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Thermodynamics of binding by calmodulin correlates with target peptide α-helical propensity

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Abstract

In this work we have examined contributions to the thermodynamics of calmodulin binding from the intrinsic propensity for target peptides to adopt an α-helical conformation. Calmodulin target sequences are thought to commonly reside in disordered regions within proteins. Using the ability of TFE to induce α-helical structure as a proxy, the six peptides studied range from having almost no propensity to adopt α-helical structure through to a very high propensity. This despite all six peptides having similar calmodulin-binding affinities. Our data indicate there is some correlation between the deduced propensities and the thermodynamics of calmodulin binding. This finding implies that molecular recognition features, such as calmodulin target sequences, may possess a broad range of propensities to adopt local structure. Given that these peptides bind to calmodulin with similar affinities, the data suggest that having a higher propensity to adopt α-helical structure does not necessarily result in tighter binding, and that the mechanism of calmodulin binding is very dependent upon the nature of the substrate sequence.

Introduction

Until about twenty years ago, the dominant view in biochemistry was that proteins require structure before they can function. In recent years however, it has become apparent that intrinsically disordered proteins (IDP's) and intrinsically disordered regions (IDR's) within proteins are extremely common and often involved in important biological functions [1-5]. IDRs have been found to play crucial roles in transcription, signaling pathways, and immune systems. IDR's have been implicated in numerous diseases states including various cardiovascular [6] and protein aggregation diseases [3], and cancer [7]. Examples of important proteins that make use of IDR's include the phosphatase calcineurin [8], many adapter proteins such as Nck [9], and transcriptional co-activators such as CBP [5].

Intrinsic disorder refers to regions of the polypeptide chain that do not fold into stable structured states, but rather, exist as dynamic ensembles of conformations [3]. The idea of dynamic ensembles does not rule out the presence of local structure, for example α-helices, but such local structure is thought to be transient. IDR's and IDP's often function by undergoing a disorder to order transition upon being bound by another protein (i.e. they fold upon binding). Recently, Dunker and co-workers [10, 11] and Tompa and co-workers [12] have suggested that regions within an IDR that possess a significant propensity to adopt local structure could act as molecular recognition features. Such features would act as binding sites for other proteins. Molecular recognition features with significant α-helix propensity appear to be most common. Despite their propensity to adopt local structure, such features are typically still dynamic and would be considered disordered.

An extremely important system that appears to take advantage of coupled folding and binding is calmodulin (CaM) and its binding targets (CaMBTs) [13]. Here CaMBT refers to the specific sequence bound by CaM in the target protein. CaM is a calcium-sensing protein that regulates the activities of many enzymes in response to changes in calcium concentration. CaM has around 300 known ligands [14]. These include important enzymes such as calcineurin [8], CaM kinase I [15] and smooth muscle myosin light chain kinase [16]. When CaM binds it induces, in most but not all cases, α-helical structure in the CaMBT sequence [17, 18]. Radivojac et al. [13] employed a bioinformatics approach to predict that unbound CaMBT sequences are often within disordered regions.

Brokx et al. [19] have studied the detailed thermodynamics of CaM binding to a series of CaMBT peptides. They demonstrated that CaM binding to some CaMBTs is enthalpically driven, whereas with the binding of others is dominated by favorable changes in entropy. They note that binding of a fully α-helical peptide to CaM should be accompanied by a small, unfavorable enthalpy of binding, ΔH_{bind}, and a large favorable entropy of binding, ΔS_{bind}, due to dehydration of hydrophobic surfaces on the peptide and CaM binding surfaces. Binding and folding a fully disordered CaMBT peptide should be accompanied by a large favorable ΔH_{bind} due in part to the formation of intrahelical hydrogen bonds, and a smaller, perhaps unfavorable, ΔS_{bind}, the latter arising from the difference between favorable dehydration of hydrophobic surfaces and unfavorable dehydration of polar groups. Based on this, Brokx et al. [19] hypothesize that the thermodynamics will be correlated in part with the extent of α-helical structure within the CaMBT prior to binding by CaM.

