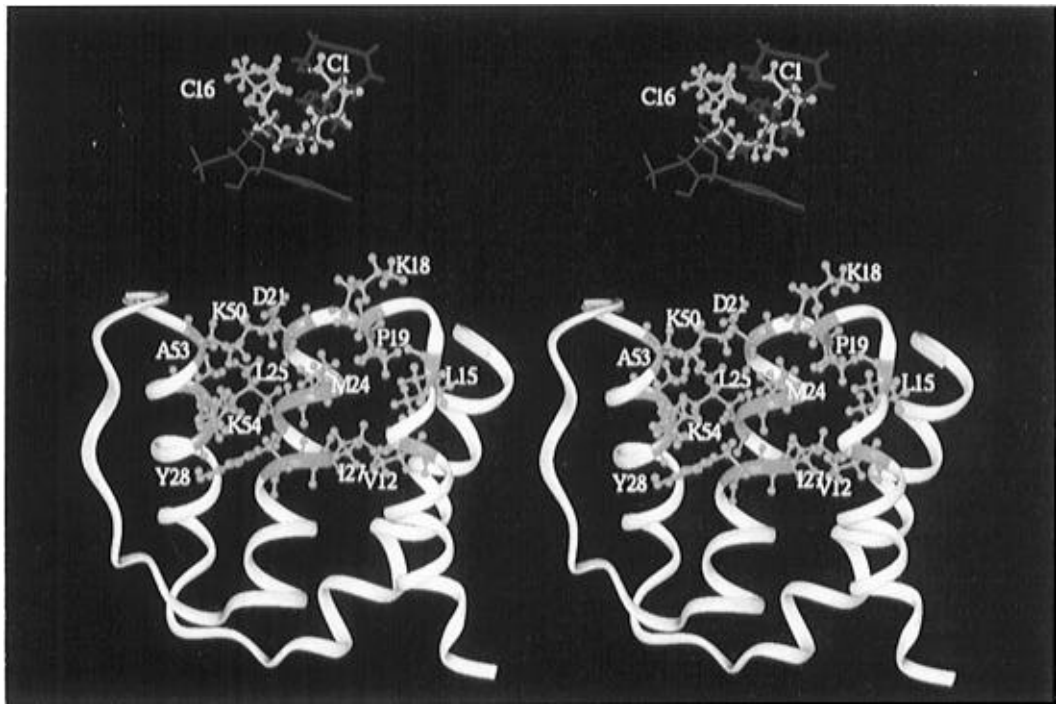

FIGURE 1: Stereoview of the three-dimensional structure of bovine recombinant ACBP in complex with hexadecanoyl-CoA. The protein is shown in a ribbon presentation through the atoms C, C^{α}, and N. Residues with van der Waals contacts to the hexadecanoyl chain are annotated and shown in a yellow ball-and-stick presentation. The ligand is shifted upward but is shown in the orientation it has in the structure of the complex. The hexadecanoyl chain is shown in yellow, the pantheteine and pyrophosphate are in green, and adenosine 3′-phosphate is in red.

protein (Kragelund *et al*., 1993). Hexadecanoyl-CoA is a complex molecule having in principle both hydrophobic and hydrophilic determinants for making favorable interactions with ACBP (Figure 1). The molecular weight of the ligand is one-tenth of that of ACBP and would be expected to have a significant influence on the protein structure when accommodating the ligated form. However, the protein scaffold is not significantly changed by ligand binding, and the four helices, their arrangement, and pattern of hydrogen bonds are essentially conserved in the complex between hexadecanoyl-CoA and ACBP (Andersen & Poulsen, 1993; Kragelund *et al*., 1993).

The binding of acyl-CoA to ACBP involves residues from all four helices and is placed in the core of a bowl-like structure. Hexadecanoyl-CoA adopts a compact arrangement from which the ω-end of the acyl chain and the 3′-phosphate of the ribose extend and form essential and specific interactions with ACBP. The compact part of the ligand is organized so that polar parts are exposed to the surface of the complex whereas the hydrophobic groups turn inward. A unique electrostatic network involving side chains of Tyr28, Lys32, and Lys54 and the 3′-phosphate plays an important role in the binding. The interactions between the two molecules have been described in detail elsewhere (Kragelund *et al*., 1993).

In order to enhance the understanding of molecular recognition phenomena and to provide a better theoretical basis for the rational design of protein functional properties by site-directed mutagenesis, it is highly desirable that detailed thermodynamic information on protein function in relation to protein structure is available. With the recent development in sensitive titration calorimetric techniques (Wiseman *et al*., 1989) that facilitate the convenient measurement of biomolecular interactions it has become possible to elucidate the energetics of many biological processes. For instance, Bhatnagar *et al*. (1994) recently used isothermal titration calorimetry to study the binding of myristoyl-CoA: protein *N*-myristoyltransferase and found that the binding of myristoyl-CoA was enthalpy driven with a large unfavorable entropy contribution and that weaker binding by shorter chain lengths was a result of reduced enthalpic interactions.

The aim of this work was to characterize the ligand binding to ACBP using isothermal titration microcalorimetry. We have previously measured the binding of acyl-CoA esters to ACBP using isothermal microcalorimetry (Rasmussen *et al*. 1994), but here we report detailed thermodynamic information on the ligand binding to ACBP. The results confirm that ACBP binds acyl-CoA esters with extremely high affinity and that the binding is strongly dependent on the length of the acyl chain. The binding was mostly enthalpically driven, but with substantial favorable entropic contributions to the free energy, except at low pH and at high temperatures. The results also indicate strong enthalpy−entropy compensation and a weak temperature dependence of ΔG° as a consequence of the large negative ΔC_p, which is caused by the presence of strong hydrophobic interactions, indicating that water localized in the binding side is displaced upon binding of the ligand. The results are discussed in relation to the three-dimensional structure of recombinant bovine ACBP−hexadecanoyl-CoA complex.

MATERIALS AND METHODS

Chemicals. Nonesterified fatty acids were from Sigma Chemical Co. (St. Louis, MO) or Larodan Fine Chemicals (Malmø, Sweden). Coenzyme A was from Pharmacia (Uppsala, Sweden).

