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The denaturation behaviour of calmodulin in sodium *n*-dodecyl sulphate, dodecyl trimethyl ammonium bromide, guanidine hydrochloride and urea

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Abstract

The denaturation behaviour of calmodulin (CaM) in sodium *n*-dodecyl sulphate (SDS), dodecyl trimethyl ammonium bromide (DTAB), guanidine hydrochloride (GuHCl) and urea was studied by fluorescence spectrophotometry at 25 and 37°C in Tris-HCl buffer, pH 7.4.

The sigmoidal denaturation curve was plotted in order to estimate the thermodynamic parameters, assuming a two-state mechanism in terms of the Pace model.

SDS and DTAB, anionic and cationic surfactants, affect CaM on a millimolar level as a result of direct interaction between CaM and surfactant as an amphipatic molecule. GuHCl and urea affect CaM on a molar level as a result of indirect interaction with the surroundings of CaM (a change in the water structure).

The thermodynamic data indicate a slight interaction in the case of SDS which induced incomplete unfolding of CaM. With DTAB, GuHCl and urea, unfolding of CaM took place to a much greater extent.

INTRODUCTION

Calmodulin (CaM), the ubiquitous intracellular Ca^{2+}-binding protein, is a multifunctional intracellular protein, which mediates in many biochemical processes [1, 2]. CaM undergoes conformational changes which produce specific interaction site(s) recognized by many different proteins. The unique ability of CaM to bind to and stimulate the activity of a large number of enzymes allows CaM to play a pivotal role in regulating cellular function [3].

The CaM molecule consists of four homologous calcium-binding domains, I, II, III and IV. Each domain contains a helix loop, a helical calcium-binding structure [4]. In the first step, calcium binds to the two

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high-affinity sites identified as the domains I and II of CaM [5]; in the second step, Ca^{2+} binds to site III and, finally, to the lower affinity site IV [6].

CaM exists as a low molecular weight monomer (MW = 16 500) [7]. The presence of calcium leads to a resistance to denaturation (boiling, 8 M urea, 1% sodium *n*-dodecyl sulphate (SDS) [8]).

Most previous studies have focused on the role of the bound calcium in modifying the structure of CaM; only a few papers have reported the $CaM-Ca^{2+}$ structure [9–11], and they did not consider the role of the $CaM-Ca^{2+}$ free structure. The purpose of denaturation studies has always been to provide additional information on the structure, properties and function of proteins; therefore, in this paper we have attempted to study the denaturation of free $CaM-Ca^{2+}$ by four denaturants, guanidine hydrochloride (GuHCl), urea, sodium *n*-dodecyl sulphate (SDS) and dodecyl trimethyl ammonium bromide (DTAB) in order to obtain thermodynamic parameters for estimating the conformational stability of free $CaM-Ca^{2+}$.

MATERIALS

Calmodulin, from bovine brain, sodium *n*-dodecyl sulphate (SDS), dodecyl trimethyl ammonium bromide (DTAB), guanidine hydrochloride (GuHCl) and urea were obtained from Sigma. All the salts used in the preparation of the buffer were analytical grades and were made up in doubly distilled water. Tris-HCl buffer (40 mM), pH 7.4, was used.

METHOD

All denaturation curves were determined by measuring the intrinsic fluorescence intensity (277 nm excitation and 310 nm emission) of solutions containing 100 $\mu g\ ml^{-1}$ calmodulin in a thermostated (25 and 37°C) 400-μl cuvette, using an RF-5000 Shimadzu fluorescence spectrophotometer. The solutions were kept at 25°C for 15 min in the presence of denaturant to reach equilibrium.

All the measurements reported refer to SDS and DTAB concentrations below the critical micelle concentration.

RESULTS AND DISCUSSION

The urea, GuHCl, SDS and DTAB denaturation curves for CaM are shown in Fig. 1. In all cases, denaturation was followed by measuring the intrinsic fluorescence (277 nm excitation and 310 nm emission) of solutions containing 6.06 μM calmodulin, as a function of the concentration of denaturant; therefore, the curves are directly comparable.

