# Intermolecular Interactions of Lysozyme and Small Alcohols: A Calorimetric Investigation

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*Received: February 6, 1997; In Final Form: April 23, 1997*[⊗]

Isothermal titration calorimetry was used to measure transfer enthalpies of hen egg white lysozyme from water to aqueous solutions of methanol, ethanol, 1-propanol, glycerol, and 2,2,2-trifluoroethanol. Excess *partial* molar enthalpies of lysozyme at infinite dilution in the alcoholic solvents, $^{\infty}H^{E}_{L}$, were calculated, and the dependence of $^{\infty}H^{E}_{L}$ on the concentration of the alcohol, was utilized to elucidate the enthalpy of alcohol−protein interactions. Results show that, at low alcohol concentrations, alcohol−protein interactions are unfavorable in terms of enthalpy (endothermic), while at higher concentrations they are favorable (exothermic). The change from endothermic to exothermic interactions happened sharply over a narrow alcohol concentration interval, and was found to occur concurrently with denaturation of the protein in some but not all cases. Comparison of the present results with previous investigations of simple binary and ternary aqueous solutions suggests that the change of sign of the interaction enthalpy is related to the water−water hydrogen bonding properties in the alcohol mixtures. It is argued that modifications by the alcohol of the percolated hydrogen bond network govern the enthalpy of alcohol−lysozyme interactions in the most water-rich samples. This suggests that occupancy by the alcohol of a binding site on the surface of the protein may not be necessary for the alcohol to affect the properties of the protein. At higher alcohol concentrations the observed interaction enthalpies are dominated by direct ("intrinsic") effects of protein−alcohol interactions, which are exothermic.

## Introduction

The energetics of weak, nonspecific interactions between proteins and small organic solutes in aqueous solution has been investigated in several recent studies.[1−6] One of the main objectives of these works has been to characterize how interactions with small solutes affect reactions in which the protein takes part (*i.e.*, elucidation of so-called linked equilibria).[7] A commonly studied example is the unfolding of globular proteins induced by such denaturants as urea or guanidinium chloride. In this case, the thermodynamics of protein−denaturant interactions for the native and denatured states is utilized to establish the effect of the denaturant activity on the native-denatured equilibrium. Studies in this field may also have a potential of identifying forces contributing to biomolecular processes such as folding, binding, and assembly. Hence, probing a biopolymer for its interaction with small solutes of different chemical composition could provide valuable insight into the nature of the driving forces governing such processes.

The thermodynamic property of most immediate interest for these purposes is the free energy of the protein−cosolvent interaction, often expressed as a preferential interaction coefficient.[2] This quantity provides direct information about the net affinity of the protein for a given cosolvent, and it can be related to effects of the activity of one component on an equilibrium involving another.[7] However, as it has been established for simple solutions, evaluation of temperature derivatives of the free energy function (*i.e.*, enthalpy and entropy) may contribute significantly to the understanding of molecular interactions. Especially so in dilute aqueous systems where solute−solute interactions are generally associated with large, nearly compensating enthalpy and entropy effects.[8]

In the present work we investigate interactions of hen egg lysozyme and small alcohols in terms of enthalpy. These systems were chosen partly because thermodynamic data for the reversible denaturation of lysozyme have been reported in detail, both in water[9] and in aqueous alcohol mixtures,[10] and partly because of the availability of information on intermolecular interactions in simple aqueous alcohol solutions (see, for example, ref 11). By utilizing a thermodynamic approach similar to the one discussed below, we have previously suggested[12−15] that binary aqueous alcohol solutions exhibit a certain threshold mole fraction, $x_A^S$, depending on the size of the hydrophobic moiety, at which the way alcohols and water mix, the "mixing scheme" of the solution, changes qualitatively. Hence, water-rich mixtures are dominated by effects reminiscent of the peculiar properties of liquid water, while beyond the threshold, the behavior of $H_2O$ resembles that of "normal" polar molecules. As it will be pointed out, this transition in the mixing scheme of the solvent strongly influences alcohol−protein interactions, and it may also to some extent affect the conformational stability of the protein molecule.

