JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# Effects of Succinylation on the Structure and Thermostability of Lysozyme

MARIJN VAN DER VEEN,*,†,‡ WILLEM NORDE,†,‡,§ AND MARTIEN COHEN STUART†

Laboratory of Physical Chemistry and Colloid Science, Wageningen University, Dreijenplein 6, 6703 HB Wageningen, The Netherlands, Centre for Protein Technology, Netherlands Organization for Applied Scientific Research (TNO)/Wageningen University, Wageningen, The Netherlands, and Department of Biomedical Engineering, University Medical Center Groningen, University of Groningen, Antonius Deusinglaan 1, 9713 AV, Groningen, The Netherlands

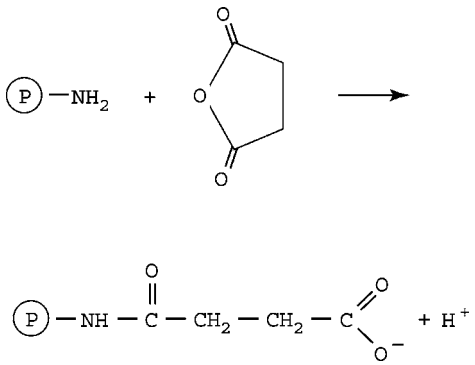
The influence of succinylation on lysozyme is studied using circular dichroism, fluorescence spectroscopy, and differential scanning calorimetry. The spectroscopic data reveal that at room temperature the structures of succinylated lysozyme and native lysozyme are similar. However, the calorimetric results show that the thermal stability of succinylated lysozyme is lower than that of native lysozyme. For succinylated lysozyme, the denaturation temperature ($T_d$) varies in the range of 325−333 K (52−60 °C) and the associated denaturation enthalpy ($\Delta_{den}H$) varies between 225 and 410 kJ/mol. For lysozyme, $T_d$ is 342−349 K (69−76 °C) and $\Delta_{den}H$ is 440−500 kJ/mol. From these data, the change in the heat capacity ($\Delta_{den}C_p$) upon thermal denaturation is derived. For lysozyme, $\Delta_{den}C_p$ is 7.5 kJ/mol/K, and for succinylated lysozyme, it is 16.7 kJ/mol/K. The value of $\Delta_{den}C_p$ for lysozyme is comparable to previously reported values. The high value of $\Delta_{den}C_p$ for succinylated lysozyme is explained in terms of an extended degree of unfolding of the secondary structure and exposure of the apolar parts of the succinyl groups. Furthermore, the Gibbs energy of denaturation, as a function of temperature, derived from the thermodynamic analysis of the calorimetric data, indicates a cold-denaturated state of succinylated lysozyme below 20 °C. However, because a denatured state at low temperatures could not be detected by CD or fluorescence measurements, the native state may be considered to be metastable at those conditions.

**KEYWORDS: Lysozyme; succinylation; thermostability; conformation**

## INTRODUCTION

The structure and structural stability of proteins play important roles in the processing and the sensorial perception of food, especially in emulsions and foams, where the formation and stabilization are only possible by adsorbing a surface active agent at the interface between the dispersed and the continuous phases. Because of the GRAS status of many proteins, they are often applied in food stuffs. In this process, the dispersion-forming and -stabilizing functionalities of a protein are strongly related to phsyical and chemical properties, in particular its structure and structural stability. With the aim to improve the protein's performance, adsorption studies are performed using modified proteins (*1*−*3*). By comparing the adsorption behavior of a modified and a native protein, one may be able to identify the influence of different types of interactions on the adsorption process. Furthermore, phenomena like the formation of protein aggregates or the formation of a protein network at the interface play a role in the stability of emulsions or foams.

In a previous paper (*4*), we reported that succinylation of lysozyme strongly influences its adsorption behavior at silica surfaces. The influence was explained in terms of electrostatic interactions of the protein with the surface. Succinylation converts primary amino groups into carboxyl groups and thereby changes the protein charge, according to the following reaction mechanism: Upon succinylation with an excess of succinic




anhydride, the isoelectric point of lysozyme shifted from 11.0 to 4.5.

Lysozyme is a structurally stable protein, and at room temperature, it is expected to undergo little, if any, confor-

* To whom correspondence should be addressed. Tel: +31-317-482585. Fax: +31-317-483777. E-mail: marijn.vanderveen@wur.nl.
† Laboratory of Physical Chemistry and Colloid Science, Wageningen University.
‡ Netherlands Organization for Applied Scientific Research (TNO)/Wageningen University.
§ University of Groningen.