In this work we set out to test the hypothesis of Brokx et al. [19]. Upon finding that five of the six CaMBTs examined possessed no detectable α-helical structure, we modified the hypothesis to state that the thermodynamics would be in part correlated with the *propensity* for the CaMBT to adopt an α-helical conformation. 2,2,2-Trifluoroethanol (TFE) is well known to induce α-helical structure in peptides possessing a propensity to be helical. Using the ability of TFE to induce α-helicity in the CaMBT's as a proxy for helical propensity, we present evidence in support of our hypothesis.

Materials and Methods

Peptides corresponding to CaMBTs from calmodulin-dependent kinase I (CaMKI), 3',5'-cyclic nucleotide phosphodiesterase (PDE) and smooth muscle myosin light chain kinase (smMLCK) were synthesized by NEO-Peptide (Cambridge, MA). CaMBTs from cerebellar nitric-oxide synthase (cNOS) and caldesmon (CaD-A) were synthesized by Pi Proteomics (Huntsville, AL). All peptides were purified to >95% homogeneity using reverse-phase HPLC, with their identities confirmed via mass spectrometry. Bee venom melittin was purchased from GenWay Biotech (San Diego, CA) and was used without further purification. Peptide sequences are given in Table I. All other reagents were purchased from Sigma (St. Louis, MO) and were of the highest purity available.

Circular dichroism (CD) spectra were collected at 20° C using a Jasco J-810 spectrapolarimeter equipped with Peltier temperature control block. Peptides were dissolved in a buffer consisting of 20 mM Tris, 200 mM NaCl, 2 mM $CaCl_2$ at pH 7.5. Peptide concentrations used for CD measurements were 100 μM. Spectra were obtained using a 1 mm pathlength quartz cuvette and

are the average of three scans taken at 50 nm.min.$^{-1}$ Reported spectra are not smoothed and have an estimated error of around ±3 %.

Results

CD spectra for the six CaMBT peptides are shown in Figure 1. In the absence of TFE five of the six have spectra indicative of disorder (Figure 1a), i.e. they have no detectable persistent secondary structure. The one exception is pPDE which, in the absence of TFE, has a CD spectrum with strong negative bands at 222 nm and 208 nm. These are indicative of a high average α-helix content.

When TFE is included in the solutions, most of the peptides gain apparent α-helix content (Figure 1). In the presence of 10% TFE, pMEL (melittin) possesses a spectrum indicating that the average α-helix content is almost as high as that for pPDE. Peptides pCAD-A, pCAMKI, pMLCK and pcNOS are more resistant to the α-helix-inducing abilities of 10% TFE (Figure 1b).

The inclusion of 40% TFE results in CD spectra that can be parsed into three categories (Figure 1c). Peptides pPDE, pMEL and pcNOS all possess high levels of α-helix content. Based on the ellipticities measured at 222 nm, $[\theta]_{222nm}$, these three peptides are essentially 100% α-helical in 40% TFE [20, 21]. Peptides pCAMKI and pMLCK have gained significant average α-helical character (estimated at ~70%), whereas pCAD-A still appears predominantly disordered in nature.

The free energy of binding to CaM, ΔG_{bind}, for each peptide is listed in Table I. The thermodynamic data were selected such that the solution conditions under which they were

measured most closely match those under which the CD data described above were collected. Notably, the ΔG_{bind} values span a relatively narrow range, with four of the six peptides having binding energies in the range -43 to -48.4 kJ.mol.$^{-1}$, indicating these peptides possess similar CaM-binding affinities. Figure 2 shows plots of enthalpy, ΔH_{bind}, and entropy, $-T\Delta S_{bind}$, measured for each peptide binding to CaM, against ellipticities measured at 222 nm, $[\theta]_{222nm}$, for each peptide in 0 and 40% TFE (the 10% TFE data is omitted for clarity). The thermodynamic data for pMLCK was taken from Wintrode and Privalov [22], with the remainder being drawn from Brokx et al. [19]. The CaMBTs most resistant to the helix-ordering action of TFE - pCAD-A, pCAMKI and pMLCK - have the most favorable changes in ΔH_{bind} (Figure 2a) and smallest, and in two cases positive, changes in $-T\Delta S_{bind}$ (Figure 2b). The peptides whose binding to CaM are most clearly entropically driven correspond to those that are most easily induced to form α-helical structure (Figure 2b).