A large number of studies have investigated effects of alcohols on the physical properties of proteins in solution. These studies have focused on various aspects, for example the way alcohols affect the stability and solubility of the native state.[16−19] However, the mechanisms through which alcohols introduce these changes remain mainly unresolved. It has been well established that typical monohydric alcohols such as methanol, ethanol, and 1-propanol generally introduce a significant destabilization of native lysozyme,[10,20,21] as well as other small globular proteins.[16,17,22] At low alcohol concentration and temperatures around 0 °C, however, the same alcohols have been found to slightly stabilize lysozyme.[10] A similar dual effect of alcohols has been reported for other proteins.[22,23]

The aim of the present study is to discuss interactions underlying such effects by utilizing a model free thermodynamic approach, as summarized earlier.[15]

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[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1997.

S1089-5647(97)00469-0 CCC: $14.00 

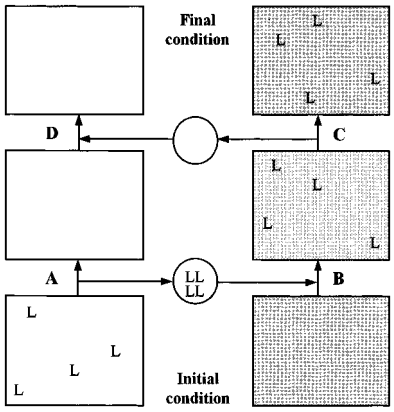


**Figure 1.** Schematic illustration of the ITC procedure. Four separate measurements (A−D) are made to determine the transfer enthalpy of lysozyme (L) at the infinite dilution, from water (white) to an alcohol solution (shaded). The heats of process A and B are obtained by titrating the concentrated lysozyme solution into water and alcohol solutions, respectively. Similarly, $q_C$ and $q_D$ are measured by titration of water and the water−alcohol−lysozyme mixture with pure water.

## Experimental Section

Lysozyme (three times recrystallized and dialyzed) and all alcohols (+99.5%) were purchased from Sigma. Alcohol solutions were prepared from aqueous buffers (10 mM glycine at pH 2.0 or 10 mM MES at pH 6.0) by weighing. After mixing, the apparent pH of the alcohol solution was measured and if necessary readjusted. Reduced and carboxymethylated (RCM) lysozyme was prepared by titrating a protein solution (*ca.* 5 mg/mL in 6 M GuHCl, 0.5 M Tris, pH 8.6 at 35 °C) with 1 M dithiothreitol. After 2 h incubation the solution of the reduced protein was cooled to 0 °C and carboxymethylated with iodoacetamide. RCM lysozyme was subsequently purified by extensive dialysis against distilled water and finally lyophilized (we are grateful to Dr. V. J. Hilser for providing this procedure).

For each series of isothermal titration calorimetry (ITC) measurements, a concentrated (70−150 mg/mL) solution of lysozyme (in glycine or MES buffer) was prepared. The solution was filtered through a 0.2 $\mu$m syringe filter and centrifuged at 20 000 g to remove any precipitate. The pH of the supernatant was readjusted, and the protein concentration determined by UV absorption at 280 nm using the extinction coefficient $\epsilon^{1\%}_{280\text{nm},1\text{cm}} = 26.5$.[24] All ITC experiments were conducted in an OMEGA instrument (Microcal, Northhampton MA).

The transfer enthalpy $Q_{trans}$, the enthalpy change associated with transfering lysozyme at the infinite dilution from water to an alcohol−water mixture, was determined by using a four-step procedure, A, B, C, and D depicted in Figure 1. $Q_{trans}$ is then calculated from the heats measured in each step ($q_A$, $q_B$, $q_C$ and $q_D$) as:

$$Q_{trans} = M/dw_L(q_A/V_A + q_B/V_B + \upsilon_w(q_C/V_C + q_D/V_D)) \quad (1)$$

where $V_i$ is the volume titrated in step i (i: A, B, C, or D), $M$ is the molecular mass of lysozyme, $d$ is the density of the concentrated lysozyme solution removed in step $A$, and $w_L$ and $\upsilon_w$ are, respectively, the weight fraction of lysozyme and the volume fraction of water in the concentrated solution. Values of $d$ and $\upsilon_w$ were estimated from the lysozyme concentrations measured by UV absorption, assuming a (concentration independent) apparent specific volume of lysozyme of 0.69 mL/g.[10] The values of $Q_{trans}$ were found constant within exeprimental uncertainty using lysozyme in the 0.2 to 2 mg/mL concentration range (*i.e.*, a protein mole fraction of about $10^{-6}$).

