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Macrocycles used as models to probe the interaction of a fatty acid derivative with its natural receptor

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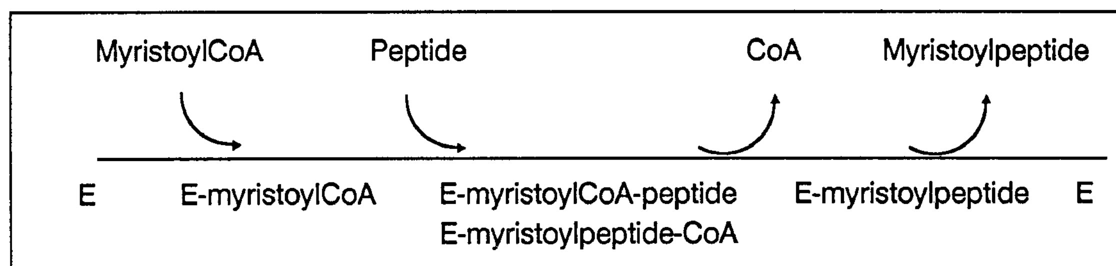
Abstract

A macrocycle-based receptor system was developed to study the interactions of fatty acids and their coenzyme A derivatives with the binding site of a receptor molecule. Although the specific compounds failed to fulfill the purpose of their design, interesting information about ammonium ion interactions with azacrowns was obtained by use of fast atom bombardment mass spectrometry.

MyristoylCoA:protein N-myristoyl transferase (Nmt, EC 2.3.1.97) is a eukaryotic enzyme that catalyzes the co-translational transfer of myristate, a rare 14 carbon saturated fatty acid, from coenzyme A to the amino-terminal Gly residue of eukaryotic proteins with diverse functions.[1] Genetic studies in the yeast *Saccharomyces cerevisiae* have shown that the 455 residue product of the *NMT1* gene is essential for vegetative growth. The acylCoA and peptide substrate specificities of *S. cerevisiae* Nmt1p have been defined using a panel of >300 fatty acid analogs, >100 peptides, and an *in vitro* enzyme assay.[2] The kinetic mechanism of this monomeric, cytosolic enzyme is ordered bi bi.

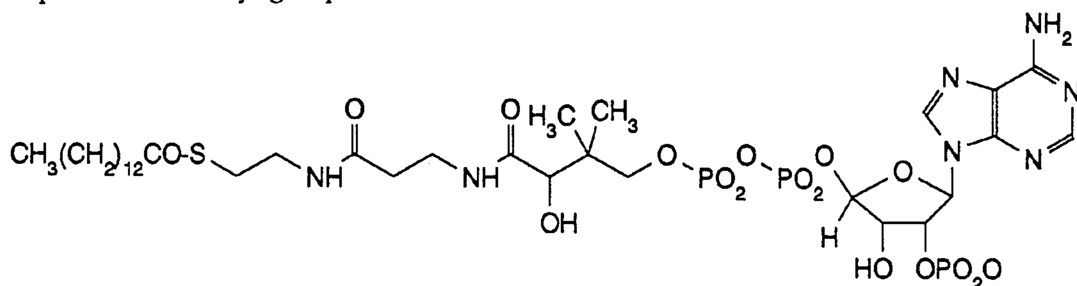


The high affinity binary Nmt1p:myristoylCoA complex has been studied using a variety of physical methods including fluorescence spectroscopy and native gel electrophoresis. A series of photopeptides has been employed in cross linking studies together with a non-hydrolyzable derivative of myristoylCoA, S-(2-oxo)pentadecylCoA, to demonstrate the ternary complex and establish that peptide substrates are unable to bind to the apo-enzyme. This result is consistent with the bi bi reaction mechanism and together with kinetic studies established that (i) cooperative interactions occur between Nmt1p's acylCoA and peptide binding sites and (ii) binding of myristoylCoA is required for synthesis of a functionally competent peptide binding site.

We have begun a series of experiments designed to create a model system based on macrocycle chemistry for characterizing the forces which participate in the binding of fatty acids and their acylCoA derivatives to proteins.