Discussion

The calcium-sensing protein CaM is of great importance given that it binds to and modulates the activity of many other proteins [14]. Brokx et al. [19] employed isothermal titration calorimetry, ITC, to measure the thermodynamics of CaM binding to their chosen target sequence peptides (CaMBTs). The CaMBT peptides chosen were either known or assumed to bind to CaM in the most common manner, with CaM wrapping around and inducing α-helical structure in the CaMBT. These authors found that, although all of the CaMBTs examined were bound by CaM with similar affinities, the binding thermodynamics ranged from being entropically driven to enthalpically driven. They hypothesized that CaM binding to a disordered peptide would lead to a large, favorable ΔH_{bind} resulting from formation of intrahelical hydrogen bonds within the

peptide. The accompanying ΔS_{bind} would be smaller, the difference between unfavorable dehydration of backbone polar groups within the peptide and favorable dehydration of hydrophobic surfaces on the peptide and CaM. On the other hand, the thermodynamics of CaM binding to a fully α-helical peptide would be dominated by a favorable ΔS_{bind} from dehydration of hydrophobic surfaces.

Radivojac et al. [13] used a bioinformatic approach to analyze CaMBTs and predicted that they most likely exist within disordered regions in the CaM target proteins. The CD spectra for the CaMBT peptides examined here in buffer, with the possible exception of that for pPDE, are largely supportive of this hypothesis (Figure 1a). The presence of significant secondary structure within the pPDE peptide does not refute the disordered region hypothesis. This helical segment could be flanked by disordered regions within 3',5'-cyclic nucleotide phosphodiesterase. Given that the most common mode of CaM binding is for it to wrap around and induce α-helical structure in its binding region [17, 18], having the binding region be readily accessible within a disordered domain would likely lead to energetically more favorable binding.

The CD spectra in Figure 1a do however pose an issue. If the CaMBTs are themselves disordered, as opposed to being flanked by disorder, then where would that leave the hypothesis of Brokx et al. [19]? Based on these CD data alone one would predict that binding of pPDE to CaM is entropically driven, while binding of the other five peptides is enthalpic in nature. Yet the data of Brokx et al. [19] indicate that binding of pPDE, pMEL and pcNOS are all entropically driven, whereas pCAMKI and pCAD-A are more enthalpically driven. Binding of pMLCK under the conditions closest to those utilized here has small favorable contributions from both ΔH_{bind} and ΔS_{bind} [22]. This led us to alter the Brokx et al. hypothesis to state that the thermodynamics would

be in part correlated with the propensity for the CaMBT to adopt an α-helical conformation. That is, the more readily α-helical structure is induced within the CaMBT, the more likely CaM binding is entropically driven.

To test this hypothesis, CD spectra of the CaMBT peptides in varying amounts of TFE were collected. TFE is well known to induce α-helical structure in peptides with a propensity to adopt this conformation. The induction of helix by TFE is used in this work as a proxy for α-helical propensity - peptides that are easily induced have a higher propensity than those that require high concentrations of TFE before adopting an α-helical conformation. Figures 1b and 1c show the CD spectra collected for the six CaMBTs in the presence of 10% and 40% TFE respectively. As noted above, the CaMBTs can be separated into three broad groups. pPDE and pMEL are part of one group, with the former being the only CaMBT to be α-helical in the absence of TFE, and the latter being very readily induced into the conformation with just 10% TFE. pcNOS can also be considered a part of this high α-helical propensity group - upon addition of 40% TFE it has a CD spectrum indicative of essentially 100% α-helix content (Figure 1c). Frederick et al.[23] have shown that both pPDE and pcNOS bind to CaM in the more usual manner, with CaM wrapped around and inducing α-helical conformations in the peptides. To the best of our knowledge a high-resolution structure of pMEL bound to CaM does not exist. However, Schulz et al. [24]have used cross-linking and mass spectrometry to derive low-resolution structures of the complex. These structures indicate that pMEL is bound by CaM in the canonical manner.

