Probing the Nature of Noncovalent Interactions by Mass Spectrometry. A Study of Protein−CoA Ligand Binding and Assembly

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Abstract: A series of noncovalent complexes formed between the 86 residue acyl CoA binding protein (ACBP) and a series of acyl CoA derivatives has been studied by electrospray ionization mass spectrometry. Conditions were found under which CoA ligands can be observed in the mass spectrometer bound to ACBP. Despite the very low dissociation constants (10^{-7} to 10^{-10} M) of the acyl CoA ligand complexes high ratios of ligand-to-protein concentration in the electrospray solution were found to increase the proportion of intact complex observed in the spectrum. Variation in the length of the hydrophobic acyl chain of the ligand (C_{16}, C_{12}, C_8, C_0) resulted in similar proportions of complex observed in the mass spectrum even though significant variation in solution dissociation constants has been measured. A substantially reduced proportion of complex was, however, found for the mutant proteins, Y28N, Y31N, and Y73F, lacking tyrosine residues involved in critical interactions with the CoA ligand. These results have been interpreted in terms of the different factors stabilizing complexes in the gas phase environment of the mass spectrometer. The complexed species were also investigated by hydrogen−deuterium exchange methods combined with mass spectrometric analysis and the results show that folding of ACBP occurs prior to complex formation in solution. The results also show increased hydrogen exchange protection in the complex when compared with the free protein. Furthermore, even after dissociation of the complex, under these nonequilibrium gas phase exchange conditions, increased protection from hydrogen exchange in the complex is maintained.

Introduction

Investigations of non-covalently-bound complexes by electrospray ionization mass spectrometry (ESI MS) are of great interest because of their relevance to molecular recognition in general and to combinatorial library searching in particular.[1] In such experiments a protein, when introduced to a library of ligands, will attach to ligands with appropriate binding characteristics. A recent demonstration of this type of experiment has been a competitive binding study of carbonic anhydrase with a number of ionic binding ligands.[2] In addition, competitive binding experiments have been used to examine the specificity of peptide binding to an SH2 domain protein.[3] The relatively small sample requirements and the rapidity of the method make MS particularly attractive to screening libraries of peptides and other ligands to examine binding interactions with proteins. The solution for analysis in ESI MS may be maintained close to physiological conditions of neutral pH and ambient temperature such that the protein remains close to its native state during introduction into the mass spectrometer. Although ESI MS has the potential for screening these libraries a number of reports in the literature suggest the observation of molecular masses corresponding to complexed species in the gas phase is not necessarily indicative of complex formation in solution. These studies have included binding of peptides without aromatic side chains in cyclodextrin inclusion complexes,[4] formation of DNA duplexes with non-complementary base pairs,[5] and the adduction of inactive enantiomers of substrates during enzyme mechanism studies.[6] Studies which address the specificity of complexes observed in the gas phase are therefore of paramount importance in establishing criteria for the study of these complexes.

Two distinct approaches are emerging for the study of noncovalent interactions involving protein molecules. The first of these involves direct observation in the ESI mass spectra, and the second involves comparison of hydrogen/deuterium exchange properties of free and bound proteins. The first approach has the potential of direct and rapid identification of protein−ligand interactions. The second approach has the potential to provide additional detailed information regarding the structure and dynamics of the protein−ligand interaction and, provided that hydrogen exchange history is preserved in the gas phase irrespective of ligand dissociation, does not rely upon the preservation of intact complex within the ion source of the mass spectrometer.

Using the first approach of direct mass measurement, a number of noncovalent complexes have been reported to remain

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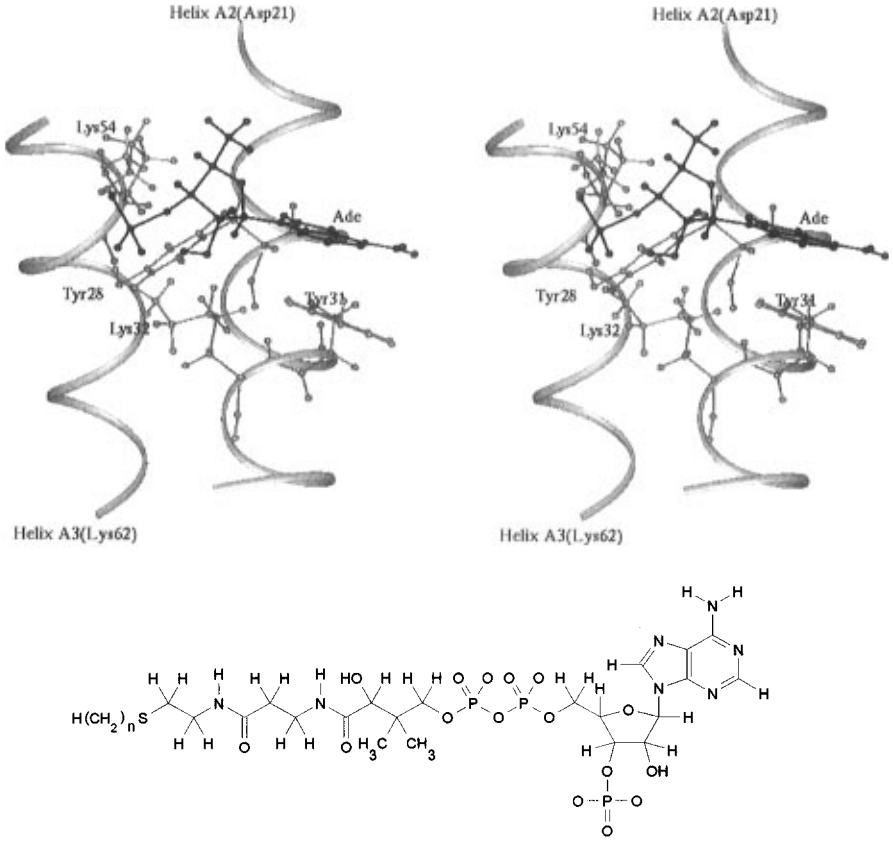