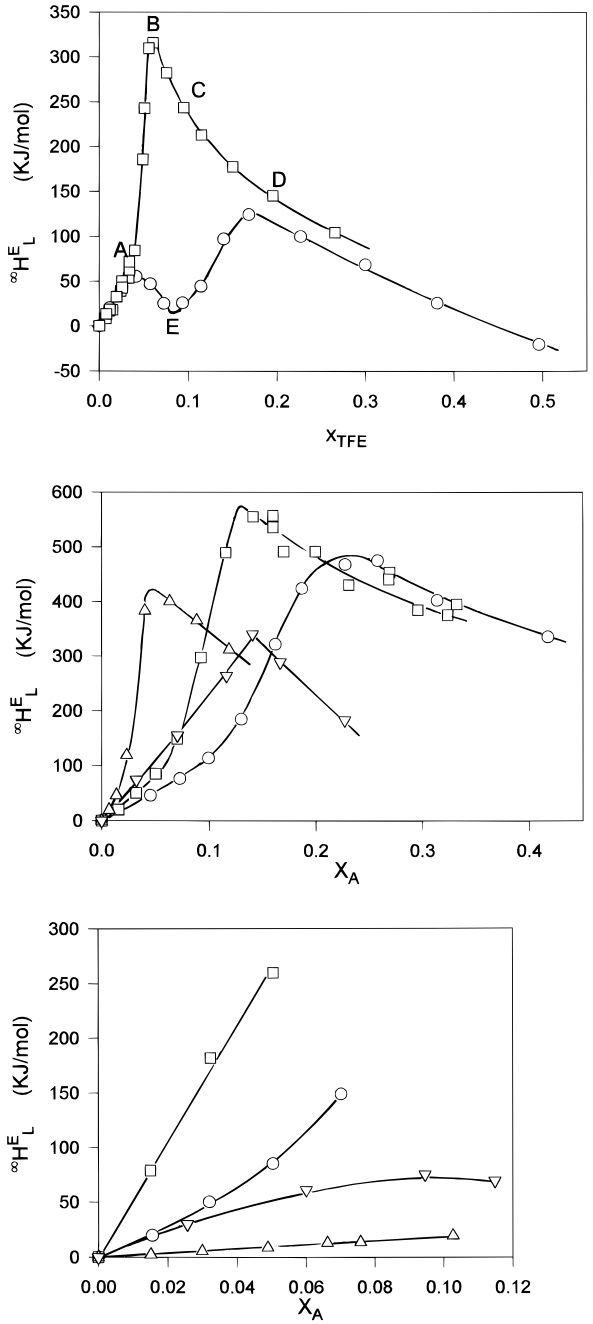


**Figure 2.** Measured values of the excess partial molar enthalpy of lysozyme, $^{\infty}H^{E}_{L}$ in alcohol solutions calculated according to eq 1 and plotted as a function of the alcohol mole fraction $x_A$: (a) for TFE solutions at pH 2.0, 30 °C (□) and pH 6.0, 20 °C (○); (b) for methanol (○), ethanol (□), and 1-propanol (△) solutions at pH 2.0, 40 °C and for ethanol solutions (▽) at pH 2.0 and 15 °C. (c) Comparison of results for lysozyme (○, △) and reduced carboxymethylated (RCM) lysozyme (□, ▽) at pH 2.0, 40 °C. The alcohols are ethanol (□, ○) and glycerol (▽, △).

Hence, we assumed that measured values of $Q_{trans}$ represent the excess *partial* molar enthalpy of lysozyme at the infinite dilution $^{\infty}H^{E}_{L}$ , in a given alcohol solution, relative to an aqueous, infinitely dilute reference state.