pCAMKI and pMLCK form the second grouping. These CaMBTs require 40% TFE in order to have significant average α-helical content (~70%). These data suggest that pCAMKI and

pMLCK have some propensity to adopt the α-helical conformation, but that the propensity is not high. Despite lower propensities to adopt α-helical conformations, according to high resolution structures of CaM bound to pCAMKI [15] and pMLCK [18], both of these complexes are of the canonical form.

pCAD-A stands alone in that even in 40% TFE it does not possess significant detectable α-helical content. This implies that pCAD-A has a very low propensity to adopt an α-helical conformation, which in turn suggests that binding to CaM should be energetically unfavorable. Yet this peptide binds to CaM with an affinity approximately in the same range as the other five CaMBTs studied (Table I). As it turns out, there is evidence that this CaMBT does not bind CaM in the canonical manner. Vogel and co-workers [25] studied the two CaM binding regions, CAD-A and CAD-B, in caldesmon and showed that, although they can bind simultaneously to CaM, only the CAD-B region becomes helical. Thus, the pCAD-A CaMBT is likely not destined to become α-helical upon binding to CaM and therefore does not require a significant propensity to adopt the CaM-bound conformation.

The ellipticity at 222nm, $[\theta]_{222nm}$, is often used as a measure of the average α-helix content of a peptide. Figure 2 shows plots of $[\theta]_{222nm}$ for each CaMBT in 0 and 40% TFE against the changes in enthalpy and entropy experienced upon CaM binding. From these plots there appears to be a correlation between average α-helix content and the enthalpic and entropic cost or gain when TFE is present. In the absence of TFE there appears to be little correlation between $[\theta]_{222nm}$ and either ΔH_{bind} or $-T\Delta S_{bind}$ (r^2=0.13 and r^2=0.14 respectively). The poor correlations appear to be largely due to the significant helical content of pPDE versus almost no detectable helix in the other peptides. As the TFE concentration is increased to 10%, there appears to be a significant

increase in correlation (r^2=0.65 and r^2=0.68 respectively; data not shown). Finally, in the presence of 40% TFE the correlations between $[\theta]_{222nm}$ and either ΔH_{bind} or $-T\Delta S_{bind}$ are r^2=0.64 and r^2=0.59 respectively. The lack of increase in correlation when moving from 10 to 40% TFE is simply a result of three peptides, pPDE, pcNOS and pMEL, possessing essentially maximal helical content in the presence of 40% TFE (Figure 2). The very similar correlation between $[\theta]_{222nm}$ and ΔH_{bind} and $-T\Delta S_{bind}$ at a given %TFE is a result of the enthalpy-entropy compensation that gives rise to the similar CaM-binding affinities measured for these CaMBTs (Table I) [19].

That the correlation between average α-helix content and $-T\Delta S_{bind}$ (or ΔH_{bind}) is not stronger is not surprising given the complexity of the various contributions to ΔG_{bind} for CaMBTs binding to CaM. These include van der Waals and electrostatic interactions, changes in solvation (including hydrophobicity), changes in conformational entropy, and even peptide length. Brokx et al. [19] were unable to find any significant correlation between the binding thermodynamics and any of those properties. Despite the lack of correlation, there is little doubt that each of these properties could be making a significant contribution to the energetics of CaM binding.

In a more recent study, Frederick et al. [23] found a strong linear correlation between the change in conformational entropy that CaM undergoes upon binding a CaMBT, and $-T\Delta S_{bind}$ (r^2=0.78). They found that changes in the internal dynamics of CaM varied significantly between the six CaM:CaMBT complexes they studied.