Figure 1. Stereoview of the lowest energy structure of recombinant ACBP in complex with C_{16} acyl CoA ligand showing the interactions between ACBP and the adenine ring and the ribose of the CoA head of the ligand. Two of the tyrosines, Y31 and Y28, which are critical for high-affinity binding of the CoA ligand, have been changed to asparagine residues in two of the mutant proteins used in this study. The primary structure of the acyl CoA ligand is shown below. Only the start of the acyl chain is shown. For the CoA ligands used in this study $n = 0$, 8, 12 and 16.

intact in the ESI experiment. These include metals, heme groups, and peptides bound to proteins[7−10] as well as multimeric protein complexes,[11] oligonucleotides,[12−16] and enzyme−substrate[17] and receptor−ligand[18] complexes. Using the second approach, hydrogen exchange measurements have been used to monitor the conformation of myoglobin in the presence of its noncovalent heme[19] and binding of peptides to an SH2 domain.[20] In addition it has been shown that hydrogen exchange properties in protein−protein complexes, such as the 800 kDa chaperone complex formed between GroEL and a protein ligand, may be monitored by dissociation of the multimeric complex to yield information about the conformation of the protein ligand.[21] Furthermore, equilibrium gas phase hydrogen exchange studies of cytochrome *c*[22] allow us to compare results from this equilibrium solution hydrogen exchange study, after transfer into the gas phase of the mass spectrometer, where significant exchange no longer takes place.

ACBP is an 86 residue protein present in a wide range of eukaryotic organisms.[23] The protein binds long-chain acyl CoAs with high affinity and is thought to have a role in the transport and storage of acyl CoAs in the cell.[24] The protein molecule adopts a four helix bundle structure which forms a bowl shape with a highly polar rim and a predominantly apolar core. The protein and acyl CoA molecules associate by a combination of electrostatic, hydrophobic, and stacking interactions, Figure 1, and have been the subject of detailed investigations by NMR,[25] ligand binding competition assays,[26] and microcalorimetry.[27] Such experiments have provided a precise picture of the amino acid residues and functional groups involved in binding. The

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Table 1. Molecular Masses of Proteins and Their Complexes Formed with CoA Ligands[a]

protein	ligand	molar ratio protein:ligand	molecular mass (Da) calcd	molecular mass (Da) measd	% complex formed	comments
WT			9913.3	9913.1 ± 0.5		peak at mass 9955.40 Da (N-terminal acetylate)
Y31N			9864.2	9864.0 ± 0.6		
Y28N			9864.2	9864.5 ± 0.9		
Y73F			9897.3	9898.1 ± 0.9		
WT	CoA	1:1	10680.3	10678.6 ± 0.1	33	binding of CoAs to ACBP
WT	C_8CoA	1:1	10793.1	10793.2 ± 0.2	25	
WT	C_{12}CoA	1:1	10849.2	10848.1 ± 3.0	22	
WT	C_{16}CoA	1:1	10905.3	10904.6 ± 0.4	20	
WT	C_{16}CoA	1:2	10905.3	10903.4 ± 0.1	28	effect of increasing ligand ratio
WT	C_{16}CoA	1:5	10905.3	10906.3 ± 0.6	35	
WT	C_{16}CoA	1:10	10905.3	10907.2 ± 3.1	40	
WT	C_8CoA	1:1:1:1	10793.1	10789.0 ± 1.5	25	competition experiment—equimolar
	C_{12}CoA	1:1:1:1	10849.2	10847.9 ± 0.4	22	mixture of ligands added to wild type
	C_{16}CoA	1:1:1:1	10905.3	10907.2 ± 3.1	10	see Figure 3
Y31N	C_{12}CoA	1:1	10800.1	10800.4 ± 0.3	<1	acyl CoAs with Y31N mutant
	C_{16}CoA	1:1	10856.2	10854.0 ± 2.0	<1	
Y31N+WT	C_{12}CoA	1:1:1	10800.1	10800.2 ± 1.0	<1	Y31N
			10849.2	10849.8 ± 1.3	15	wild type
Y31N+WT	C_{16}CoA	1:1:1	10856.2	10854.2 ± 1.0	2	Y31N
			10905.3	10901.3 ± 1.0	23	wild type, see Figure 4a
Y28N+WT	C_{12}CoA	1:1:1	10800.1	10801.3 ± 1.2	1	Y28N
			10849.2	10849.2 ± 1.4	15	wild type, see Figure 4b
Y73F+WT	C_{12}CoA	1:1:1	10833.2	10835.2 ± 1.8	3	Y73F
			10849.2	10850.1 ± 2.0	20	wild type, see Figure 4c

protein	ligand	molar ratio protein:ligand	calcd	measd	real time	no. of protected sites	heat to 70 °C
				Hydrogen-Exchange Experiments			
WT	C_{16}CoA	1:1	10905.3	10967.9 ± 1.2	280 mins	61 for ligated protein	9914.1 ± 1.2
			9913.3	9971.9 ± 3.0	280 mins	59 for dissociated protein	
WT	C_8CoA	1:1	10793.1	10853.8 ± 2.0	300 mins	61 for ligated protein	
			9913.3	9971.4 ± 2.5	300 mins	58 for dissociated protein	9913.8 ± 0.9
WT			9913.3	9958.6 ± 1.0	300 mins	45 for *apo* protein	9914.9 ± 1.4
				Pulse-Labeled-Samples			
WT	C_{16}CoA	1:1	10905.3	10947.8 ± 1.2	500 ms	43 sites in native state of ligated protein	
			9913.3	9956.6 ± 1.3	500 ms	43 sites in native state of dissociated protein	
WT			9913.3	9919.8 ± 0.4	0 ms	6 sites due to D content of labeling pulse	
			9913.3	9944.3 ± 1.0	500 ms	31 sites protected in native state	