Preparation of Recombinant Bovine ACBP. Recombinant bovine ACBP was purified from *Escherichia coli* harboring

the ACBP expression plasmid pKK223ACBP-EI as previously described (Mandrup *et al*., 1991). The protein concentration was determined using an extinction coefficient of 16 900 M^{-1} cm^{-1} at 280 nm.

Synthesis of Acyl-CoA Esters. Short-chain acyl-CoA esters (C_2-C_4) were synthesized and purified as previously described (Stadtman, 1957). Medium- and long-chain acyl-CoA (C_8-C_{14}) were synthesized and purified as described by Rosendal *et al*. (1993). The concentrations were determined using an extinction coefficient of 14 700 M^{-1} cm^{-1} at 260 nm.

Microcalorimetry. All microcalorimetry experiments were carried out in binding buffer (25 mM ammonium acetate, pH 6.0) or as indicated in the figures. Ligand binding to ACBP was analyzed in a Microcal Omega titration microcalorimeter (Northampton, MA) equilibrated to the desired temperature with a circulating bath. Unless otherwise indicated the temperature was 27 °C. In a typical experiment, the protein solution, approximately 25 μM, was placed in the calorimeter cell and was stirred at 400 rpm. The sample was titrated with a 0.5 mM ligand solution using 25−30 aliquots of 4 μL added at 3-min intervals from a 100-μL stirring syringe. The reference cell was filled with 0.02% sodium azide in water, and the instrument was calibrated by standard electrical pulses. Thermogram data were integrated using the ORIGIN software supplied by MicroCal, Inc., and isotherms were analyzed as described previously (Wiseman *et al*., 1989; Sigurskjold *et al*., 1991). All experiments were repeated two to four times.

Calculation of Surface Areas and van der Waals Contacts. Accessible surface area (ASA) was calculated as described by Lee and Richards (1971) using the program X-PLOR version 3.1 (Brünger, 1992). Values of van der Waals radii were as described by Chothia (1976), and in the case of phosphorus a radius of 1.80 Å was used as described by Bondi (1964). The water probe had a diameter of 1.4 Å. All hydrogen atoms were removed from the structures before the surface area calculations. The total (A_{total}), nonpolar (A_n, carbon atoms bound to hydrogen), and polar (A_p) ASAs were calculated. The ASA was calculated for the following four sets of structures: 29 solution structures of apo-ACPB as determined by Andersen and Poulsen (1993), which reside in the Brookhaven data base (2abd); 20 solution structures of holo-ACBP (complex between ACBP and hexadecanoyl-CoA) as determined by Kragelund *et al*. (1993), which reside in the Brookhaven data base (laca), 50 random hexadecanoyl-CoA structures, and 50 random dodecanoyl-CoA structures, both random sets generated by X-PLOR using standard setup. The random structures were generated from random coordinates, followed by molecular dynamics and subsequent minimization using a force field only containing terms for covalent geometry and a simple repulsive term for nonbonded interactions. In the case of the holo-ACBP/hexadecanoyl-CoA complex the areas were calculated for the ACBP part, the hexadecanoyl-CoA part, the hexadecanoyl chain, and the complete complex.

van der Waals contacts between side-chain atoms of ACBP and atoms of the hexadecanoyl chain were calculated for 20 NMR structures of the complex between ACBP and hexadecanoyl-CoA (Kragelund *et al*., 1993) using X-PLOR version 3.1 (Brünger, 1992). A simple repulsive potential was applied with a maximum repel constant of 1.0. The number of contacts from the hexadecanoyl chain to ACBP was for the individual methylene groups and the single carbonyl and methyl group averaged and accumulated along the acyl chain.

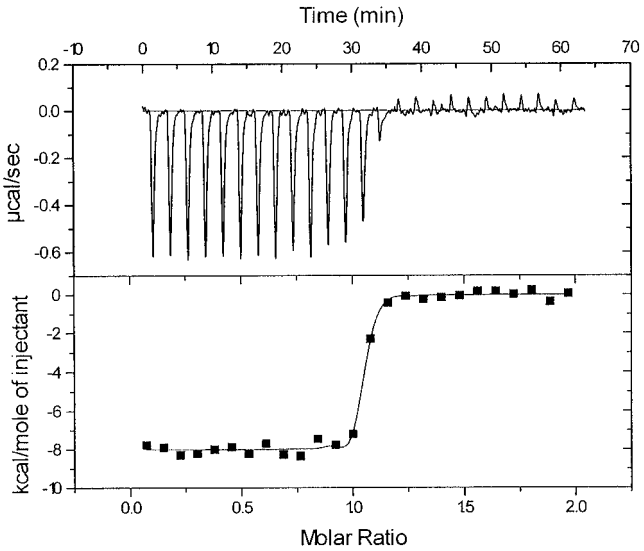

FIGURE 2: Binding isotherm for the titration of ACBP with dodecanoyl-CoA at 27 °C. An 20 μM solution of ACBP was titrated with 25 4-μL injections of 0.5 mM dodecanoyl-CoA. The area under each injection signal was integrated and plotted in the bottom panel. The solid line represents a nonlinear least squares fit of the reaction heat for the injection with the assumption of a single binding site.