The changes in fluorescence intensity for SDS and DTAB are much higher than those for GuHCl and urea. In addition, the effective surfactant

concentrations were found to be in the millimolar range for SDS and DTAB and in the molar range for GuHCl and urea. The reason for this is probably because GuHCl and urea preferentially take part in hydrogen bonding, affecting on the surroundings of CaM and breaking protein hydrogen bonds [12]. This enhances the solubility of non-polar molecules, including that of the amino-acid side chains, in proportion to their accessible surface area, diminishing the magnitude of the hydrophobic effect by up to one third [13]. This effect should be sufficient to produce unfolding of the protein, as shown by the sigmoidal shape in Fig. 1(c) and (d). Because the surfactants, SDS and DTAB, produce conformational changes at the relatively low concentrations of much less than 1%, this behaviour apparently reflects attractive forces between the protein molecules and the surfactant ions (molecules) which are of the same order of magnitude as the attractive forces between the surfactant ions themselves. For this reason, they combine with native proteins in multiple equilibria [14, 15], i.e. many equivalents (moles) per mole of protein. On unfolding, proteins bind even more surfactant. The mechanism of surfactant denaturation involves the binding of the surfactant ions to sites on the protein molecule, which results in unfolding and further bindings, often in a cooperative fashion [16, 17]. This means that the initial stage in the interaction involves the binding of the surfactant ion to oppositely charged sites on the surface of the native protein molecule, although the ionic interaction is modulated by hydrophobic effects [18].

A commonly used method for estimating the conformational stability of a protein is an analysis of the urea and GuHCl denaturation curves [19–22]. Except for a few studies that were made in this laboratory, surfactant denaturation curves are seldom used for this purpose [23–25]. Studying the changes in physical property, i.e. the fluorescence intensity, of a protein when it unfolds seems to reveal a great deal of information concerning its physical state. Figure 1 shows the greater change in fluorescence intensity of calmodulin when SDS and DTAB are used, as compared to GuHCl and urea, compare Fig. 1(a) and (b) with Fig. 1(c) and (d).

Many small globular proteins have been found to approach closely a two-state mechanism [26], $N \rightleftharpoons D$, in which only the native state N and the denatured state D are present in significant concentrations in the transition region. The values of Y characteristic of the native state Y_N and the denatured state Y_D can be obtained in the transition region by extrapolation from the linear portions of the denaturation curve at low and high denaturant concentration, as shown in Fig. 1.

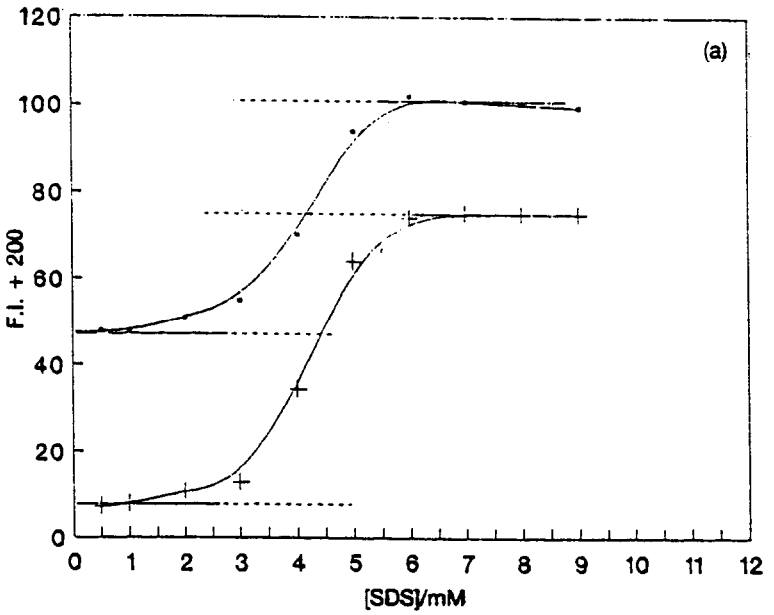
By assuming a two-state mechanism for unfolding, the fraction of unfolded protein F_D can be calculated using [27]