Dissociation constants[b]

protein	ligand	K_D	ref	protein	ligand	K_D	ref
WT	C_8CoA	$0.2 \pm 0.1 \times 10^{-6}$ M	23	Y31N	C_{12}CoA	$0.9 \pm 0.1 \times 10^{-7}$ M	25
WT	C_{12}CoA	$0.6 \pm 0.6 \times 10^{-9}$ M	23	Y28N	C_{12}CoA	$0.2 \pm 0.3 \times 10^{-7}$ M	25
WT	C_{16}CoA	$<10^{-10}$ M	25	Y73F	C_{12}CoA	$1.2 \pm 0.1 \times 10^{-7}$ M	32

[a] Each measurement is the average of at least two measurements and represent masses obtained from raw data with minimal smoothing. Maximum entropy methods were used to obtain the proportion of complex formed in each experiment. [b] Binding constants for ACBP and mutant proteins with CoA ligands determined by microtitration calorimetry, pH 6.0 in 1mM ammonium acetate.[28]

specificity of the interaction in solution has been established since the protein does not bind strongly to free CoA or to long-chain fatty acids (C_{14} to C_{22}). Furthermore, measurement of dissociation constants for the complexes in 25 mM ammonium acetate buffer, pH 6, Table 1, has shown a distinct preference for long-chain acyl CoA esters (C_{14} to C_{22}).[28]

In this article we focus on the noncovalent complexes formed between the pH stable thioether analogs of acyl−coenzyme A esters (acyl CoAs) and acyl−coenzyme A binding protein (ACBP). The presence of a number of well-defined interactions, a range of acyl CoA ligands which form complexes with differing dissociation constants, and the existence of mutant proteins known to form less stable CoA−protein complexes make this an ideal system for assessing the role of different interactions in preserving protein−ligand complexes and their hydrogen−exchange information in the gas phase of mass spectrometric experiments.

Materials and Methods

Materials. Recombinant bovine ACBP and mutant proteins were produced and purified as described previously.[29] Thioether analogues of the acyl CoAs were synthesized as reported previously.[26] Free CoA was obtained from Pharmacia, Uppsala, Sweden. In all MS experiments ultrapure water (ELGA maxima system) and deuterium oxide (Fluorochem) were adjusted to the required pH with formic acid (Fisons) and acetonitrile (HPLC grade) was obtained from Rathburn. All reported pH values are direct pH-meter readings.

Methods. Complexes were formed by addition of appropriate quantities of a 200 μM solution of thio acyl CoA solution in water at pH 5 to a 20 μM solution of protein at the same pH. The concentration of protein was calculated from UV absorption at 280 nm using an absorption coefficient of 15.4 mM^{-1} cm^{-1} as determined for native bovine ACBP.[28] The concentration of the ligands was calculated from UV absorption at 260 nm using an absorption coefficient of 14.7 mM^{-1} cm^{-1}. Complexes were formed by addition of protein to ligand.

MS. All mass spectra were obtained on a Platform Mass Spectrometer (Micromass). Spectra were calibrated against hen egg white

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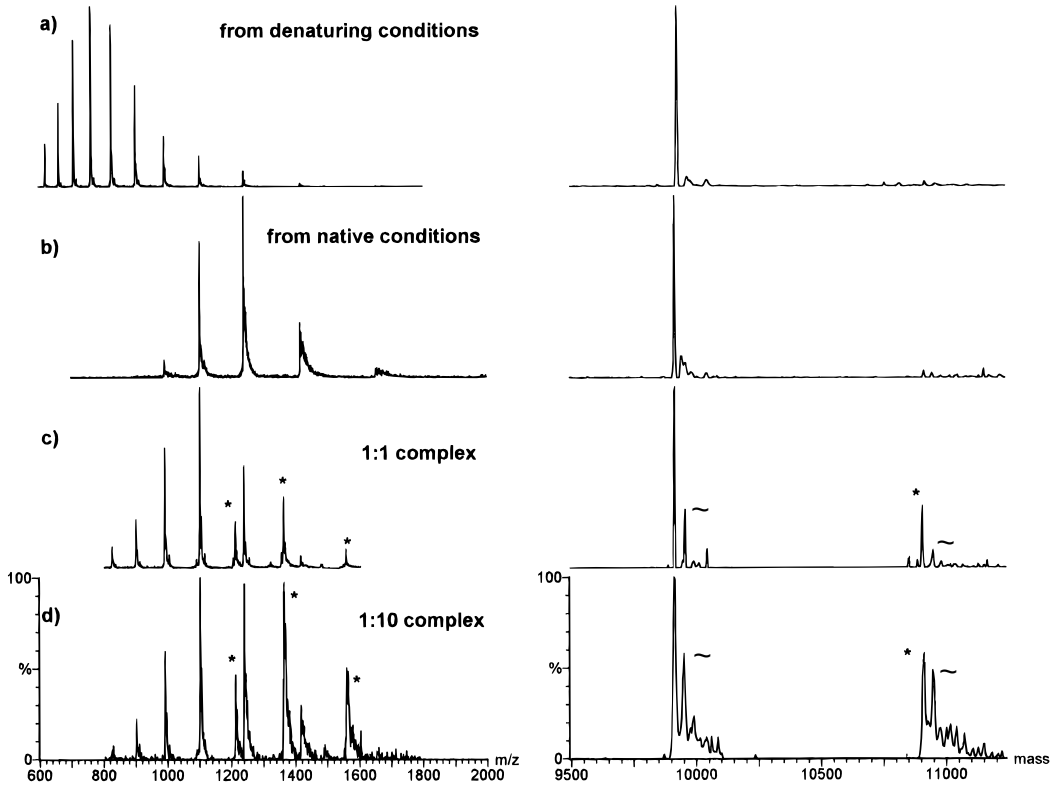