In this work we have focused on the contribution of the intrinsic propensity for each CaMBT peptide to adopt an α-helical conformation to the thermodynamics of CaM binding. Using the

ability of TFE to induce α-helical structure as a proxy, the six CaMBTs studied range from having almost no helical propensity (pCAD-A) through to a very high propensity (pPDE; Figure 1). Even if we discount pCAD-A given that it likely binds CaM in a non-helical conformation [25], the remaining five CaMBTs span a significant range of propensities. Our data indicate there is some correlation between these deduced propensities and the thermodynamics of CaM binding (Figure 2). It has been suggested that molecular recognition features, such as CaMBTs, possess significant propensity to adopt local structure [10-12]. Although that would certainly appear to be the case for pPDE, pMEL and even pcNOS, the other two of the CaMBT peptides studied known to be α-helical when bound by CaM appear to have much weaker propensities. This despite all CaMBT peptides studied having similar CaM-binding affinities (Table I). This finding implies that, at least for CaMBTs, molecular recognition features may possess a broad range of propensities to adopt local structure.

It is often assumed that pre-organization of ligand structure "prepays" some of the cost of conformational entropy lost upon binding. Thus, ligands that are predominantly in the bound conformation prior to complex formation would have a higher binding affinity than those that are not. In studies of ligands binding to the SH2 domain from Grb2, Martin and co-workers [26, 27] have found that this common assumption does not always hold. Furthermore, Bachmann et al. [28] used kinetic analyses to study the binding of the S-peptide to ribonuclease S. Specifically they asked to what extent the S-peptide adopted α-helical structure in the transition state of the binding reaction. These authors did not detect any α-helix in the transition state, suggesting that the S-peptide folded *after* binding to ribonuclease S. Experiments in which mutations were introduced that altered the helical propensity of the S-peptide reinforced this conclusion.

Does CaM bind to pre-organized CaMBTs, as might be implied by the molecular recognition feature hypothesis [10-12], or do CaMBTs undergo folding after binding more akin to the S-peptide binding to ribonuclease S [28]? As noted by Kiefhaber et al. [29], whether a given binding reaction fits the folding-after-binding or pre-organized ligand models can really only be determined via characterization of the transition state. The structural and thermodynamic data discussed here can only provide suggestions as to which model, if either, CaM binding to a given CaMBT follows. Of the CaMBT peptides studied here, only pPDE possesses significant α-helical structure in the unbound state (Figure 1a), implying that it might be binding to CaM in a pre-organized conformation. This binding is accompanied by a large favorable ΔS_{bind}, presumably due to burial of hydrophobic surface area [19]. At the other extreme, pCAD-A has very little α-helical character even in the presence of 40% TFE (Figure 1c), with its binding to CaM being driven by a favorable ΔH_{bind}, possibly arising from formation of hydrogen bonds. These data imply that CaM binding pCAD-A might adhere to the folding-after-binding model, although as noted above, pCAD-A may not be α-helical when bound to CaM [25]. It is more difficult to assess what might be happening with the other CaMBTs. Importantly, the CaMBTs studied here bind to CaM with similar affinities (Table 1) [19], suggesting that CaM binding can follow either model depending on the nature of the CaMBT. Alternatively, there is a continuum of binding mechanisms with the two models defining the ends of the spectrum. Future studies that characterize the transition states of CaM binding to CaMBTs are required to distinguish between these possibilities.

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Figure Legends

Figure 1: Far UV CD spectra collected at 20°C for the six CaMBTs studied. **a.** In buffer, **b.** in the presence of 10% TFE, and **c.** with 40% TFE.