Denaturation of lysozyme in mixed buffer/TFE solvents was studied by differential scanning calorimetry (DSC). A DASM 93 calorimeter developed at the biocalorimetry center, Johns Hopkins University, Baltimore, MD, was loaded with lysozyme solution (0.6 mL, 1.5−2.5 mg/mL). The solvent was mixtures of buffer (10 mM Glycine, pH 2.0) and TFE at concentrations of 0−25% (w/w). The sample was heated at 1 °C/min from 0 to 85 °C using the mixed solvent as reference, and the results

**TABLE 1: Comparison of the Present Results and Literature Data for the Denaturation of Lysozyme in Aqueous Solutions of Methanol (MeOH), Ethanol (EtOH), 1-Propanol (PrOH), and 2,2,2-Trifiuoroethanol (TFE)**

| | | | present results | | | literature data | | |
|---|---|---|---|---|---|---|---|---|
| alcohol | $T_d$ °C | pH | $\Delta H_d$[a] kJ/mol | $x_A^{d}$ [a] | $x_A^{S}$ [a] | $\Delta H_d$ kJ/mol | $x_A^{d}$ | refs |
| none | 40 | 2.0 | 270[b] | - | - | 300 | | 9,10 |
| TFE | 30 | 2.0 | 320[c]/350[b] | 0.050[c]/0.046[b] | 0.044[f] | N/A | 0.059[d] | 29 |
| TFE | 20 | 6.0 | 180[c] | 0.125[c] | 0.044[f] | N/A | 0.11 | 19 |
| MeOH | 40 | 2.0 | 400[c] | 0.170[c] | 0.16[e] | 390 | 0.15 | 10 |
| EtOH | 40 | 2.0 | 430[c] | 0.103[c] | 0.125[e] | 422 | 0.085 | 10 |
| 1-PrOH | 40 | 2.0 | 375[c] | 0.033[c] | 0.070[e] | 395 | 0.032 | 10 |

[a] $\Delta H_d$ and $x_A^{d}$ indicate the enthalpy and alcohol concentration (in mole fraction units) of denaturation under the listed pH and temperature conditions. $x_A^{S}$ is the location of the singular behavior discussed in the text. [b] DSC. [c] ITC. [d] At 27 °C. [e] At 25 °C (ref 14). [f] Unpublished results.

were analyzed with respect to temperature and enthalpy of denaturation.

## Results and Discussion

The data of $^{\infty}H^{E}_{L}$ are shown in Figure 2. The curves generally include three distinct regions. In Figure 2a, for example, $^{\infty}H^{E}_{L}$ for pH 2 and 30 °C increases gradually with the trifluoroethanol (TFE) mole fraction in the most water-rich composition range (*i.e.* from 0 to point A). At an intermediate composition region (from point A to B) a sharper increase in $^{\infty}H^{E}_{L}$ is observed, and at still higher alcohol concentrations (from B to D), the plot is characterized by negative slopes. The $^{\infty}H^{E}_{L}$ functions for methanol, ethanol, and 1-propanol solutions at pH 2 and 40 °C (Figure 2b) follow a similar pattern. Values of the enthalpy change associated with the sharp increase in $^{\infty}H^{E}_{L}$ (from point A to B in Figure 2a, for example) were estimated from the curves and listed in Table 1, together with the alcohol mole fraction at the midpoint of the increase. Comparison of these values with literature data on the enthalpy of lysozyme denaturation ($\Delta H_d$, measured by DSC) and the transition midpoint ($x_A^{d}$) under the same conditions (Table 1) seems to justify the suggestion that the sharp increase at intermediate alcohol concentrations (point A to B in Figure 2a) is caused by heat absorbed during denaturation of the protein.

Thus, the results shown in Figure 2a suggest that point A represents the onset of denaturation at pH 2, 30 °C, and point E the onset at pH 6, 20 °C.

The contribution of protein denaturation was subtracted from the measured $^{\infty}H^{E}_{L}$ values. The extent of the denaturation process ($\alpha$) was estimated as a function of the alcohol concentration by extrapolating the pre- and posttransition data to the transition region, and evaluating the fraction of the total measured denaturation enthalpy adsorbed at a given alcohol concentration. Results are plotted in Figure 3. These data then allow calculation of a "corrected" partial molar enthalpy of lysozyme, $^{\infty}H^{E}_{L,corr}$, which is devoid of effects due to denaturation.

$$^{\infty}H^{E}_{L,corr} = {}^{\infty}H^{E}_{L} - \alpha\Delta H_d \qquad (2)$$

where $\Delta H_d$ is the molar denaturation enthalpy of lysozyme. Values of $^{\infty}H^{E}_{L,corr}$ are plotted against the alcohol mole fraction in Figure 4, and it appears that all curves consist of a region with positive slope in the water-rich composition range and a region with negative slope at higher alcohol concentrations. The change in slope occurs sharply over a narrow alcohol concentration range. For TFE (Figure 4a), the maxima of the $^{\infty}H^{E}_{L,corr}$ functions (at $x_{TFE} = 0.040$ under both sets of conditions) turned out to coincide with the onset of denaturation for pH 2, 30 °C, while the protein did not denature until $x_{TFE} = 0.11$ at pH 6, 20 °C. In the light of this, it is striking that both plots showed maxima at the same loci.