Figure 2. Positive ESI mass spectra of ACBP under a variety of experimental conditions. The charge state spectra on the left have been mass transformed using a maximum entropy method[30] to give molecular ions shown on the right. The charge states and molecular ions marked with an asterisk represent the ACBP-C_{16} acyl-CoA gas phase complex and peaks marked ~ are assigned as alkali metal adducts of the protein and complex and arise from the difficulty in obtaining salt free CoA ligands. The other peaks in the spectrum arise from free protein in the gas phase. Experimental conditions: (a) the running solvent and protein sample solution contained 50% acetonitrile, 50% water, and 1% formic acid and the source temperature was set at 50 °C. (b−d) The running solvent and protein solution were 100% water at pH 5, with pH adjusted with formic acid. ESI mass spectra were obtained without source heating and with cooling of the interface, solvent delivery system and protein samples before analysis, see Experimental Section. The voltages in the ESI interface were optimized for the maximum proportion of complex rather than for overall signal intensity. Optimal conditions were found to be +2.8 kV on the electrospray needle and +0.4 kV on the counter electrode and the voltage difference between the nozzle and skimmer (ΔCS) was maintained at 5 V throughout. ESI mass spectra were also obtained at pH 6.0 in 1 mM ammonium acetate to mimic more closely the conditions for the solution binding measurements. The overall quality of the spectra, however, deteriorated at this pH and significantly higher proportions of complex were not observed

lysozyme and spectra shown represent the raw data with minimal smoothing and are the average of 10 scans. The percentage of complex reported represents the fraction of the total ion current measured in each spectrum between *m/z* 800 and 1600 and peaks not fully resolved to the baseline were analyzed by maximum entropy methods.[30] The ESI interface was cooled by immersing the nebuliser gas in an ice−salt bath at −10 °C and the spectrometer was operated without source heating. Samples were cooled on ice before introduction and the solvent delivery system was cooled by immersion in an ice−salt bath. The protein concentration was 20 μM for all spectra and 10 μL portions were introduced into the ESI interface via a rheodyne injector.

Hydrogen−Deuterium Exchange Labeling. Samples for hydrogen exchange measurements were prepared by dissolving *apo* protein or ligated protein in D_2O at pH 5 using 2 steps of lyophilization. The proteins were then re-suspended in D_2O at pH 5 and diluted 100 fold into water at pH 5 to initiate hydrogen exchange. After 3 days the hydrogen exchange sample solutions were heated to 70 °C to remove residual deuterium. Hydrogen exchange kinetics were plotted using Sigma Plot (Jandell Scientific Ltd.). Hydrogen exchange pulse labeled samples were prepared by dissolving lyophilized protein in 99.9% D_2O, 6 M GuDCl, 0.02 M deuterated sodium acetate, pH 5.3, to a concentration of 10 mg/mL.[31] Refolding was initiated by an 11-fold dilution into refolding buffer (0.02 M sodium acetate, pH 5.3, 5 °C) and the protein was allowed to fold for defined folding time intervals. Deuterons not trapped in persistent structure were exchanged for protons by applying an 8.5 ms labeling pulse at pH 11 (0.2 M boric acid). The pH of the solution was then lowered to pH 5.0 by addition of acetic acid and samples were washed with 5 volumes of formic acid in water at pH 5 and analyzed by mass spectrometry as described above. Refolding in the presence of the ligand was examined using thio C_{16} acyl CoA, present in a 1:1 molar ratio in the refolding buffer.

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Results

Observation of the Noncovalent Complex. The mass spectra of ACBP obtained under a variety of ESI conditions are shown in Figure 2. The spectra from these different conditions are clearly distinct, indicating that the protein retains a history of its solution environment into the gas phase. The spectrum obtained from standard ESI MS conditions, 50% acetonitrile in water with 1% formic acid and a source temperature of 50 °C, is shown in Figure 2a. These standard ESI conditions, in general, denature the protein but allow accurate mass determination and confirm the molecular weight of the protein as 9913.1 ± 0.5 in close agreement with that anticipated from the amino acid sequence of the protein, Table 1. The spectrum obtained from aqueous conditions and lower source temperatures (25 °C), Figure 2b, exhibits a marked change in the charge state distribution from that observed in Figure 2a, with a shift of the charge state maximum from +13 to +9. This reduction in charge has been found to be a feature of spectra obtained from solutions in which protein remains in a native-like conformation during introduction to the ESI source.[21]

The spectrum obtained, under identical conditions to those of Figure 2b above, but after addition of a molar equivalent of thio C_{16}−acyl CoA to a solution of ACBP, is shown in Figure 2c. An additional series of peaks is observed, labeled with asterisks, whose mass corresponds to the sum of thio C_{16}−acyl CoA ligand and ACBP, Table 1. When this protein complex was analyzed from standard ESI conditions, ions corresponding to the mass of the complex were not observed even for increased ratios of protein to ligand (see below). The observation of ligated protein was found to be sensitive to voltages in the ESI interface, with a reduction in complexed species observed for increases in the nozzle-to-skimmer voltage. Furthermore, observation of the complex was dependent on the ESI source temperature such that the proportion of complexed species was reduced to zero by increasing the temperature from 20 to 80 °C. The proportion of complexed species was, however, unchanged in the spectrum after dilution of the protein−ligand concentration from 20 to 5 μM.