10.1021/jf058029f CCC: $30.25 
Published on Web 06/16/2005

mational alteration by mild modifications. This is observed more often when small modifications or mutations are introduced in proteins. Tian et al. (*5*) found identical secondary structures for several mutants of bacteriophage T4 lysozyme. Kosters et al. (*6*) reported similar circular dichroism (CD) spectra for ovalbumin that was chemically modified with different methods. In contrast with these results, Lakkis et al. (*7*) observed shifts in the CD and fluorescence spectra when modifying casein, bovine serum albumin (BSA), and whey protein isolate with alkyl, acetyl, and succinyl groups, except for the alkylation of BSA.

Here, we present a detailed analysis of the influence of succinylation on the structure and the structure stability of lysozyme. The conformations, i.e., the secondary and tertairy structures, of both lysozyme and succinylated lysozyme were probed with CD and fluorescence spectroscopy. The amount of the secondary structure was assessed by far-UV CD, and the tertiary structure was probed by near-UV CD and fluorescence. The thermostability of the structure of lysozyme and succinylated lysozyme was determined with differential scanning calorimetry (DSC), and the experimental data allow for a thermodynamic analysis of protein denaturation as a function of temperature (*8*).

## MATERIALS AND METHODS

**Materials.** Hen egg white lysozyme was purchased from Sigma (L-6876) and used without further purification. All other chemicals were of analytical grade.

**Modification of the Protein.** Lysozyme was dissolved in 20 mM phosphate buffer of pH 8. A large excess (more than 10 times the molar ratio) of succinic anhydride was added in small portions; the pH was kept between 8 and 9 by adding 0.1 M NaOH. Following this, the protein solution was dialyzed against water for 4 days at 4°C and then freeze-dried. The mass of the protein after modification was measured by matrix-assisted laser desorption/ionization time-of-flight. There were three fractions of modified lysozyme, succinylated at 8, 9, or 10 sites, respectively. The isoelectric point of the modified protein was 4.5 as determined by gel electrophoresis (*4*).

**Fluorescence Spectroscopy.** Fluorescence was measured with a Varian Cary Eclipse Fluorimeter equipped with a Peltier element for temperature control. The excitation wavelength was 280 nm, and the fluorescence emission was measured between 300 and 400 nm. The excitation and emission slits were set at 2.5 nm. The measurements at constant temperature were made at 20 °C. For the temperature scans, the temperature was changed at 0.1 °C/min. We did not use any external fluorescent probe.

**CD.** CD was measured using a Jasco J-715 spectropolarimeter. The far-UV CD measurements were performed in a quartz cuvette of 1 mm with a protein concentration around 3 $\mu$M in 10 mM phosphate buffer. The sample was scanned from 260 to 190 nm. The near-UV CD measurements were performed in a 1 cm quartz cuvette with a protein concentration around 30 $\mu$M. The sample was scanned over a wavelength range from 310 to 260 nm. For both methods, the spectra presented were an average of eight scans. A data pitch of 0.1 nm was used, and the scan rate was 20 nm/min. The bandwidth was 1.0 nm. The cell was thermostated with a Peltier element at 20 °C unless specified otherwise. The concentration of the proteins was determined with UV absorption at 280 nm.

**DSC.** DSC of the protein solutions was performed at a scan rate of 1 °C/min on a MicroCal VP-DSC with fixed cells of 0.5 mL. The proteins were dissolved in buffers containing $10^{-3}$ M phosphate for the high pH values and $10^{-3}$ M acetate for the low pH values. The ionic strength was adjusted to a value of $10^{-2}$ M using NaCl. The protein concentration was 5 g/L. The solution was degassed because air bubbles may alter the pressure during heating. Buffer against buffer measurements were performed and used for baseline correction.