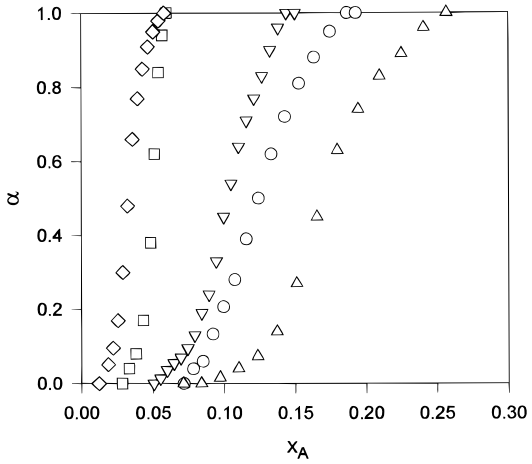


**Figure 3.** Extent of denaturation of lysozyme as a function of the alcohol mole fraction estimated from the enthalpy data in Figure 2. Symbols represent (△) methanol; (▽) ethanol; (◇) 1-propanol, all at pH 2.0 and 40 °C; (□) TFE at pH 2.0, 30 °C; and (○) TFE at pH 6.0, 20 °C.

As we suggested earlier,[15,25] the composition dependence of a partial molar quantity provides model-free information on molecular interactions. Thus, positive slopes for the lowest alcohol concentrations show that interaction between lysozyme and the investigated alcohols are unfavorable in terms of enthalpy (*i.e.*, endothermic). The relative magnitude of this endothermic interaction was evaluated from Figure 4 in the range $0 < x_A < 0.02$. The values of slopes (we call enthalpic interaction function, $H_{LA}$ [25]) are listed in Table 2. These results show that alcohol−protein interactions in the most water-rich concentration region are more endothermic the larger the nonpolar surface area of the interacting molecules.

Thus, the value of $H_{LA}$ increases gradually through the sequence methanol, ethanol, and 1-propanol. Moreover, the interaction function for ethanol with native lysozyme is about a third of that of ethanol with reduced carboxymethylated (RCM) lysozyme, which is completely unfolded under these conditions[26] and thus has a larger exposed nonpolar surface area than the native protein. Comparison between the interaction functions for the two $C_3$ compounds glycerol ($H_{LA} = 0.17 \times 10^3$ kJ/mol) and 1-propanol ($H_{LA} = 3.3 \times 10^3$ kJ/mol) further supports the suggestion that alcohol−lysozyme interactions become increasingly unfavorable in terms of enthalpy with increasing size of (solvent exposed) nonpolar moieties on either the alcohol or protein molecule. Negative slopes at intermediate and high alcohol concentrations (Figure 4) signify enthalpically favorable (exothermic) lysozyme−alcohol interactions in this concentration range.

The changeover from unfavorable to favorable lysozyme−alcohol interaction occurs at mole fraction $x_A^{S}$, (Table 1) exactly where the transition of mixing scheme occurred in respective binary aqueous alcohols.[8,14] This hints that the interaction