The fraction of bound complex observed in the mass spectrum was calculated from the relative ion currents measured for this and the uncomplexed species. The complex formed from aqueous solution containing equimolar C_{16} thio−acyl CoA and ACBP, however, shows only an ion current of ca. 20%. Under the solution conditions used here, however, NMR measurements at pH 5 (unpublished data) and the dissociation constant measured by microcalorimetry, Table 1, indicate that the ligand is effectively 100% bound to the protein prior to electrospray; dissociation of the complex during the experiment has therefore taken place. Increasing the ratio of protein:ligand stepwise from 1:1 to 1:10, however, increases the proportion of complex observed in the ESI mass spectrum; ca. 40% of the total ion current for the bound protein was observed with a 10-fold molar excess of thio C_{16} acyl−CoA ligand, Figure 2d. This shows that the proportion of complex present in the gas phase depends not only on the ESI conditions but also upon the relative concentration of CoA ligand in the solution prior to ESI, and suggests the possibility of nonspecific gas phase interactions.

Variation of the Chain Length of the CoA Ligand. In order to investigate the effect of the solution binding constant of the ligand on the ratio of complexed to uncomplexed ACBP in the ESI spectrum, the mass spectra of different CoA−protein complexes were recorded, Table 1; the dissociation constants of the three acyl CoA ester ligands are shown in Table 1. An interaction between ACBP and free CoA was observed by NMR and was estimated to be relatively low compared to that of the acyl CoA ligands.[32,33] The large differences in dissociation constants, $K_d = 10^{-6}$, 10^{-8}, and 10^{-10} M for the C_8, C_{12}, and C_{16} CoA−protein complexes, respectively, has been attributed to the increasing hydrophobic effect of the hydrocarbon chain.[20] These large differences in dissociation constants are not, however, reflected in the proportion of complexed to uncomplexed species observed in the ESI mass spectra; in each case a similar ratio of the complexed to free protein is observed irrespective of the ligand. Indeed binding of free CoA to ACBP was detected in the ESI mass spectrum of a 1:1 mixture of free CoA and protein, Table 1. In a competition experiment, designed to select the most tightly binding of the three acyl CoA ligands, an equimolar mixture of the ligands was added to the protein such that the total concentration ratio of protein: ligand was 1:3, and the mass spectra were recorded for the complexes formed. Under a variety of mass spectrometric conditions complexes were observed involving all three ligands within the same spectrum, Figure 3. Close examination of the relative intensities for the three acyl CoA ligand complexes reveals a small but reproducible increase in intensity of the C_8

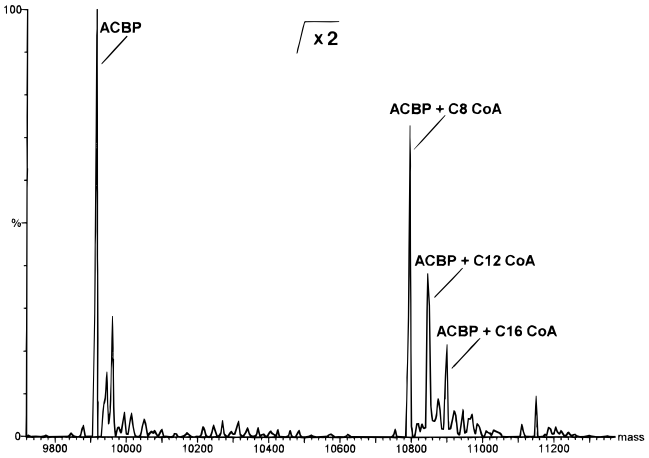

Figure 3. ESI mass spectrum of ACBP after addition of thio analogues of C_8, C_{12}, and C_{16} acyl CoA ligands. The complex mixture was formed by addition of equimolar quantities of C_8, C_{12}, and C_{16} CoA ligands to ACBP at the same concentration (20 pmol/μL). The ESI mass spectra were obtained under the same conditions as described for Figure 2b.

CoA−protein complex in the gas phase over that of the C_{12} and C_{16} CoA protein complexes. This result may be attributed to increased efficiency of collision induced dissociation in the gas phase, the larger CoA ligands being more susceptible to dissociation in the gas phase, by collisions with residual gas, thus providing one possible explanation for the slight increase in gas phase stabilty $C_{16} < C_{12} < C_8$ CoA ligand−protein complexes.

In a further experiment designed to examine the effect of the acyl chain on the stability of the gas phase complex, free CoA was added to ACBP and spectra recorded, Table 1. A similar proportion of complexed and uncomplexed protein was, however, observed for equimolar mixtures of free CoA and ACBP as for the ligands containing a fatty acid chain. This result shows that in the gas phase the presence of the fatty acid chain does not affect the proportion of complex detected in the mass spectrum. This confirms the conclusion that the interactions stabilizing this gas phase complex are located in the CoA region of the ligand molecule.