RESULTS AND DISCUSSION

The binding of acyl-CoA esters to acyl-CoA binding protein was studied using isothermal titration calorimetry (Wiseman *et al*., 1989). Figure 2 shows raw data obtained by titrating a 25 μM solution of recombinant bovine ACBP (25 mM ammonium acetate, pH 6.0) with a ligand solution containing 0.5 mM dodecanoyl-CoA in the same buffer. There are 25 equivalent 4-μL injections of ligand into the protein solution, reaching the point of saturation at a molar ratio of 1, consistent with previous data (Rosendal *et al*., 1993). As stated by Wiseman *et al*. (1989), the product, termed the *c*-value, between the affinity constant, K_A, and the concentration of binding protein should not exceed 1000 and should preferably be between 10 and 100. Calculation of the *c*-value would yield values of approximately 5000 for dodecanoyl-CoA and tetradecanoyl-CoA. However, the upper value of 1000 was not rigorously determined by Wiseman *et al*. (1989). The binding constant is primarily determined by the slope of the isotherm in the equivalence point. If the total protein concentration is called $[P]_0$ and the total ligand concentration is called $[L]_0$, then the slope is given by

$$\frac{\partial[PL]}{\partial[L]_0} = \frac{1}{2} - \frac{[L]_0/[P]_0 + 1/c - 1}{4\sqrt{{}^1/_4(1 + [L]_0/[P]_0 + 1/c)^2 - [L]_0/[P]_0}} \quad (1)$$

In the equivalence point ($[L]_0/[P]_0 = 1$) eq 1 reduces to

$$\frac{\partial[PL]}{\partial[L]_0} = \frac{1}{2} - \frac{1}{4\sqrt{{}^1/_4 + c}} \quad (2)$$

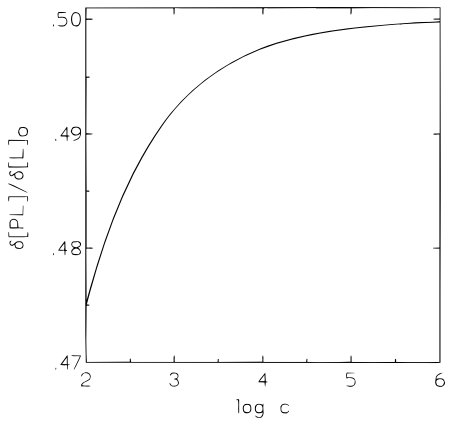

FIGURE 3: Dependency of the *c*-value ($c = K[P]_0$) of the slope of the isotherm in the equivalence point.

Table 1: Thermodynamic Data on the Temperature Dependence of the Binding of Dodecanoyl-CoA to ACBP at pH 6.0[a]

T (K)	nd	K (M^{-1})	$-\Delta G°$ (kJ mol^{-1})	$-\Delta H°$ (kJ mol^{-1})	$T\Delta S°$ (kJ mol^{-1})
285	2	$(1.4 \pm 0.9) \times 10^8$	45.0 ± 1.3	19.5 ± 0.4	25.5 ± 1.4
290	2	$(1.4 \pm 1.1) \times 10^8$	45.2 ± 2.1	23.3 ± 0.7	21.9 ± 2.3
295	2	$(3.3 \pm 1.9) \times 10^8$	48.1 ± 1.4	27.2 ± 0.6	20.9 ± 1.5
300[b]	3	$(1.7 \pm 1.2) \times 10^8$	47.3 ± 1.6	34.1 ± 0.8	13.2 ± 1.8
300	3	$(2.0 \pm 0.7) \times 10^8$	47.6 ± 0.9	30.4 ± 0.5	17.2 ± 1.1
305	2	$(1.1 \pm 0.3) \times 10^8$	46.9 ± 0.7	37.0 ± 0.9	10.0 ± 1.1
310	2	$(6.2 \pm 2.7) \times 10^7$	46.2 ± 1.1	42.1 ± 1.3	4.2 ± 1.7
315	2	$(7.4 \pm 1.6) \times 10^7$	47.4 ± 0.6	44.4 ± 0.8	3.1 ± 1.0

[a] Values are shown as means ± errors derived from the standard deviations obtained from the nonlinear regression analysis, using the law of error propagation for the duplicate and triplicate determinations. nd: number of determinations. [b] *c*-value ≈ 1200.

Figure 3 shows a plot of eq 2 for *c*-values between 10^2 and 10^6. Clearly, there is a significant change in the slope also between 1000 and 10000, although it is much smaller than between 100 and 1000. It is thus possible to extract a binding constant from isotherms in this high range if the experiment is carefully designed with respect to concentrations and injection volumes in order to ensure that points are recorded in the transition region. The binding constant will, however, be determined less accurately, of course. However, titrating a 5 μM solution of recombinant bovine ACBP with a 0.2 mM dodecanoyl-CoA solution (*c*-value ≈ 1200) yielded a binding constant of $(1.73 \pm 1.16) \times 10^8$ M^{-1}, not significantly different from the value obtained with a *c*-value of 5000 (Table 1).

Temperature Dependence. The temperature dependence of the binding of dodecanoyl-CoA to ACBP at pH 6.0 is shown in Table 1 and in Figure 4. A plot of $\Delta H°$ as a function of temperature shows clearly a linear relationship, and from the slope the heat capacity change ΔC_p is obtained as -871 ± 41 J mol^{-1} K^{-1} ($r = -0.995$). The heat capacity of interactions involving proteins is determined by hydrophobic and hydrophilic interactions both for protein folding and protein−ligand interactions (Livingstone *et al*., 1991; Murphy & Gill, 1991; Murphy & Freire, 1992; Murphy *et al*., 1993; Privalov & Makhatadze, 1992; Spolar *et al*., 1992). The contributions from polar and nonpolar interactions to the heat capacity change have been found to be proportional to changes in polar and nonpolar solvent-accessible surface areas (ASA) according to the relation (Murphy & Freire, 1992; Privalov & Makhatadze, 1992; Murphy *et al*., 1993):

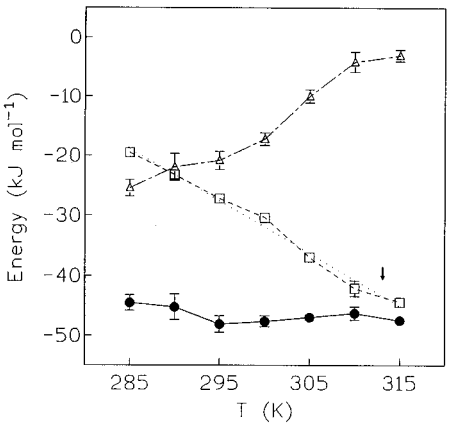

FIGURE 4: Temperature dependence of the binding of dodecanoyl-CoA to ACBP at pH 6.0: $\Delta G°$ (●, solid line), $\Delta H°$ (○, dashed line), and $-T\Delta S°$ (△, dotted line). The arrow indicates the enthalpy at the temperature where $\Delta S°$ equals 0; see text for details. The straight line for $\Delta H°$ has a slope ($=\Delta C_p$) of -871 ± 41 J mol^{-1} K^{-1}.