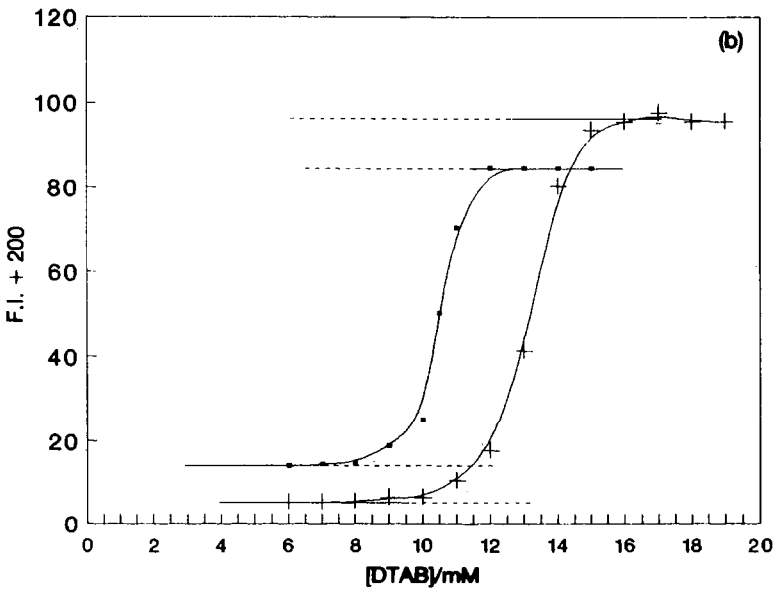
$$F_D = (Y_N - Y_{obs})/(Y_N - Y_D) \quad (1)$$

and the difference in free energy between the folded and unfolded conformations ΔG is calculated using

$$\Delta G = -RT \ln \frac{(F_D)}{(1-F_D)} = -RT \ln \frac{(Y_N - Y_{obs})}{(Y_{obs} - Y_D)} \quad (2)$$

where Y_{obs} is the observed variable parameter.