Mutation of the Protein Binding Site. The binding of CoA ligands to ACBP involves stacking of the adenine ring with the aromatic ring of the tyrosine residue Y31. A mutant in which Y31 has been replaced by an asparagine residue has been shown by microcalorimetric titration to have a dissociation constant for the C_{12} CoA in solution reduced by a factor of 40,[32] Table 1. The mass spectrum of the mutant protein electrosprayed from aqueous solution at pH 5 confirmed the calculated mass, Table 1. Addition of an equimolar quantity of the C_{12} CoA ligand to the mutant protein showed only a very weak series of ions, <1% of the total ion current, corresponding to complex formation. The difference in mass between the wild type and the Y31N mutant enables the two proteins to be readily resolved in the mass spectrum of a 1:1 mixture of the two proteins, hence the two proteins experience identical mass spectrometry conditions. The spectrum obtained from a competition experiment in which a molar equivalent of C_{16}CoA ligand is added to the mixture of proteins is shown in Figure 4a. The mass difference observed shows complexed species predominantly for wild type protein with a ratio of intensities of 25:1 in favor of wild type to mutant protein. Similar results were obtained when the C_{12} acyl CoA ligand was examined in this competitive experiment with a ratio of wild type and mutant protein complexes of 30:1.

(32) Personal communication, Jen Knudsen.

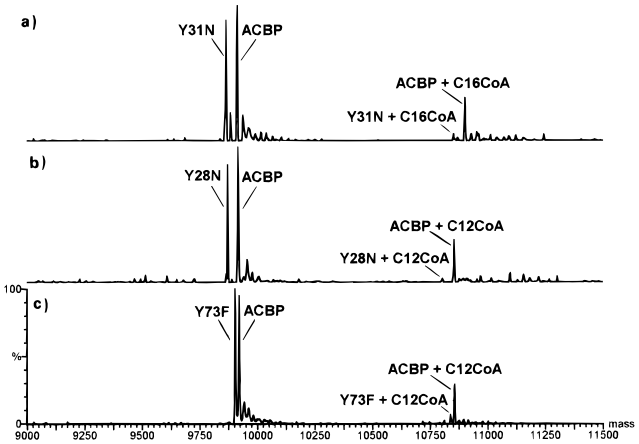

Figure 4. Mass transformed mass spectra, using maximum entropy methods, from competitive binding experiments of Y31N, Y28N, and Y73F mutant proteins with wild type protein and acyl CoA ligands. For all spectra wild type ACBP and mutant were mixed in equimolar ratios (20 μM concentration) and a molar equivalent of either thio C_{16} acyl CoA (upper trace) or thio C_{12} acyl CoA ligand (middle and lower trace) was added to the mixture of mutant and wild type protein.

The phosphate of the CoA ligand is involved in a network of two salt bridges with residues Lys32 and Lys54 and a hydrogen bond to the hydroxyl group of Tyr28. A second mutant protein, Y28N, in which this tyrosine residue is replaced with an asparagine residue, was also examined by ESI MS, Table 1. In a similar competitive binding experiment to that described above, Y28 mutant protein and wild type protein were mixed in equimolar quantities and 1 molar equiv of the C_{12} ligand was added, Figure 4b. Comparison of intensities of complex remaining intact again showed a significantly reduced proportion, by a factor of 40,[32] of complexed species for the Y28 mutant protein compared with that for the wild-type protein. This reduction in intensity for the complexed species is in accord with the reduction in binding affinity measured by microtitration calorimetry for this point mutation.

The adenine ring of the CoA ligand is further stabilized by the hydroxyl group of Tyr 73 forming hydrogen bonds to the adenine ring.[25] A mutant protein, Y73F, in which this tyrosine residue is replaced by a phenylalanine residue has been shown by microcalorimetry to have reduced binding affinity for the C_{12}CoA ligand.[32] Furthermore, increased stability to unfolding by GuHCl has been measured for this mutant in relation to wild type ACBP.[33] Equimolar quantities of wild type ACBP and Y73F mutant were mixed and an equimolar quantity of C_{12} CoA ligand added to the protein mixture. The mass spectrum, Figure 4c, shows a substantially higher proportion of complex with wild type ACBP than with Y73F mutant, Table 1. Thus, in spite of the increased stability of this mutant, ligand binding in the gas phase reflects the reduction in binding affinity for the Y73 mutant protein measured in solution.

Hydrogen−Deuterium Exchange Measurements. Samples of free ACBP and of ACBP complexed with C_{16}, C_8, and C_0 acyl CoA ligands were solvent exchanged with D_2O such that all labile sites in the proteins were occupied with deuterium atoms. The exchange of D for H was then measured by the decrease in mass with time following dissolution of the deuterated protein into water having the natural isotopic composition. A comparison of the number of sites protected from solvent exchange measured over 100 h for the *apo* protein and ligated proteins is shown in Figure 5a. These results demonstrate an increased protection against hydrogen exchange