Figure 2: Plots of $[\theta]_{222nm}$ measured for the CaMBTs in 0 and 40% TFE against **a.** ΔH_{bind} and **b.** $-T\Delta S_{bind}$. Values of ΔH_{bind} and $-T\Delta S_{bind}$ were obtained from Brokx et al. [19] for all peptides except pMLCK, the data for which was obtained from Wintrode and Privalov [22]. Thermodynamic data used was chosen such that conditions under which it was measured most closely resembled those used to obtain the CD data.

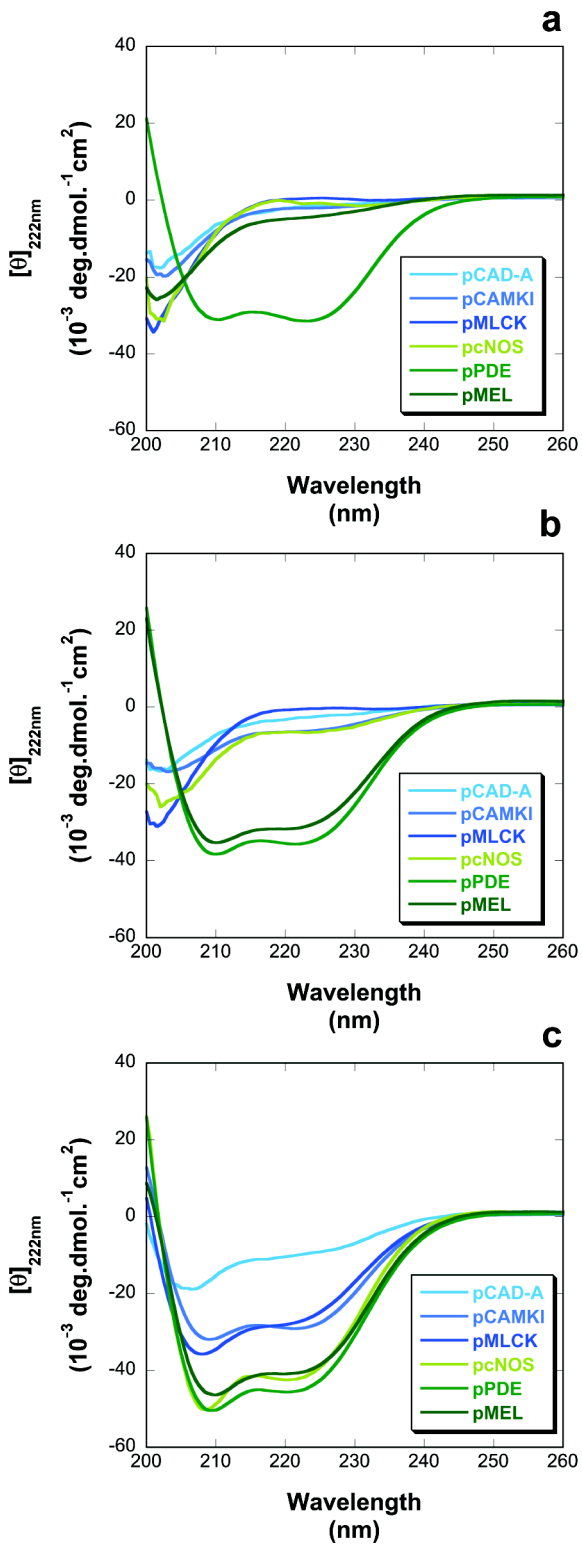
Table I: Sequences of the CaM-binding peptides and their free energies of binding to CaM.

Peptide	Sequence	ΔG_{bind} (kJ.mol^{-1})
pCAD-A	GVRNIKSMWEKGNVFSS	-35.1[a]
pCAMKI	AKSKWKQAFNATAVVRHMRKLQ	-43.0[a]
pMLCK	ARRKWQKTGHAVRAIGRLSS	-34.2[b]
pcNOS	KRRAIFKKLAEAVKFSAKLMGQ	-46.3[a]
pPDE	QTEKMWQRLKGILRCLVKQL	-45.9[a]
pMEL	GIGAVLKVLTTGLPALISWIKRKRKQQ	-48.4[a]

[a] Calculated from K_a values measured by Brokx et al. [19].