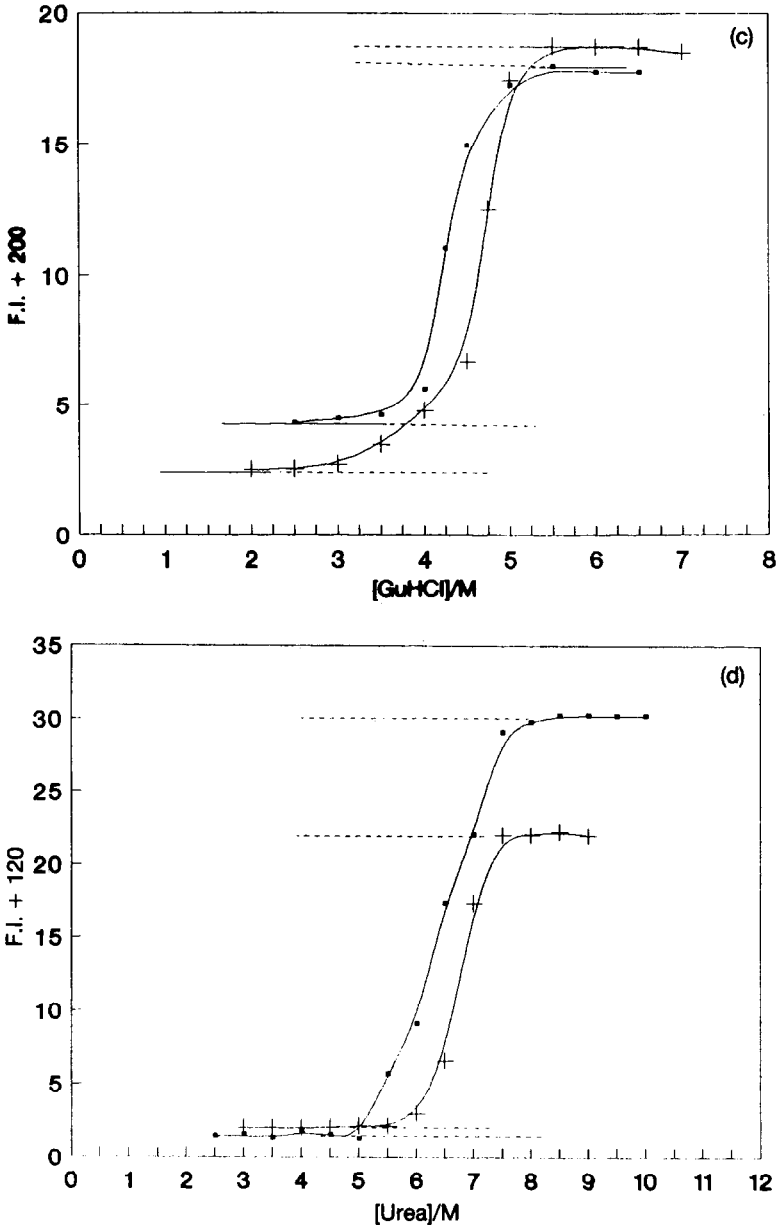


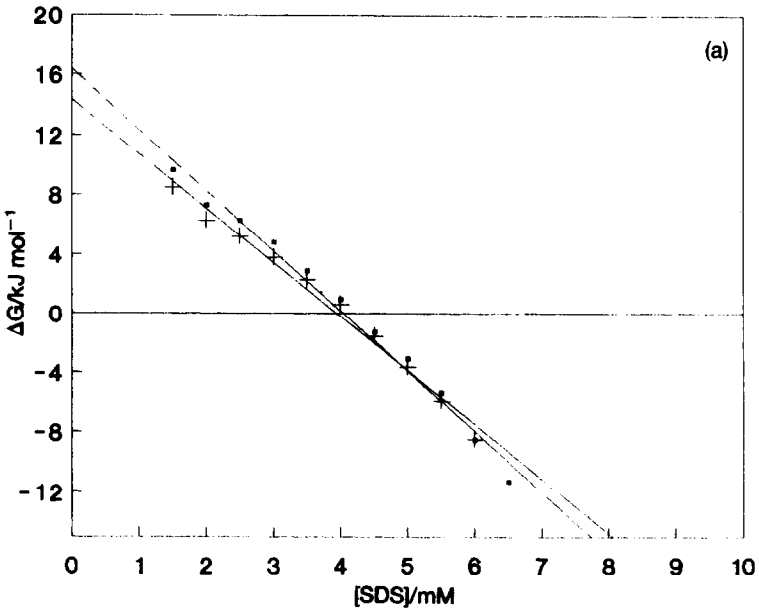
Fig. 1. Denaturation curves for calmodulin; fluorescence intensity (F.I.) versus denaturant concentrations. (a) SDS; (b) DTAB; (c) GuHCl; (d) urea: ■, 37°C; +, 25°C.