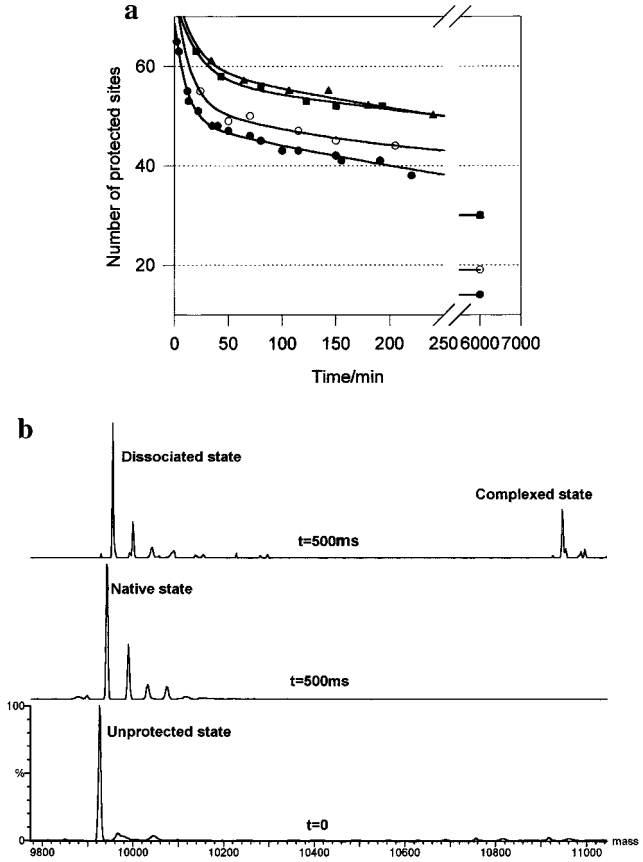

Figure 5. (a) Comparison of hydrogen−deuterium exchange kinetics for *apo* ACBP and ACBP ligated with thio C_{16} acyl CoA, C_8 acyl CoA, and free CoA. The hydrogen exchange of the *apo* protein is represented by ●, with ▲, ■, and ○ representing the ligated protein with thio C_{16}, C_8 CoA and CoA ligands, respectively. (b) Folding and assembly of the thio C_{16} CoA complex. Protein samples were refolded for 500 ms from guandinium chloride in the absence of ligand (lower two traces) and in the presence of an equimolar quantity of C_{16} CoA ligand (upper trace). ESI mass spectra were obtained from identical conditions as those described for Figure 2b above. The number of sites protected from exchange in each case are the following: unprotected state 6, arising from the deuterium content of the labeling pulse, native state 31, and dissociated and ligated states 43.

for the protein when bound to the acyl CoA ligands with 54 and 53 deuterons remaining unexchanged after 150 min in both protein complexes formed with C_{16} and C_8 acyl CoAs compared with 46 for protein bound to free CoA in solution and 42 at the same time point for the *apo* protein. After much longer times, 24 h, of exposure to the exchange environment both acyl CoA complexes retain 31 deuterons compared with 19 for the free CoA complex and 14 for the *apo* protein. This level of protection from hydrogen exchange is consistent with NMR measurements for the *apo* and C_{16} acyl CoA bound proteins.[34] Although 2D NMR measurements were performed at pH 6.65, as opposed to pH 5 for mass spectrometry, additional protection in the C_{16} acyl CoA complex after 24 h was calculated from individual rate constants to be equivalent to 18 amides, in close agreement with the additional 17 amides measured in the mass spectrum of the acyl CoA complexes.

The ESI mass spectra recorded for these proteins undergoing hydrogen exchange show that peaks corresponding to the complexed and dissociated protein contain essentially the same number of sites protected from exchange as a result of complexation. A small but reproducible increase of the number

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of sites protected from solvent exchange was recorded for the complexed protein corresponding to 2 deuterons. This increase is intriguing and may possibly be attributed to slowly exchanging hydrogens present in the buried adenine ring of the bound acyl CoA ligand, which do not therefore contribute to the mass of that fraction of the protein which has undergone gas phase dissociation. Otherwise, in the gas phase the protein molecules, whether attached to the ligand or not, contain closely similar numbers of protected sites showing that they retain a history of their hydrogen exchange behavior in the solution prior to ESI.

In a further set of experiments the rate of folding and assembly of the protein−ligand complex was examined using pulse labeled hydrogen exchange and MS.[35] This technique monitors the formation of persistent structure during protein folding by monitoring protection from exchange of labile amide deuterons involved in secondary structure. The overall folding kinetics observed for ACBP in the presence and absence of the C_{16} acyl CoA ligand were found to be closely similar; no intermediate species was observed in either case.[36] Moreover the increased levels of hydrogen exchange protection in the ligated protein support the findings of real time hydrogen exchange measurements where increased protection was observed in the complexed and dissociated protein when compared with *apo* protein, Figure 5b.

Discussion

The nature of events occurring in the final stages of the ESI process is not well understood. Two models have, however, been proposed. In one the ESI droplet shrinks during solvent evaporation until only single ions remain; this is known as the charged residue model.[37] The second model involves desorption of ions from the surface of the droplet before the ultimate droplet size is reached; this is known as the ion evaporation model.[38] These two models have been refined to take into account experimental observations, particularly for macromolecular complexes, where highly solvated molecular ions are thought to desorb from initial droplets and are subsequently desolvated.[39] The lack of a definitive mechanism for ESI, however, makes distinction between specific and nonspecific noncovalent interactions a difficult problem. The dependence of the proportion of complexed species on the relative concentrations of protein and ligand has been observed previously in other systems[4,5,20] and attributed to an increased concentration of ligand in the electrospray droplet. This situation might be expected to contribute to nonspecific associations since the probability of droplets containing more ligand to protein could lead to the production of nonspecific protein−ligand interactions. Since both mechanisms of ESI ion formation are likely to be sensitive to the concentration of ligand, a pre-requisite of all such studies is a low concentration of protein and ligand in solution.

A range of electrostatic, hydrogen bonding, and nonpolar stacking interactions is involved in the binding of ACBP to its ligands. Hydrophobic interactions, however, appear to be critical to the preservation of this complex in solution since ACBP has a relatively low affinity for free CoA. A large proportion of the binding energy can therefore be attributed to burial of the long hydrocarbon acyl chain.[26] In the latter stages of the ESI process, water will be stripped from the protein molecules, giving rise to a situation very different from the solution conditions. Where hydrophobic interactions have been shown to play a major role in the solution state of other systems, for example in the leucine zipper peptides and the receptor−ligand interaction,[4,13,40] the fraction of complexed species in the gas phase is low (typically ∼10−20%) since such interactions are in large part attributed to the role of solvent. By contrast little or no dissociation of protein metal ion complexes in the gas phase has been observed in the mass spectra of the metal complexes[7,8] and varying amounts of DNA duplex have been reported in a number of DNA MS studies.[12−16] Moreover, protein−heme and some protein−protein interactions remain intact under the appropriate ESI conditions.[8,10] The specific role of electrostatic interactions in stabilizing gas phase complexes has been suggested from a study of spermine and a spermine binding peptide where, despite the relatively low binding affinity measured in solution, a stable gas phase complex was observed with little gas phase dissociation.[41] In addition, the determination of dissociation constants for oligonucleotide−serum albumin complexes has been reported and the strong dependence of the solution phase K_D (measured by ESI MS) on the ionic strength of the buffer was suggested to arise from electrostatic forces involved in the binding of this complex.[42]