$$\Delta C_p = c_p \Delta A_p + c_n \Delta A_n \quad (3)$$

where c_p and c_n are the respective polar and nonpolar proportionality factors per unit area, respectively, and ΔA_p and ΔA_n are the corresponding changes in the ASAs. The proportionality factors have been determined experimentally from protein unfolding and transfer processes of model compounds (Murphy & Freire, 1992; Privalov & Makhatadze, 1992; Spolar *et al*., 1992; Murphy *et al*., 1993). From Murphy and Freire (1992) the following relations are obtained: $c_p = +1.1 \pm 0.1$ J mol^{-1} K^{-1} $Å^{-2}$ and $c_n = -1.9 \pm 0.1$ J mol^{-1} K^{-1} $Å^{-2}$, respectively. Thus a large negative heat capacity change indicates that a large nonpolar surface area has been removed from contact with water.

From Makhatadze and Privalov (1993) one has the following relationship, applicable to protein folding, between the enthalpy and the change in polar ASA:

$$\Delta H = h^* \Delta A_p + \Delta C_p (T - T_H^*) \quad (4)$$

where h^* is the enthalpy per unit surface area ($=-146 \pm 13$ J mol^{-1} $Å^{-2}$) at the temperature T_H^* (=376 K) at which the specific enthalpy of folding for globular proteins converges toward a common value. At this temperature the nonpolar enthalpy is 0. Combination of eqs 3 and 4 makes the calculation of polar and nonpolar ASAs possible, assuming that noncovalent ligand binding by proteins is governed by the same type of interactions as protein folding and that eqs 3 and 4 are obeyed. There is some experimental indication that this is a fair assumption (Freire, 1993; Murphy *et al*., 1993); however, a number of counter examples have also been reported (Varadarajan *et al*., 1992; Ladbury *et al*., 1994). These cases may involve larger conformational changes of the proteins concurring with binding which may contribute significantly to ΔC_p (Jin *et al*., 1993; Spolar & Record, 1994). It has been shown by Kragelund *et al*. (1993) that ACBP does not change conformation significantly upon ligand binding, and calculations of the ΔA_p and ΔA_n from the above relations result in 665 and 849 $Å^2$, respectively.

The results from the accessible surface area calculations are shown in Table 2, and it can be seen that the acyl chain in the complex has practically no surface contact. Assuming that the dodecanoyl-CoA/ACBP complex has the same

Table 2: Accessible Surface Areas

	A_{total} (Å^2)	A_n (Å^2)	A_p (Å^2)
apo-ACBP	5277 ± 110	3289 ± 82	1988 ± 85
apododecanoyl-CoA	1176 ± 50	674 ± 37	502 ± 31
apohexadecanoyl-CoA	1263 ± 52	760 ± 37	503 ± 33
holo-ACBP	4657 ± 57	2818 ± 45	1838 ± 76
holohexadecanoyl-CoA	346 ± 25	118 ± 18	227 ± 23
holohexadecanoyl	6 ± 7	5 ± 7	1 ± 2
ACBP/hexadecanoyl-CoA	5002 ± 60	2937 ± 47	2065 ± 74

surface area as the hexadecanoyl-CoA/ACBP complex, which is reasonable since the acyl chain does not have any surface contact, the changes upon ligation can be calculated as $\Delta A_n = 1026 \pm 101$ Å^2 and $\Delta A_p = 425 \pm 117$ Å^2. Inserted in eq 3, this gives $\Delta C_p = -1471 \pm 251$ J mol^{-1} K^{-1}. Inserted in eq 4, this gives $\Delta C_p = -1376 \pm 187$ J mol^{-1} K^{-1}. Compared to the measured ΔC_p of -871 ± 41 J mol^{-1} K^{-1}, these numbers are somewhat on the large side. This is clearly because the random apododecanoyl-CoA structures were calculated in extended conformations, thereby making A_n much higher than can be expected in solution where the hydrophobic chain will curl to achieve minimum water surface area. Furthermore, there may be a small difference in ΔA_n for the protein surface between binding of dodecanoyl-CoA and hexadecanoyl-CoA which also will contribute to the overestimation. The overestimated A_n of apododecanoyl-CoA can be calculated from eq 4, since $\Delta\Delta C_p$ is 505 ± 191 J mol^{-1} K^{-1} and $\Delta\Delta A_n = 677 \pm 263$ Å^2. This number is equivalent to A_n of apododecanoyl-CoA, showing that apododecanoyl-CoA must be folded to protect the hydrophobic parts from contact with water.

A plot of $\Delta H°$ vs $T\Delta S°$ values for the binding of dodecanoyl-CoA at different temperatures (not shown) shows a strong enthalpy−entropy compensation with a slope near unity ($=0.922 \pm 0.042$) and an enthalpy intercept (enthalpy at the temperature where $\Delta S°$ equals 0) of -43.9 ± 1.4 kJ mol^{-1} ($r = 0.995$). The weak temperature dependence of $\Delta G°$ and the strong enthalpy−entropy compensation are a direct consequence of the large ΔC_p. As pointed out by Ha *et al*. (1989), since $(\partial\Delta H°/\partial T)_p = \Delta C_p$ and $(\partial(T\Delta S°)/\partial T)_p = \Delta C_p + \Delta S°$, then, if $|\Delta C_p| \gg |\Delta S°|$, the changes in $\Delta H°$ and $T\Delta S°$ with temperature will be roughly the same ($=\Delta C_p$). They will also both have the same sign as ΔC_p and hence compensate each other. Therefore, the large ΔC_p compared to $\Delta S°$ is the thermodynamic origin of the weak temperature dependence of $\Delta G°$, and since the presence of strong hydrophobic interactions is responsible for the large ΔC_p, it is concluded that the hydrophobic effect causes the weak temperature dependence of the free energy. Similar strong enthalpy−entropy compensations have been observed in a number of other protein−ligand binding systems (Szewczuk & Mukkur, 1977a,b; Mukkur, 1978; Herron *et al*., 1986; Sigurskjold & Bundle, 1992; Sigurskjold *et al*., 1994).