RESULTS AND DISCUSSION

The structure of coenzyme A is shown in the figure below. It has many polar residues as part of its structure so it is easy to imagine how binding might occur by a variety of hydrogen bond or dipolar interactions, although little about this question is known with any certainty. Myristic acid, on the other hand, presents the enzyme with a cylinder about 14Å long that is non-polar except for the carboxyl group.



Myristic acid thioester of coenzyme A

(Myr-CoA)

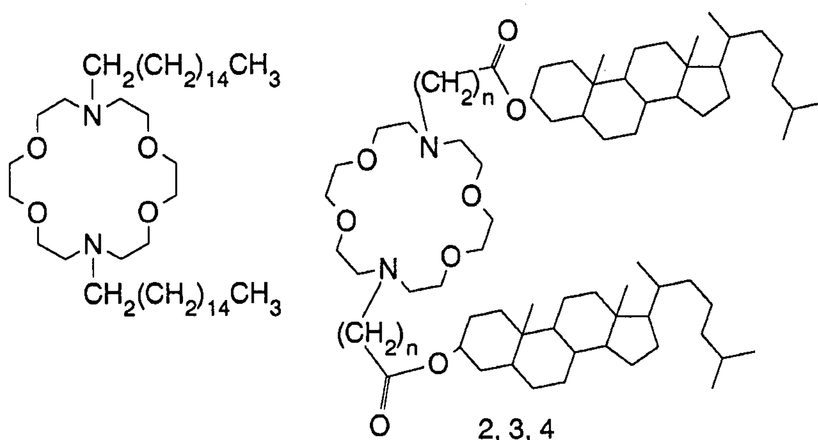
Two important forces can obviously contribute to the overall free energy of binding (ΔG): van der Waals interactions between the hydrocarbon surfaces (ΔH) and loss of water from the binding site when the myristic acid derivative is bound (ΔS). The binding constant (K) can be assessed and it directly reflects the free energy of complexation (ΔG=RT lnK). It is also true that $\Delta G = \Delta H - T\Delta S$. What is unknown and what is not easily assessed is whether binding of the fatty acid is dominated by the enthalpic (ΔH) or entropic term ($T\Delta S$), if indeed one dominates the other.

One obvious way to assess the importance of water displacement from the enzyme's active site is to undertake direct thermodynamic measurements on the natural system. This could presumably be accomplished either by calorimetry or by determining the equilibrium constant at a variety of temperatures (the van't Hoff method). Because of the obvious difficulty associated with such an undertaking, we decided to prepare a model system that might give relevant information about such binding.

Design rationale Our approach to this problem was to prepare a simple receptor molecule that might have a stronger interaction with myristic acid than with a shorter carboxylic acid such as acetic acid. We envisioned measurement of the equilibrium binding constant between the receptor and the substrate. In principle, the observed log K_S value should be greater when hydrocarbon contacts were greater than when they were either weak or absent. We selected a macrocyclic polyether as the basic binding site for the receptor system. By using a crown ether, it would be possible to bind either a metallic cation, perhaps of the form $M^{+-}O_2C(CH_2)_nCH_3$, or an ammonium salt of the form $CH_3(CH_2)_nNH_3^+$.