The many well characterized interactions present in the protein−ligand interaction described here allow some conclusions as to the relative importance of the different contributions to the stability of the gas phase relative to the solution phase protein ligand complexes. Absence or reduction in the length of the acyl chain, while having a profound effect on solution binding,[26] has little or no effect on the proportion of complexed and uncomplexed species observed in the gas phase. By contrast, replacement of the tyrosine residues involved in nonpolar stacking and hydrogen bonding interactions leads to a substantial reduction in the proportion of complexed species. This is consistent with stacking and hydrogen bonding interactions being maintained to a greater extent than hydrophobic interactions during removal of water molecules in the ESI process. Furthermore, even when increasing the stability of the protein to unfolding, in the case of the Y73F protein mutant, the proportion of complexed protein remained lower than that observed for wild type protein. This suggests that overall protein stability is less important than the hydrogen bonding interactions of Y73 and the adenine ring of the CoA ligand in stabilizing this protein−ligand complex in the gas phase. The results from this study suggest that the nature of the interaction stabilizing a protein−ligand complex can differ substantially in the gas and solution phases, and suggest that the potential of MS for selecting ligands with particular binding characteristics may be an important feature of this technique. For example, the ability to discriminate between binding stabilized by ionic as opposed to hydrophobic interactions may prove useful in selecting a subset of ligands with particular binding characteristics.

The results presented here also support conclusions that the ability of MS to monitor hydrogen−deuterium exchange phenomena represent a powerful means for probing structure and complex formation.[19−21] Since *apo* and ligated ACBP share the same overall fold[25] the increased protection from exchange

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(36) The two amides protected from hydrogen exchange in the acyl CoA ligand observed in the real time hydorgen exchange experiments were not observed in the pulse-labeled experiment since CoA ligand was added in protonated refolding buffer.

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observed by both MS and NMR must arise from interactions with the CoA ligand. Furthermore, this increased protection from exchange was observed in pulse labeling experiments in the presence of CoA ligand. It is important to note that the two acyl CoA protein complexes, with substantially different dissociation constants, do not show a significantly different degree of hydrogen exchange protection.. The dissociation constants of the acyl C_8 and C_{16} CoA protein complexes used in this study, however, are such that the protein is overwhelmingly in the complexed state and hence hydrogen exchange measurements of the more slowly exchanging amides will reflect the ligated rather than *apo* protein.[19] By contrast, the higher dissociation constant estimated for the free CoA protein complex suggests that an equilibrium exists between *apo* and CoA bound protein in solution. The level of hydrogen exchange protection measured for the CoA protein complex, intermediate between that of the *apo* and the acyl CoA bound proteins, may be attributed to this solution equilibrium effect. Similar effects on the hydrogen exchange protection have been observed in the study of peptide binding to an SH2 domain where increasing the molar ratio of peptide leads to increasing levels of hydrogen exchange protection in the protein until an equimolar ratio of peptide to protein was obtained.[20]

Hydrogen exchange protection measurements are therefore particularly valuable in distinguishing between nonspecific attachments in the gas phase and noncovalent interactions present in solution prior to ESI. The strength of this approach is three-fold. Firstly, nonspecific attachments in the gas phase will not give rise to significant changes in hydrogen exchange protection since the latter reflects solution conditions. Secondly, the results presented here suggest that even weakly bound ligands in solution, such as the free CoA protein−ligand complex, lead to an increased hydrogen exchange protection of the protein complex over that of the *apo* protein. Thirdly, the history of the complex is retained by virtue of the hydrogen exchange labeling even in the event of gas phase dissociation. This is demonstrated by both real-time and pulse labeled hydrogen exchange experiments in which the extent of hydrogen exchange protection was found to be identical for both dissociated and complexed protein in the gas phase. The lack of hydrogen deuterium exchange equilibration in this system, in contrast to a cytochrome *c* gas phase hydrogen exchange study,[22] suggests a relatively short time elapses from the protein−ligand complex being in solution to the formation of gas phase protein ions. These results indicate therefore that either dissociation of the complex occurs late in the electrospray process, presumably during the desolvation of the protein−ligand ions, or that dissociation of the complex occurs prior to solvent evaporation but that the time scale of subsequent events is rapid such that loss of hydrogen exchange protection is negligible.

In conclusion, the protein−ligand interactions examined here have features which are important when comparing solution binding properties with observations in the gas phase. Careful control of the electrospray conditions is essential not only for observation of protein−ligand complexes in the gas phase but also for preserving hydrogen exchange information. The correlation between solution and gas phase binding observed for mutant proteins in competition with the wild type protein implies that some interactions are similar in gas phase and solution complexes. The lack of a correlation between the fraction of complex observed in the gas phase and solution binding affinities for ligands differing only in their hydrocarbon chain length indicates the need for caution in a simplistic use of mass spectrometry for screening purposes. The fact that different types of intermolecular forces appear to be of different relative importance in maintaining complexes in the gas and solution phases suggests, however, that their comparison can provide important insight into the nature of the interaction between a protein and its ligands.

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