Figure 5 shows a van't Hoff plot of the binding of dodecanoyl-CoA to ACBP. The van't Hoff equation

$$\frac{\partial(\ln K)}{\partial T} = \frac{\Delta H}{RT^2} \tag{5}$$

is usually integrated to give a linear form in reciprocal temperature, if ΔH is considered temperature-independent:

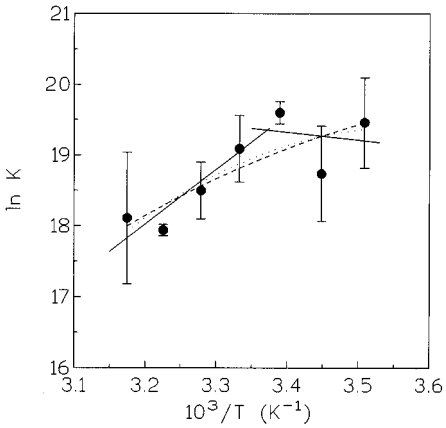

FIGURE 5: van't Hoff plot of the binding of dodecanoyl-CoA to ACBP. Straight lines have been obtained under the assumption that $\Delta C_p = 0$. The curved line has been fitted assuming a temperature-independent ΔC_p.

$$\ln K = \ln A - \frac{\Delta H_{vH}}{RT} \tag{6}$$

where K is the binding constant, A is an intercept value related to entropy, ΔH_{vH} is the so-called van't Hoff enthalpy, R is the gas constant, and T is the absolute temperature.

The van't Hoff plot of dodecanoyl-CoA binding to ACBP displays straight lines at low and high temperatures (Figure 5), respectively, that have been obtained from linear regression to the linear van't Hoff equation. The plot displays a relatively strong curvature, although it is doubtful whether the slope of the curve actually changes sign. The straight line at the higher temperatures (the five leftmost points) corresponds to $\Delta H_{vH} = -64 \pm 12$ kJ mol^{-1} ($r = 0.949$) and the three rightmost at lower temperatures to $\Delta H_{vH} = +9 \pm 64$ kJ mol^{-1} ($r = -0.142$). Using the above linear version of the van't Hoff equation assumes implicitly that the enthalpy has no temperature dependence ($\Delta C_p = 0$). This is obviously inappropriate in the present case.

If the enthalpy is assumed to depend linearly on temperature, i.e., with a temperature-independent non-zero heat capacity change

$$\Delta H_{vH} = \Delta H_r + \Delta C_p(T - T_r) \tag{7}$$

where ΔH_r is the enthalpy at a reference temperature T_r, then the van't Hoff equation can be written in the form

$$\int_{K_r}^{K} d\ln K = \frac{\Delta C_p}{R}\int_{T_r}^{T}\frac{T - T_r}{T^2}\,dT \tag{8}$$

where K_r is the association constant at T_r. Solution of this yields

$$\ln K = \ln K_r + \frac{\Delta C_p}{R}\left[-(1 + \ln T_r) + T_r\left(\frac{1}{T}\right) - \ln\left(\frac{1}{T}\right)\right] \tag{9}$$

In the derivation of eq 9, the reference temperature has been chosen so that $\Delta H_r = 0$. The curved dotted line in Figure 5 has been obtained from a fit to this equation ($\ln K_r = 19.37 \pm 0.58$; $T_r = 281.4 \pm 25.2$ K). From this curve the heat capacity change can be estimated as -1800 J mol^{-1} K^{-1}, indicating a somewhat stronger temperature dependence of the van't Hoff enthalpy than the calorimetric enthalpy. The dashed curve in Figure 5 corresponds to ΔC_p kept

constant at -871 J mol^{-1} K^{-1}. It is clear that this line is not significantly different and estimation of the heat capacity is very uncertain by the measurement.

Similar differences between calorimetric enthalpies and van't Hoff enthalpies and nonlinear van't Hoff plots are quite common (Hearn *et al*., 1971; Hinz *et al*., 1979; Ross & Subramanian, 1981; Fukuda *et al*., 1983; Mukkur, 1984; Wiesinger & Hinz, 1986; Sigurskjold & Bundle, 1992; De Cristofaro & Landolfi, 1994; Sigurskjold *et al*., 1994; Naghibi et al., 1995; Liu & Sturtevant, 1995). A very pronounced example of a biphasic van't Hoff plot with two straight lines for a simple system was reported recently by Mandal and Jayakumar (1994), who studied the micelle formation of a tetrapeptide. This system has a very sharp transition around 40 °C with large values of ΔH_{vH} of opposite sign on each side. This likely reflects the rather complex temperature dependence when both polar and nonpolar interactions as well as associated solvent rearrangements of the amphiphilic species are involved in the binding process in aqueous solution.

Calorimetry measures both the enthalpy change of the binding reaction, ΔH_{int}—the intrinsic enthalpy change—and the heats, ΔH_{con}, of all possible concomitant reactions which may accompany the binding reaction but do not directly influence the intrinsic binding constant K. Such reactions could, for example, involve changes in protonation, hydration, or conformation. To a first approximation the van't Hoff enthalpy is equal to the intrinsic enthalpy and $\Delta H_{cal} \cong \Delta H_{vH} + \Delta H_{con}$. Therefore, a large difference between the van't Hoff and calorimetric enthalpies could be caused by a large, temperature-dependent value of ΔH_{con}. This could very well originate from changes in hydrophobic hydration [see Naghibi et al. (1995) and Liu and Sturtevant (1995)] in which release of water molecules from hydrophobic surfaces upon binding results in loss in enthalpy (due to stronger hydrogen bonds of structured water) and gain in entropy—a phenomenon known to be very temperature dependent (Privalov & Gill, 1988; Muller, 1992; Makhatadze & Privalov, 1993; Privalov & Makhatadze, 1993). It is also possible that the discrepancies originate from intermediates or that two different mechanisms are dominant at different temperatures.