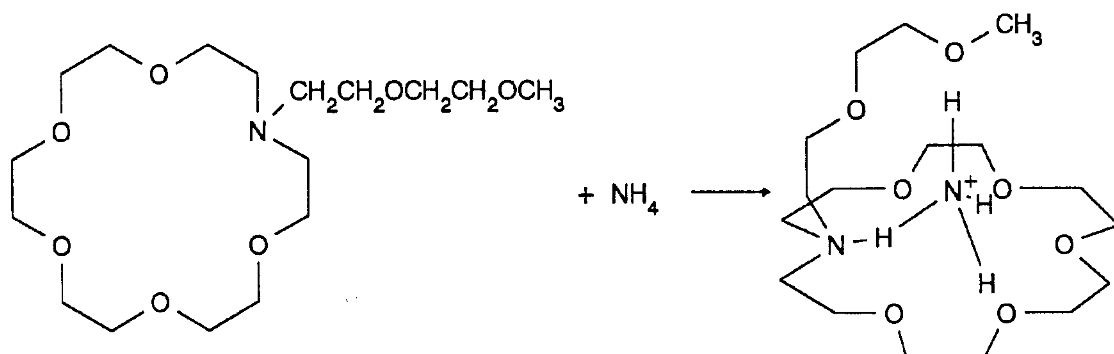
Previous studies involving diaza-18-crown-6[3] suggested the possibility that attachment of two hydrocarbon sidechains to the macroring might afford a receptor that could interact with the $CH_3(CH_2)_n-$ chain in either a bound ammonium salt or metal carboxylate. The next question was what sort of residue could effectively interact with a hydrocarbon chain. An *N,N'*-dialkyldiaza-18-crown-6 compound such as **1** or the corresponding trialkyltriaza[4] compound both seemed possible although the latter would likely be more difficult to prepare. The problem with both of these compounds is that the hydrocarbon chains are very flexible and the probability of significant contact with a bound amphiphilic salt is small.

An examination of Corey-Pauling-Koltun (CPK) molecular models suggested that the *alpha* surface of a steroid such as cholestanol is very nearly flat. Of course, the *beta* surface is undulated by the presence of angular methyl groups making it less useful so there is some statistical problem with this choice. Nevertheless, either a steroid or a polyaromatic compound seemed to hold forth the greatest promise. The polyaromatic has the advantage that aromatic amino acids are known to line the cavity of certain squid axon cation channels[5] and intestinal fatty acid binding protein.[6] The disadvantage is that a single extended surface of aromatic rings such as found in anthracene or phenanthrene is usually characterized by very poor solubility. The synthesis of *bis*(steroid) **2** was patterned on the preparation of steroidal lariat ether compounds[7] that have been found to be of use in the formation of membranes.[8]



Solution ammonium ion binding In principle, either a myristic acid carboxylate salt or an ammonium salt of the form $R{-}NH_3^+$ should be able to form complexes with crowns generally and with the steroidal crown compound in particular. We previously used ammonium salt complexation in methanol solution to demonstrate intramolecular sidearm participation in binding by lariat ethers.[9] In this study, a series of 15- and 18-membered ring aza-lariat ethers alkylated at nitrogen by $(CH_2CH_2O)_nCH_3$ sidearms were complexed by NH_4^+. Molecular models (CPK) suggested that binding would be generally weak for 15-membered ring structures and stronger for lariat ethers containing 18 atoms in the macroring. Further, it appeared that for the 18-membered ring structures, three N–H•••H hydrogen bonds would form to the ring and the remaining (perpendicular) hydrogen bond would form to the second oxygen of the $(CH_2CH_2O)_nCH_3$ sidechain. Peak binding was observed for *N*-2(-2-methoxyethoxy)ethoxyaza-18-crown-6, **3**. It was also interesting to note that the binding constant for **3** in anhydrous methanol was approximately 4.8 log units. If this represents four hydrogen bonds, then each interaction is valued at 1.2 log units. The 15-membered ring compounds, which models suggested should not be able to form more than three hydrogen bonds, showed peak binding near 3.6 log units.

Hydrogen bonding to crown ethers Hydrogen bonded complexes of macrocyclic polyethers



have been known almost from the discovery of these remarkable compounds. We have recently shown that although such interactions are well documented, important questions about the orientation of N—H bonds in aza-macrocycles remain.[10] It appears likely that ammonium ion ($R—NH_3^+$) N—H•••N hydrogen bonds form in the case of aza- and triaza-18-crown-6 derivatives (an NHN bond is symmetry required in diaza) but the evidence for such interactions is scant.