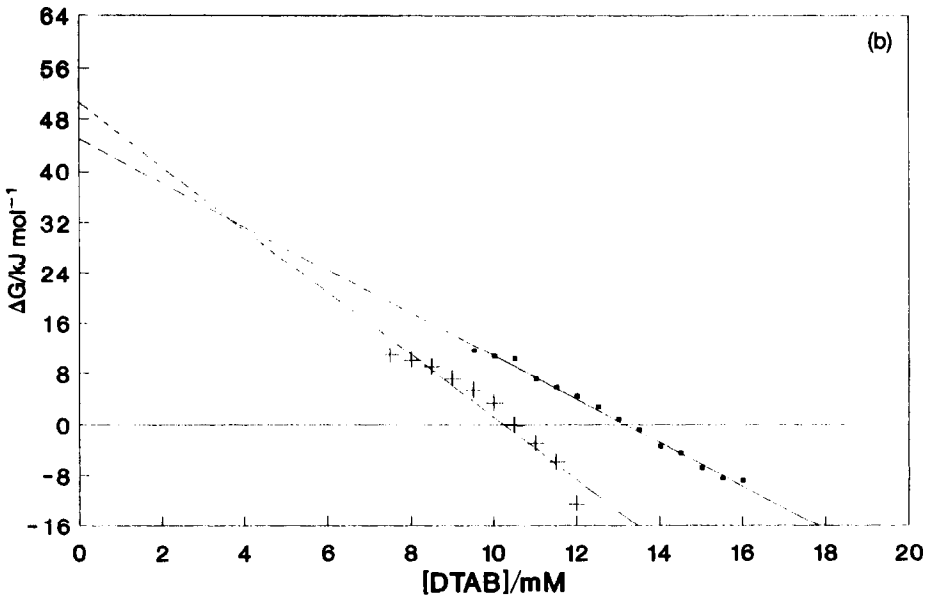
For all of the results reported here, ΔG was found to vary linearly with denaturant concentration, and a least-squares analysis was used to fit the data to the equation

$$\Delta G = \Delta G(H_2O) - m(\text{denaturant}) \quad (3)$$

where $\Delta G(H_2O)$ is the value of ΔG in the absence of denaturant and m is a measure of the dependence of ΔG on denaturant concentration [20]. The midpoint of the denaturation curve, $(\text{denaturant})_{1/2}$, is $\Delta G(H_2O)/m$, because $\Delta G = 0$ for $(\text{denaturant})_{1/2}$.

Figure 2 shows the plots of ΔG versus denaturant concentration which can be used to estimate $\Delta G(H_2O)$ from the intercept, m from the slope, and