In a bimolecular association both species will lose translational, rotational, and conformational entropy to various degrees (Finkelstein & Janin, 1989). The only two sources of favorable entropy are those associated with solvent displacement, i.e., the release into bulk solvent of molecules solvating the surfaces that interact in the binding process, and the increased vibrational entropy from the increase in vibrational modes upon association (Sturtevant, 1977; Finkelstein & Janin, 1989). The vibrational entropy gain never exceeds the losses from restrictions in translation, rotation, and conformation (Tidor & Karplus, 1994), so only when the solvent displacement is efficient can the overall entropy change become favorable to binding. Since ligand binding by ACBP is entropy-assisted, this is a strong indicator of efficient solvent displacement and, hence, of good structural complementarity between the two molecular surfaces. Water molecules can play an important role in molecular recognition processes as mediators of hydrogen bonds and by providing structural complementarity, but such associations are strongly enthalpy driven with large unfavorable entropy contributions (Ysern *et al*., 1994).

From the very interesting recent analysis by Spolar and Record (1994) the entropy was assumed to consist of three contributions

$$\Delta S^\circ = \Delta S_{HE} + \Delta S_{rt} + \Delta S_{conf} \qquad (10)$$

where ΔS_{HE} is the contribution from the hydrophobic effect, ΔS_{rt} is the contribution from rotational and translational changes, and ΔS_{conf} is the contribution from conformational changes. Vibrational entropy changes were ignored in this analysis. The first term is expected to be positive, whereas the two last terms both are negative (Ross & Subramanian, 1981). The enthalpy intercept of the enthalpy−entropy compensation plot gives the enthalpy when $\Delta S^\circ = 0$. This enthalpy is -43.9 kJ mol^{-1}, and from Figure 4 it is determined that this occurs at $T = 313.6$ K (40.5 °C). At this temperature the hydrophobic entropy exactly cancels the entropy penalties from rotational, translational, and conformational restrictions. The hydrophobic contribution as a function of temperature can be estimated from (Spolar & Record, 1994):

$$S_{HE} = 1.35\Delta C_p \ln(T/386) \qquad (11)$$

which in the present case gives $\Delta S_{HE} = +244$ J mol^{-1} K^{-1} ($-T\Delta S_{HE} = -76.5$ kJ mol^{-1}). The factor 1.35 stems from an assumption that the ratio $\Delta A_n/\Delta A_p$ is the same as for protein folding which is a reasonable assumption, as outlined above. Also from Spolar and Record (1994), the entropy loss from rotational and translational restrictions is estimated as $\Delta S_{rt} = -209$ J mol^{-1} K^{-1} ($-T\Delta S_{rt} = +65.5$ kJ mol^{-1}) for a bimolecular association. This means that the rest of the entropy at this temperature, where the total entropy is zero, is approximately -35 J mol^{-1} K^{-1} ($-T\Delta S_{conf} = +11.0$ kJ mol^{-1}), which as expected is unfavorable and mainly represents increased conformational restrictions. This is a relatively small value, and thus the association is thus best described as a "rigid body" binding rather than an "induced fit" mechanism, since the latter is usually characterized by conformational entropy changes in the range 75−2400 J mol^{-1} K^{-1} (Spolar & Record, 1994). This conclusion is corroborated, as far as the protein structure is concerned, by the NMR structures of the apo- and holoproteins that are essentially similar (Andersen & Poulsen, 1993; Kragelund *et al*., 1993). The small ΔS_{conf} contribution presumably originates from the conformational restrictions obtained in the bound form of the ligand. From NMR studies on the solution conformation on free coenzyme A and derivatives only approximately 30% of the molecules were found to adopt flexible, preferable conformations (Lee & Sarma, 1975). These preferable conformations appear in some way to resemble the semicurled structure found for hexadecanoyl-CoA in the binding site of ACBP. With the high content of hydrophobic groups in the hexadecanoyl-CoA one should not expect a fully unfolded conformation in the free form of the molecule.

Chain Length Dependence. Figure 6 shows the chain length dependence of binding acyl-CoA to ACBP at 27 °C. The relations are linear up to $n = 14$ for ΔG°, $T\Delta S^\circ$, and ΔH°. The slopes of the straight lines in the linear region give contributions per methylene group: $\Delta\Delta G^\circ(-CH_2-) = -1.66 \pm 0.16$ kJ mol^{-1}, $\Delta\Delta H^\circ(-CH_2-) = -1.92 \pm 0.12$ kJ mol^{-1}, and $-T\Delta\Delta S^\circ = +0.26 \pm 0.23$ kJ mol^{-1}. The

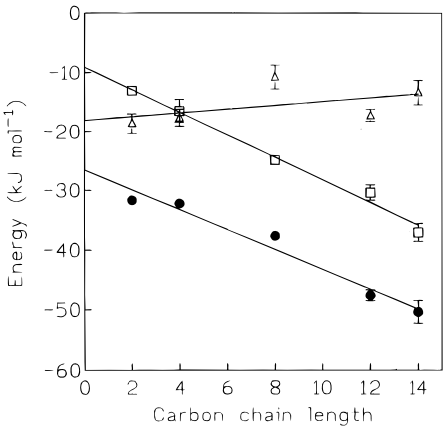

FIGURE 6: Chain length dependence of the binding of acyl-CoA to ACBP at 27 °C and pH 6.0: $\Delta G°$ (●), $\Delta H°$ (○), and $-T\Delta S°$ (△).

increased binding with increasing chain length is largely an enthalpy effect, and the entropic contributions are small, although there is a slight tendency toward more unfavorable entropy with increasing chain length. The maximum binding is known to occur at a chain length of C_{20} (Rosendal *et al*., 1993), and extrapolations to this chain length yield values of −60.0, −47.4, and −12.6 kJ mol^{-1} for $\Delta G°$, $\Delta H°$, and $-T\Delta S°$, respectively. This corresponds to an association constant of 3.2×10^{10} M^{-1}, outside the window of direct measurement by titration calorimetry (Wiseman *et al*., 1989). However, this value is in the same range as the binding affinity between yeast ACBP and hexadecanoyl-CoA (Knudsen *et al*., 1994). Ligands with chain length $n \geq 16$ could not be measured directly.