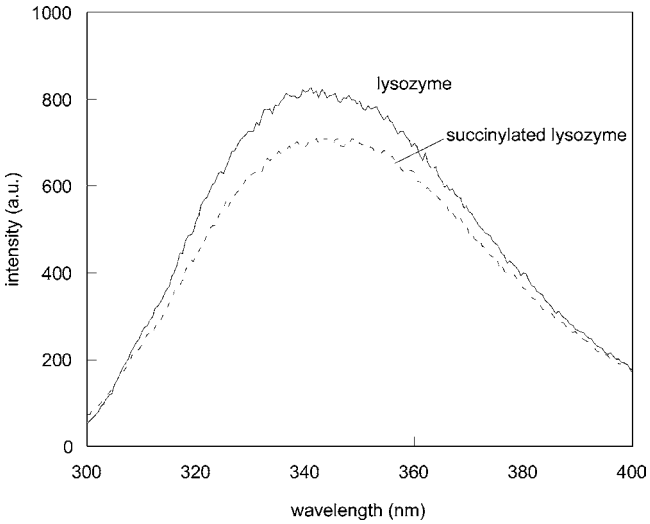



**Figure 1.** Fluorescence emission spectrum of lysozyme and succinylated lysozyme.

## RESULTS AND DISCUSSION

**Structure of Lysozyme and Succinylated Lysozyme.** The fluorescence and CD spectra of lysozyme and succinylated lysozyme were measured and compared. For the fluorescence measurements, the six tryptophan residues present in lysozyme were used as intrinsic fluorophores. A wavelength of 280 nm was used to excite the tryptophans, and the emission of light was measured between 300 and 400 nm. In **Figure 1**, the fluorescence spectra of lysozyme and succinylated lysozyme are shown. At room temperature, the position of the maxima of the emission spectra of the two proteins coincide, indicating that the tryptophans are in a similar environment. The intensity of the fluorescence is somewhat higher for native lysozyme. The reason for this difference in intensity cannot unambiguously be explained. On one hand, the intensity of the fluorescence of the tryptophans depends on the polarity of the moieties surrounding this residue. On the other hand, if there is a change in the surrounding polarity, the peak maximum is expected to shift as well, which is not observed. Possibly, the decrease in intensity is caused by a reduced contribution from the tyrosine residues that have reacted with succinyl. Anyway, the fact that the positions of the peak maxima of lysozyme and succinylated lysozyme coincide suggests that the tertiary structure of lysozyme is not significantly affected by succinylation.

The tertiary structure is further probed by near-UV CD spectroscopy. In the near-UV region, the CD spectrum of proteins is determined by aromatic side chains and disulfide bonds. The environment of aromatic compounds influences the CD spectrum, and the CD spectrum is therefore characteristic for the tertiary structure of the protein (*9*). The near-UV CD spectra of the native and the modified protein are shown in **Figure 2**. The largest parts of the spectra overlap; only around 280 nm is the ellipticity of succinylated lysozyme less than that of the native protein. Around 280 nm, tyrosine gives a peak in near-UV CD spectra (*9*). This is a clear indication that tyrosine residues are succinylated.

Secondary structure elements such as $\alpha$-helices and $\beta$-sheets have dichroic activity in the wavelength range from 190 to 260 nm (*9*). In **Figure 3**, the far-UV CD spectra of lysozyme and succinylated lysozyme are given. The spectra are very similar for both proteins, indicating only a slight difference, if any, in secondary structure upon succinylation. This is observed more often when small modifications or mutations are intro-

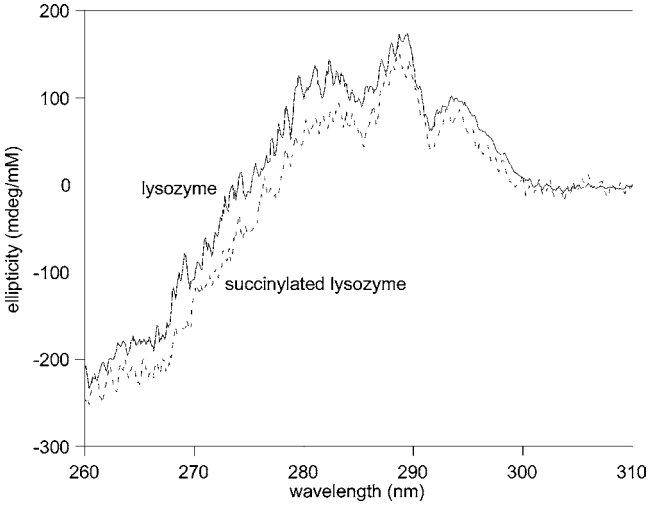



**Figure 2.** CD spectrum of lysozyme and succinylated lysozyme in the near-UV region.

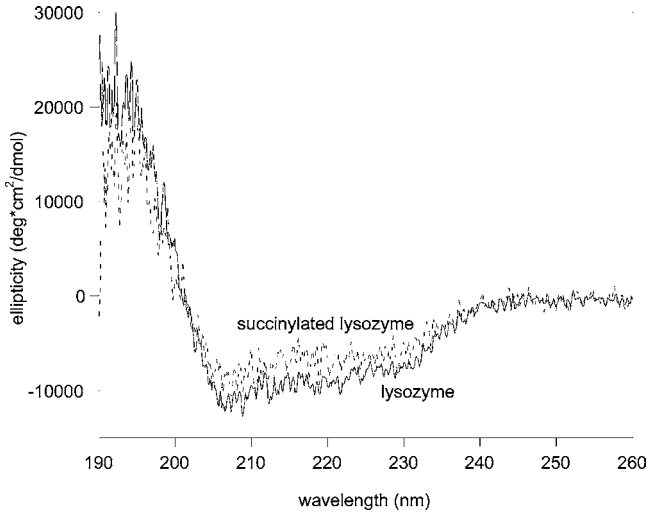



**Figure 3.** CD spectrum of lysozyme (thick line) and succinylated lysozyme (thin line) in the far-UV region.

duced in proteins (*5*, *6*). In conclusion, because the fluorescence and CD spectra of lysozyme and succinylated lysozyme are hardly different, the secondary and tertiary structures of the proteins at 20 °C are considered to be similar, if not identical.