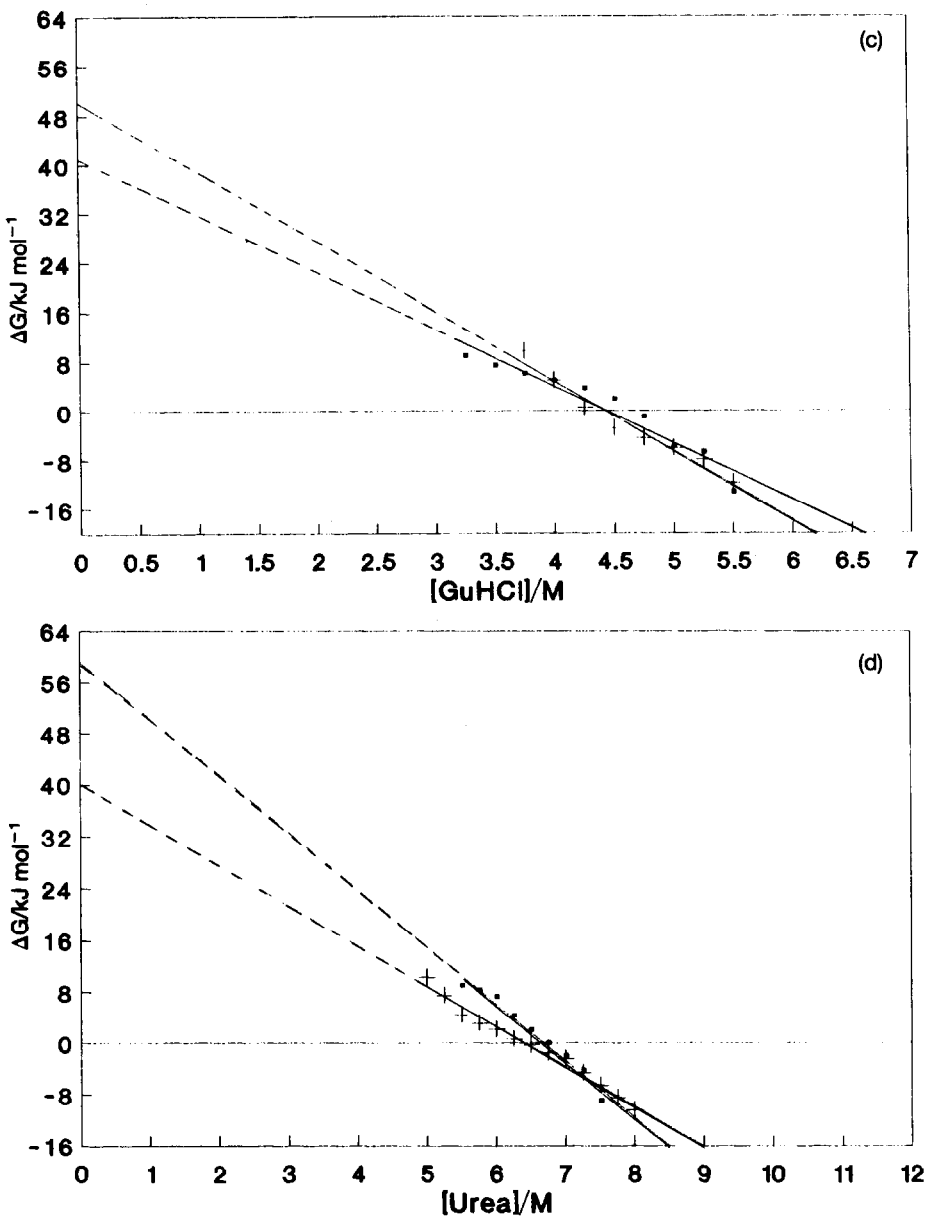
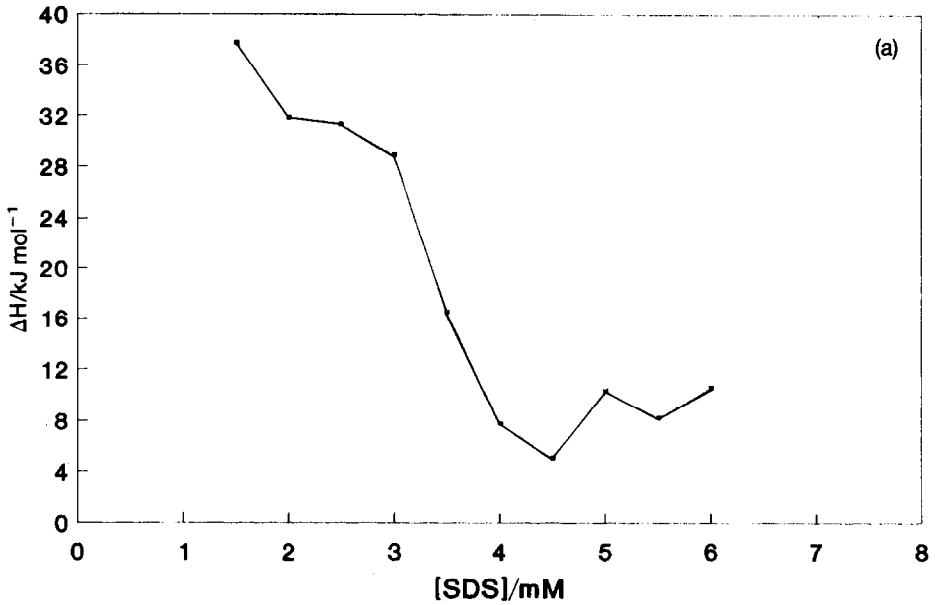