Chain length dependencies are usually more pronounced in the decrease of entropy compensated by increased enthalpic interactions [see, for example, Ambrosino *et al*. (1987) and Sigurskjold *et al*. (1991)]. The increased enthalpy of binding probably originates from van der Waals interactions, and it is consistent with the rigid body binding model where the conformational entropy is small. Possible adverse entropy changes as a function of increased chain length are probably largely compensated by increased hydrophobic contributions. The number of van der Waals contacts between side-chain atoms of ACBP and atoms of the acyl chain of hexadecanoyl-CoA has been calculated in a set of 20 NMR structures (Figure 7). A significant variation is seen not only for the number of contacts for each carbon group along the chain but also for a particular group within the set of 20 structures (Figure 7a,b). Generally, the number of contacts increases with the length of the acyl chain, and this tendency is more pronounced when the number of contacts is accumulated along the chain (Figure 7c). It should, however, be noted that, in the structure calculation of the complex between ACBP and hexadecanoyl-CoA, the middle part of the acyl chain was unconstrained, which could give rise to a small bias in the actual number of contacts present in the complex. Interestingly, in the complex with ACBP, the acyl chain is almost completely covered both by the remaining part of the CoA head and by residues from the protein. Enthalpy-driven hydrophobic interactions have been observed before and are sometimes called the nonclassical hydrophobic effect (Seelig & Ganz, 1991).

The intercepts represent the binding of coenzyme A itself and have the values $\Delta G° = -26.8 \pm 1.5$, $\Delta H° = -9.0 \pm 1.1$, and $-T\Delta S° = -17.7 \pm 2.1$ kJ mol^{-1}. The coenzyme A lacks the carbonyl group of the acyl moiety, and any specific interactions between this carbonyl and ACBP will lead to overestimations of the values. From hydrogen exchange data obtained on both the free and ligated ACBP, amides of Thr17 and Ala20 in the loop between A1 and A2 were found to belong to the group of amides that exchange more slowly in the complex than in the free form of the protein (Kragelund *et al*., 1995b). The amide of both Thr17 and Ala20 could be involved in hydrogen bonding to the carbonyl of the acyl chain, but no spectral information could be obtained to confirm this. It is known that free fatty acids do not bind to ACBP (Rasmussen *et al*., 1990; Rosendal *et al*., 1993). The binding of the coenzyme A moiety is thus entropy driven, reflecting the predominantly hydrophobic nature of the compound. That the 3′-phosphate of the ribose is essential for binding (Rosendal *et al*., 1993) was confirmed by measuring the binding of dephosphododecanoyl-CoA to ACBP. The 3′-phosphate accounts for about 40% of the binding energy. This decrease in affinity is caused by decreases in entropic and in enthalpic contributions (Table 3).

Ionic Strength Dependence. The binding of dodecanoyl-CoA was determined at various sodium chloride concentrations, and Figure 8 shows the thermodynamic functions. The affinity decreases with higher salt concentration, and at concentrations ≥100 mM a plateau for $\Delta G°$ is reached around 7 kJ mol^{-1} smaller than its strongest binding. This decrease in affinity is caused entirely by a decrease in the entropic contributions, since $\Delta H°$ is constant. Ionic interactions are expected to contribute little to the enthalpy in aqueous solution (Ross & Subramanian, 1981), the favorable energy being primarily a desolvation effect. Ionic interactions in the more "dehydrated" interior of the protein would have much larger enthalpy contributions. Thus the ionic interactions screened by high salt concentrations are probably long range in nature and exposed to solvent. There are only a few potential ionic groups located within or close to the binding site which are not already involved in salt bridges. The side chains of three lysines, Lys13, Lys16, and Lys50 are all placed in the vicinity of and possibly involved in coordinating the two phosphates of the pyruvate group of the CoA head. A screening of these charged groups could be one possible reason for the small but significant decrease in binding detected.

pH Dependence. Figure 9 shows the pH dependence of the binding of dodecanoyl-CoA (Figure 9A) and tetradecanoyl-CoA (Figure 9B) to ACBP. The binding is reduced at lower pH values. The changes are more pronounced for the longer side chain, and again a rather strong enthalpy−entropy compensation is observed. At low pH the interaction becomes strongly enthalpy driven with a large unfavorable contribution from entropy. The unfavorable change in entropy slightly exceeds the favorable change in enthalpy rendering the overall binding somewhat weaker. The change in ionization presumably leads to increased ionic or polar interactions, but these also seem to obscure the good complementarity leading to great losses in entropy and weaker binding. ACBP is known to unfold in a two-state reaction by decreasing pH and to be highly unfolded at pH 2.25 (Kragelund *et al*., 1995a). The midpoint of this transition is at pH 2.85, starting unfolding at pH 3.5 at 298 K. The decrease in binding affinity is observed at one pH