[b] Calculated from K_a values measured by Wintrode and Privalov [22].

Figure 1: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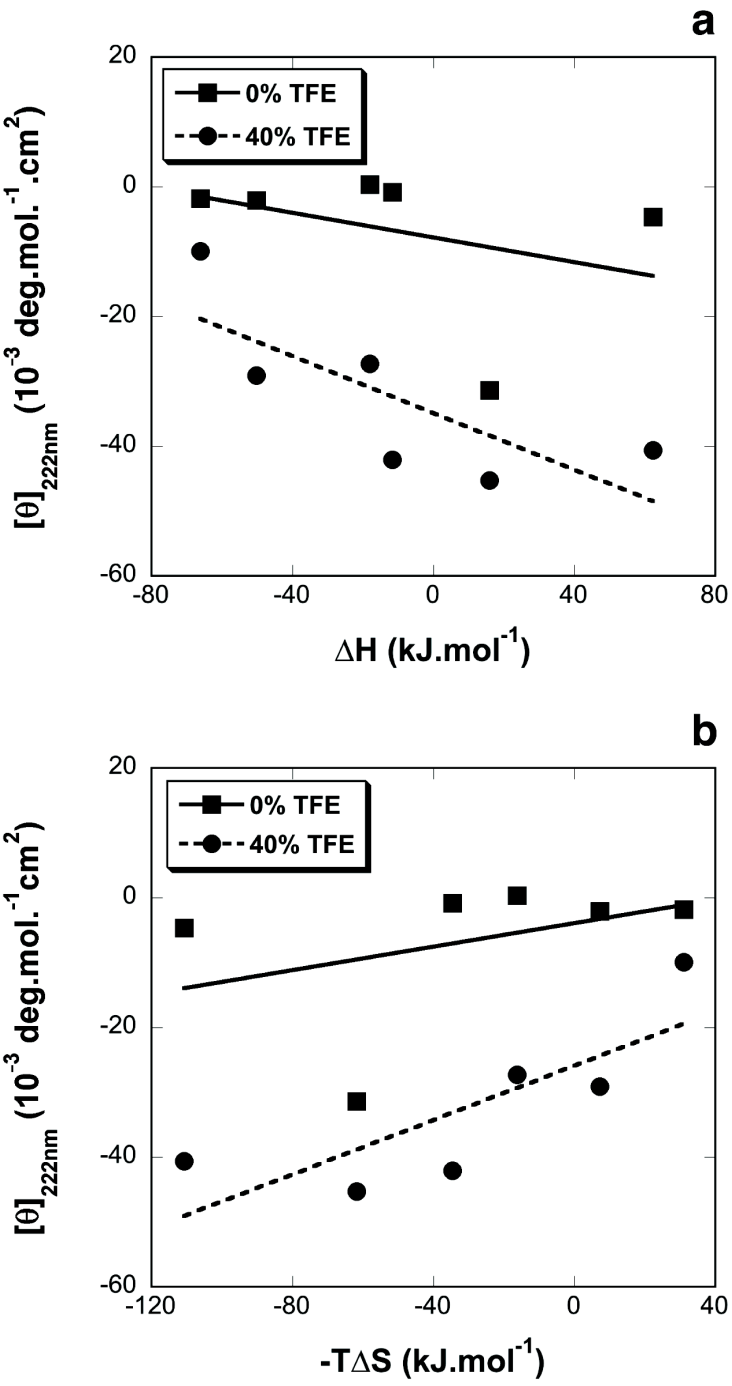
260

Figure 2:


a
0% TFE
40% TFE
$[\theta]_{222nm}$ (10^{-3} deg.mol.$^{-1}$.cm^2)
ΔH (kJ.mol^{-1})
b
0% TFE
40% TFE
$[\theta]_{222nm}$ (10^{-3} deg.mol.$^{-1}$.cm^2)
$-T\Delta S$ (kJ.mol^{-1})