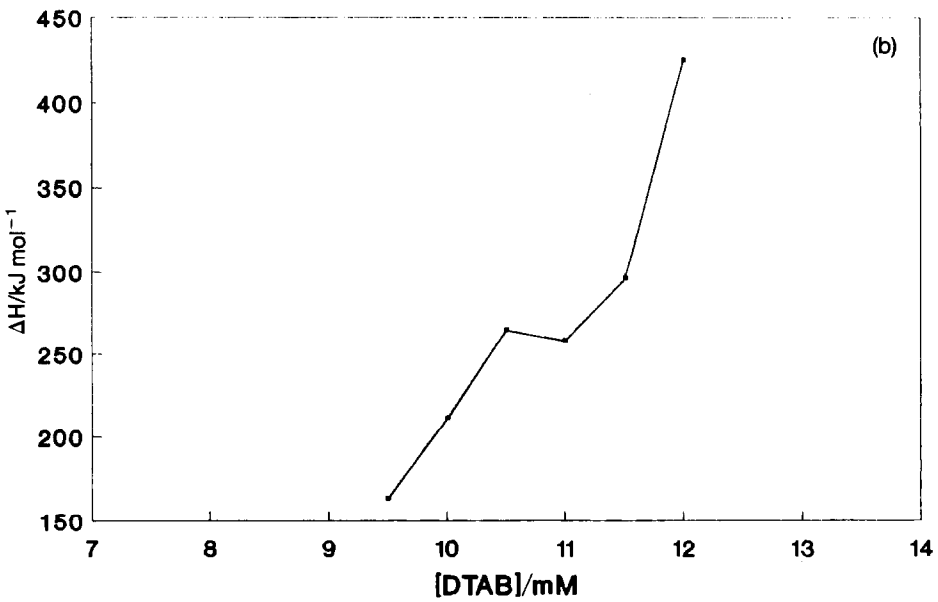
Fig. 2. Free energy ΔG calculated from measurement in the transition region using eqn. (2) (the data used were from Fig. 1) versus denaturant concentration. (a) SDS; (b) DTAB; (c) GuHCl; (d) urea: ■, 37°C; +, 25°C.

$(\text{denaturant})_{1/2}$ at $\Delta G = 0$ of the linear plots. All data from Fig. 2 are tabulated in Table 1.

The value of $\Delta G(H_2O)$ indicates the extent of unfolding or conformational stability of the system. The value of $\Delta G(H_2O)$ for CaM–SDS complexes is markedly different from those with DTAB, GuHCl and urea.

These results show that CaM–DTAB complexes caused more unfolding than the CaM–SDS complexes. This is probably because CaM is highly acidic ($pI = 4.1$) and carries a large negative charge above its pI [28], and because DTAB is a cationic surfactant and has a positive charge on the ammonium group. CaM–SDS complexes may achieve some structure and