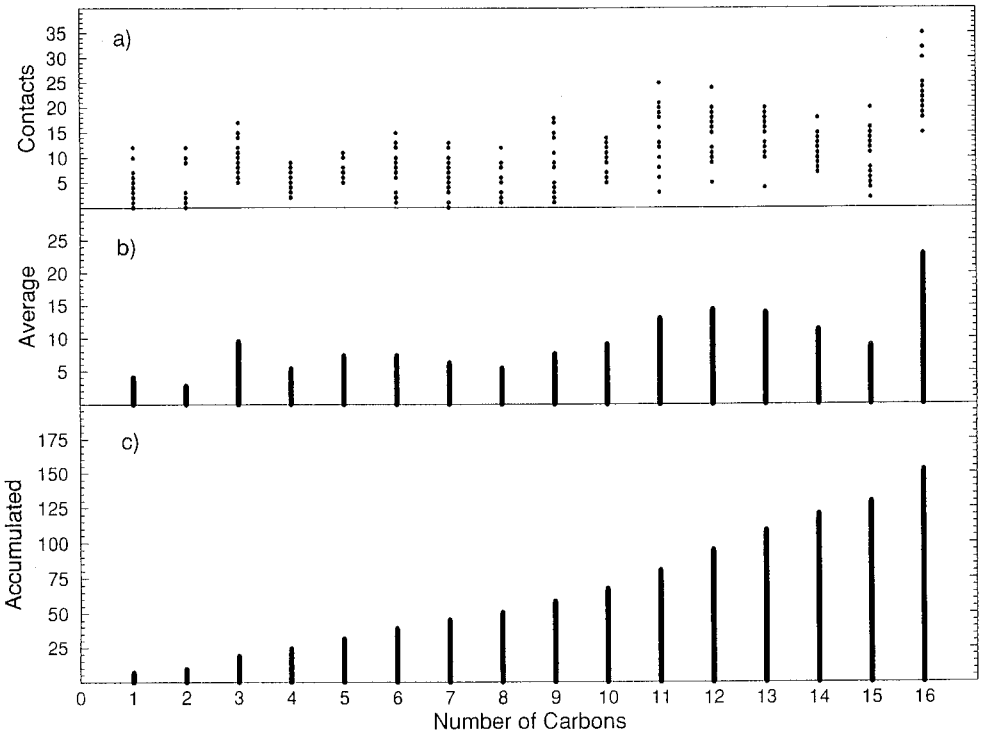

FIGURE 7: van der Waals contacts between side-chain atoms of ACBP and atoms of the acyl chain of hexadecanoyl-CoA. Contacts were calculated for 20 NMR structures of the complex between ACBP and hexadecanoyl-CoA using X-PLOR version 3.1 (a, b). The accumulated number of contacts increases with the length of the acyl chain (c).

Table 3: Thermodynamic Data on the Chain Length Dependence of the Binding of Acyl-CoA to ACBP at 27 °C and pH 6.0[a]

n	nd	K (M^{-1})	$-\Delta G°$ (kJ mol^{-1})	$-\Delta H°$ (kJ mol^{-1})	$T\Delta S°$ (kJ mol^{-1})
2	3	$(3.3 \pm 1.1) \times 10^5$	31.7 ± 0.8	13.1 ± 1.5	18.7 ± 1.7
4	3	$(4.4 \pm 1.2) \times 10^5$	32.3 ± 0.6	14.6 ± 1.3	17.7 ± 1.4
8	3	$(3.6 \pm 0.9) \times 10^6$	37.6 ± 0.7	26.9 ± 1.9	10.7 ± 2.0
12	3	$(2.0 \pm 0.7) \times 10^8$	47.6 ± 0.9	30.4 ± 0.5	17.2 ± 1.1
12, dephospho	2	$(3.5 \pm 1.4) \times 10^5$	31.6 ± 0.8	20.5 ± 1.7	11.1 ± 1.8
14	3	$(5.9 \pm 4.9) \times 10^8$	50.4 ± 1.9	37.0 ± 0.8	13.3 ± 2.1

[a] Values are shown as means ± errors derived from the standard deviations obtained from the nonlinear regression analysis, using the law of error propagation for the duplicate and triplicate determinations. nd: number of determinations.

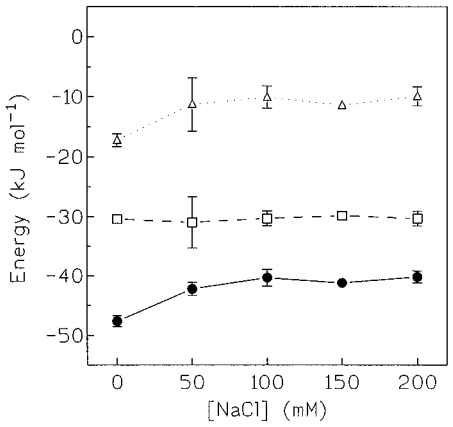

FIGURE 8: Ionic strength dependence of the binding of dodecanoyl-CoA to ACBP at 27 °C and pH 6.0: $\Delta G°$ (●, solid line), $\Delta H°$ (○, dashed line), and $-T\Delta S°$ (△, dotted line).

value only, i.e., pH 3.6, which is very close to the unfolding transition. As the complementarity in the binding site at this pH is declining, it is possible that either parts of the protein structure undergo substantial dynamics or that the entire protein molecule gets more compact as a result of charge repulsion. As a result, the ligand might be more difficult to restrain in the binding site at the same strength as at the

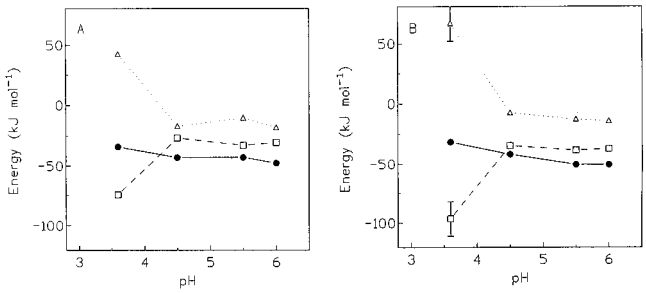

FIGURE 9: pH dependence of the binding of dodecanoyl-CoA (A) and tetradecanoyl-CoA (B) to ACBP at 27 °C: $\Delta G°$ (●, solid line), $\Delta H°$ (○, dashed line), and $-T\Delta S°$ (△, dotted line).

higher pH values, but more experiments are needed to confirm what causes the decrease in binding observed.

CONCLUSION

From titration calorimetry measurements on the binding of different ligands to ACBP, important information has been obtained that can explain aspects of ligand binding to ACBP. Though the three-dimensional structures of both free and ligated ACBP are solved by NMR spectroscopy, and specific interactions between the ligand and ACBP are known, it has now been established that the binding of acyl-CoA is

hydrophobically driven through an enthalpy−entropy compensation. The binding resembles that of a "lock-and-key" model, which is further confirmed by the fact that the protein structure is essentially unchanged when the ligand is bound. The significant contribution to binding from the 3′-phosphate of the ribose, known from earlier work (Rosendal *et al*., 1993) and shown in the structure to be involved in substantial electrostatic interactions (Kragelund *et al*., 1993), has now been shown to account for almost half of the binding energy for dodecanoyl-CoA. Also, for the first time surface areas have been calculated for sets of NMR structures and have, together with data from calorimetry, provided convincing experimental evidence for the mechanism of ligand binding to ACBP.

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