Analysis by fast atom bombardment mass spectrometry Fast atom bombardment mass spectrometry (FAB/MS) is emerging as an excellent method to assess complexation interactions between crown ethers and various cations.[11] The method involves the analysis of gas phase ions but due to the nature of desorption process, the spectra generally reflect the solution interactions although ionization processes may occur in the selvedge region.[12]

Our first thought about how to assess any hydrophobic interaction that might occur between the steroid's *alpha* face(s) and the cation's hydrocarbon chain was to use *bis*(steroid) **2** and sodium myristate. Unfortunately, this suffered from the obvious difficulty that the resulting crown•Na^{+-} OOC—R complex would be neutral in the presence of the carboxylate anion and therefore undetectable. The free Na^+•crown complex would give an ion current but the absence of the counteranion would negate the entire premise of the experiment.

We shifted our attention to alkylammonium salts such as $R—NH_3^+$ on the presumption that a complex of the form $R—NH_3^+$•crown would be charged and the integral alkyl (R) group would interact (or not) with **2**. Specifically, we studied ethyl-, nonyl-, and tetradecylammonium chlorides in the presence of **2**. Our expectation was that a tripod complex between the azacrown and ammonium ion would form and then the steroidal sidechains of **2** would interact with R, leading to more stable and therefore more prominent ions, as R increased in size. When a mixture of ethylammonium chloride ($CH_3CH_2NH_3^+Cl^-$), 18-crown-6, and *m*-nitrobenzyl alcohol were subjected to FAB mass spectral conditions, an intense ion was observed at m/z 310 that corresponds to [18-crown-6•$CH_3CH_2NH_3$]$^+$. Similarly dominant ions were detected at m/z 408 and m/z 478 respectively when either nonylammonium or tetradecylammonium cations were combined with 18-crown-6 in the FAB matrix, suggesting ready formation of the complexes [18-crown-6•$CH_3(CH_2)_nNH_3$]$^+$ where n = 8 or 13. When the same complexation experiment was performed on a 1:1:1 mixture of ethyl-, nonyl-, and tetradecylammonium chlorides, the m/z 478 ion dominated the spectrum, the m/z 408 ion was about half as intense, and the ion corresponding to the ethylammonium complex was weak. This order, $C_{14} > C_9 > C_2$, of complex stability seems reasonable if the alkyl groups are aligning at the gas-liquid interface of *m*-nitrobenzyl alcohol. The longer alkyl groups will surely dominate the surface and this should be reflected in the intensities of the ions observed.

Thus the assessment of sidearm interactions by this comparative mass spectral experiment was compromised by the alkylammonium salt surface interactions. Nevertheless, we conducted a systematic study of *bis*(steroid)-crown derivatives (**2-4**) in *m*-nitrobenzyl alcohol with tetradecylammonium chloride. We were surprised to find that the dominant process in each case was proton transfer. In the mass range where complexation was expected, only very minor peaks were present. Amplification of this region showed ions that did not correspond in an obvious way to the expected complexes. For example, in the case of **3**, an ion was observed at m/z 1443. This is *two* daltons less than expected for **3**•$C_{14}H_{29}NH_3^+$. There is no chemical process obvious to us that can account for this. Moreover, two other ions, major in the amplified region, having m/z 1368 and 1383, respectively. These are 62 and 77 daltons less than expected for **3**•$C_{14}H_{29}NH_3^+$. We were unable to attribute these peaks either to matrix ions or any known or expected contaminant in the system.

Essentially the same phenomenon was observed for **4**. Again, the protonated ion dominated, a peak was observed at a mass corresponding to [**4**•$C_{14}H_{29}NH_3^+$]-2H. Finally, the peaks at mass values of 62 and 77 daltons less than expected were also present. A control experiment was run to assess the effect of the steroid sidechains. Exactly the same experiment was conducted using *N,N'*-bis(hexadecyl)diaza-18-crown-6 and tetradecylammonium chloride in *m*-nitrobenzyl alcohol.

We were surprised to see that the results of this experiment exactly paralleled those described above. At the present time, we cannot account in detail for these remarkable phenomena. Nevertheless, these preliminary studies show clearly that simply designed receptors, especially in conjunction with a sensitive technique such as FAB/MS may reveal details of weak interaction that are of considerable biological importance.

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