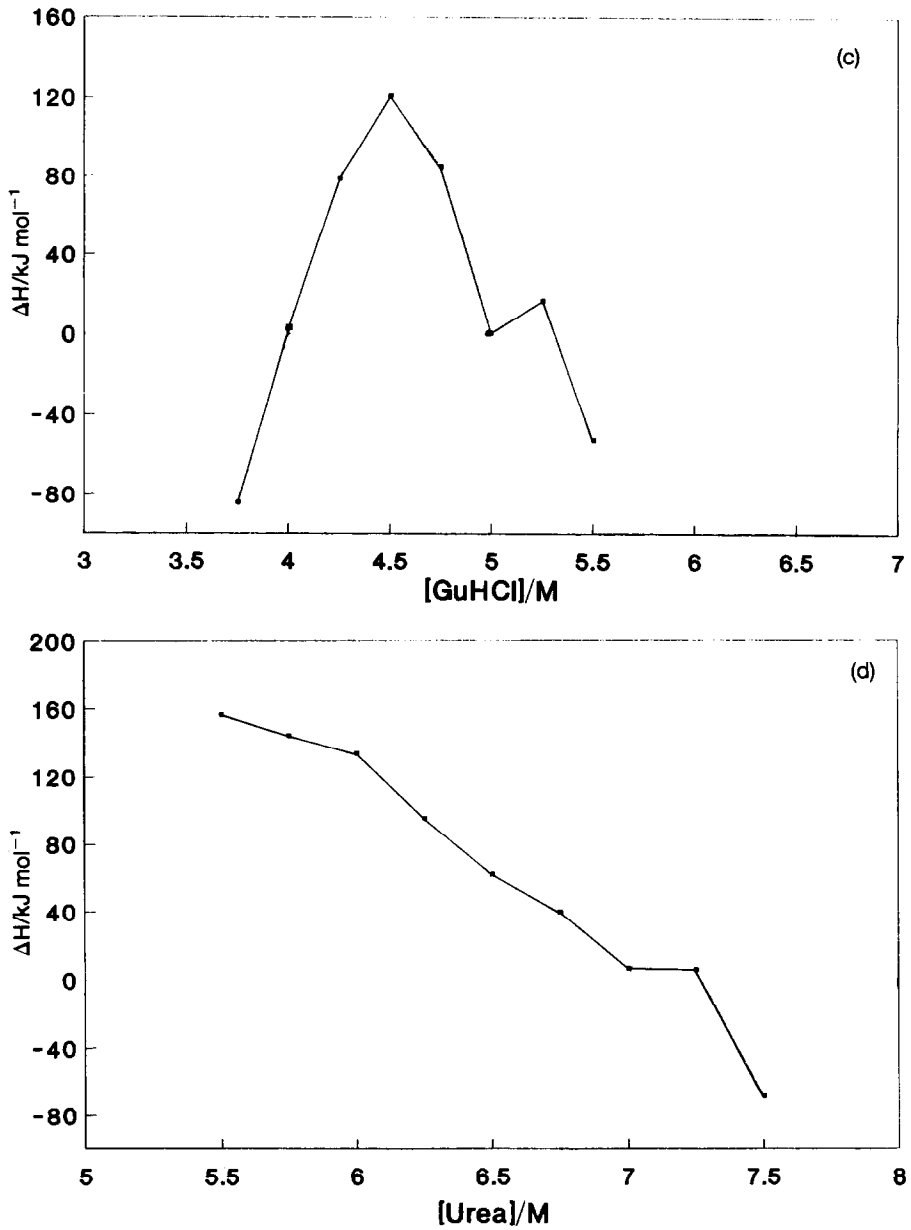


Fig. 3. Enthalpy of interaction versus denaturant concentration obtained from the Gibbs–Helmholtz equation at 25°C and 37°C. (a) SDS; (b) DTAB; (c) GuHCl; (d) urea.

thus could not be unfolded completely. The value of m shows the high cooperativity for surfactants related to GuHCl and urea, but the value of $\Delta G(H_2O)$ indicates the net conformational stability for CaM.

The enthalpy of denaturation was calculated from the Gibbs–Helmholtz equation [29] at two temperatures, 25 and 37°C, and is shown in Fig. 3. The

TABLE 1

Parameters characterizing the SDS, DTAB, GuHCl and urea denaturation of calmodulin at pH 7.4

Denaturant	$\Delta G(H_2O)$/ (kJ mol^{-1})	$[D]_{1/2}$/M	m/ (kJ mol^{-1} M^{-1})
SDS (25°C)	16.2	4.1×10^{-3}	3.95×10^{3}
SDS (37°C)	14.2	4.0×10^{-3}	3.55×10^{3}
DTAB (25°C)	45.14	13.1×10^{-3}	3.44×10^{3}
DTAB (37°C)	50.58	10.25×10^{-3}	4.93×10^{3}
GuHCl (25°C)	40.96	4.4	9.31
GuHCl (37°C)	50.16	4.42	11.32
Urea (25°C)	58.52	6.7	8.73
Urea (37°C)	40.13	6.4	6.27

enthalpy of interaction of CaM–SDS is also much lower than for the others. This indicates that DTAB breaks many hydrogen bonds, like GuHCl and urea, and changes the structure of the water around the CaM. The enthalpy data indicate a slight interaction for SDS, and a stronger interaction for DTAB. This is because of the negative charge distributed on the SDS head group and the positive charge concentrated on the large ammonium head group of DTAB.

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