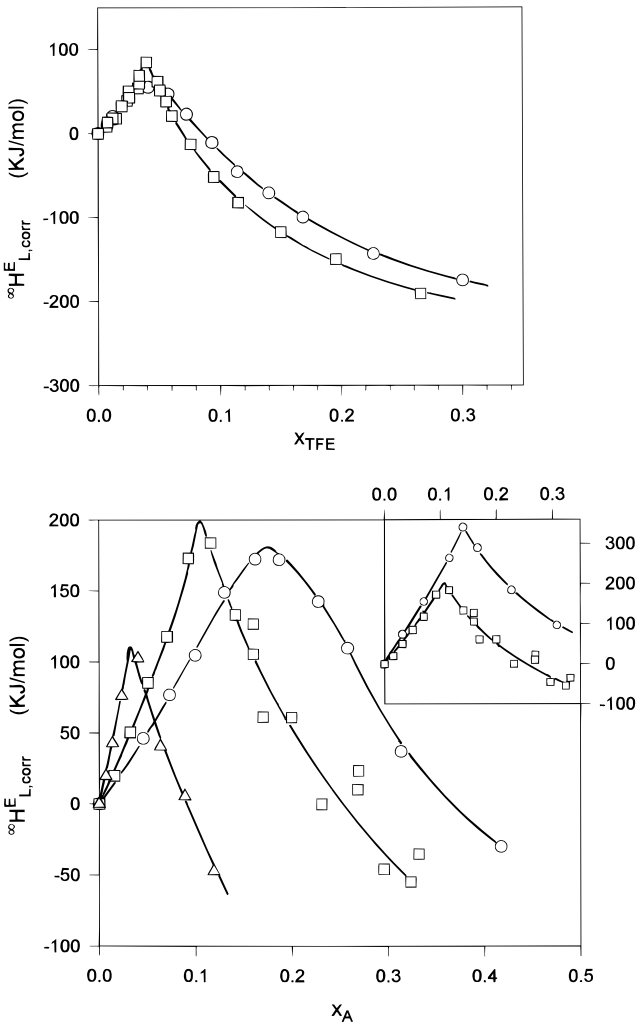


**Figure 4.** Values of $^{\infty}H^{E}_{L,corr}$ calculated according to eq 2 and plotted against the alcohol mole fraction: (a) for TFE solutions at pH 2.0, 30 °C (□) and pH 6.0, 20 °C (○); (b) for methanol (○), ethanol (□), and 1-propanol (△) solutions at pH 2.0, 40 °C. Effects of temperature is illustrated in the inset showing $^{\infty}H^{E}_{L,corr}$ for ethanol solutions at pH 2.0, 40 °C (□) and pH 2.0, 15 °C (○).

**TABLE 2: Estimates of the Lysozyme−Alcohol Enthalpy Interaction Function ($H_{LA}$) for the Most Water-Rich Concentration Range ($0 < x_A < 0.02$)[a]**

| protein | alcohol | pH | $T$ (°C) | $H_{LA}$ (kJ/mol)[a] |
|---|---|---|---|---|
| lysozyme | TFE | 2.0 | 30 | $1.6 \times 10^3$ |
| lysozyme | TFE | 6.0 | 20 | $1.6 \times 10^3$ |
| lysozyme | 1-PrOH | 2.0 | 40 | $3.3 \times 10^3$ |
| lysozyme | EtOH | 2.0 | 40 | $1.6 \times 10^3$ |
| lysozyme | MeOH | 2.0 | 40 | $1.1 \times 10^3$ |
| lysozyme | glycerol | 2.0 | 40 | $0.17 \times 10^3$ |
| RCM−lysozyme | EtOH | 2.0 | 40 | $5.4 \times 10^3$ |
| RCM−lysozyme | glycerol | 2.0 | 40 | $1.3 \times 10^3$ |

[a] All values of $H_{LA}$ are positive suggesting endothermic protein−alcohol interactions. Interactions become increasingly infavorable in terms of enthalpy the larger the nonpolar moiety of the alcohol.

between lysozyme (at its infinite dilution) and an alcohol is strongly affected by the nature of mixing scheme, and thus it is likely that the unfavorable interaction is mediated through the still percolated hydrogen bond network.[25,27] For $x_A > x_A^S$, on the other hand, $H_2O$ acts as a normal polar solvent with very small thermal effect of solute hydration,[15,25] and the favorable lysozyme−alcohol intercations are dominated by direct effects. It has been previously suggested that amphiphilic alcohols exert their effects on the properties of proteins through binding to the protein surface.[17,28] The present results show, however, that in water-rich solvents, this "binding" is associated with an unfavorable enthalpy change and, more importantly, that the effects may in fact be mediated through the percolated hydrogen bond network, rather than necessarily induced through the occupancy of a site on the protein surface.

**Acknowledgment.** This work was supported by the Danish Science Research Council (P.W.) and by the Natural Science & Engineering Research Council, Canada (Y.K.). Most of the experimental work presented here was done at the Biocalorimetry Center, Johns Hopkins University, Baltimore, MD. We are grateful to Dr. Ernesto Freire and his co-workers for their help and acknowledge the support from the NIH (Grant RR04328) to the center.

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