**Thermal Stability of Lysozyme and Succinylated Lysozyme.** Although their three-dimensional structures are essentially equal at 20 °C, the thermal stabilities of these structures may be different for the two proteins. We monitored temperature-induced structural changes by measuring the fluorescence while heating the protein sample. Upon denaturation, the protein (partly) unfolds and it is expected that the tryptophan residues become more exposed to an aqueous, polar environment. As a result, the peak maximum of the fluorescence shifts toward higher wavelength. In **Figure 4**, the ratio of fluorescence emission intensities at 360 ($I_{360}$) and 320 ($I_{320}$) nm is plotted against the temperature. By taking the ratio $I_{360}/I_{320}$, the shift becomes more pronounced. A transition is observed in the temperature range 50−70 °C for succinylated lysozyme and between 70 and 80 °C for native lysozyme. For both proteins, $I_{360}/I_{320}$ is 1.4 for the native state and 2.0 for the denatured state and it implies that the tryptophan residues indeed become exposed to a more polar environment upon denaturation. The denaturation temperature decreases as a result of succinylation, meaning that the modified protein is less stable. Furthermore, the transition is more gradual for succinylated lysozyme, indicating either that the transition is less cooperative or that the population of succinylated protein molecules is less homogeneous with respect to stability.

Another way to determine the structure stability of a globular protein is to measure the amount of energy required to unfold that structure. This energy may be measured by DSC. In a DSC apparatus, the protein is heated at the same rate as a blank and the measured differential heat is the heat required to denature the protein. In **Figure 5**, the denaturation thermograms of lysozyme and succinylated lysozyme at pH 5 are shown. The peak area corresponds with the enthalpy of denaturation ($\Delta_{den}H$), and the temperature at the peak maximum is identified with the denaturation temperature ($T_d$). In this experiment, we find that succinylation lowers $T_d$ by 20 °C and reduces $\Delta_{den}H$ by 200 kJ/mol.

To allow for a more complete thermodynamic analysis of the protein structure stability, DSC thermograms are taken at various pH values, for both lysozyme and succinylated lysozyme. Variation of pH causes variation in the protein structure stability and, hence, in the values for $T_d$ and $\Delta_{den}H$. In our measurements, the $T_d$ of lysozyme varied between 342 and 349 K (69−76 °C) and the $\Delta_{den}H$ varied between 440 and 500 kJ/mol. For succinylated lysozyme, the $T_d$ varied from 325 to 333 K (52−60 °C) and the $\Delta_{den}H$ varied between 225 and 410 kJ/mol. The complete set of data is given in **Table 1**. The denaturation temperatures as measured with DSC compare well with the transition temperatures as observed with fluorescence (**Figure 4**). Furthermore, the denaturation peaks of succinylated lysozyme are broader and reflect a more gradual thermal denaturation transition of the succinylated protein sample, thus corroborating our observations with fluorescence.

Heating the protein sample to a temperature well above the thermal denaturation temperature causes irreversible behavior. It has been proven (*10*) that the irreversibility is caused by intermolecular aggregation of unfolded lysozyme and that the unfolding as such, reflected by the peak in the DSC thermogram, proceeds reversibly. We checked the reversibility of the denaturation for succinylated lysozyme by heating the sample to the denaturation temperature and keeping it at that temperature for 20 min. After that, the sample still had a denaturation peak at the denaturation temperature.

The calorimetric enthalpy may then be compared with the Van't Hoff enthalpy, $\Delta_{den}H^{vH}$, which may be derived from the thermograms as well (*11*). These data are included in **Table 1**. Similar values for $\Delta_{den}H$ and $\Delta_{den}H^{vH}$ point to a two-state denaturation process with no thermodynamically stable intermediate states. Thus, it is concluded that except for pH 11 lysozyme essentially denatures by a two-state process ($\Delta_{den}H/\Delta_{den}H^{vH}$ deviates no more than 10% from unity). The same holds for succinylated lysozyme except, perhaps, at pH 4 and pH 3.5. Then, assuming a reversible two-state process, the enthalpy of denaturation at any temperature can be calculated according to:

$$\Delta_{den}H(T) = \Delta_{den}H_{T_d} + \int_{T_d}^{T} \Delta_{den}C_p \, dT \qquad (1)$$

where $\Delta_{den}H_{T_d}$ is the denaturation enthalpy at $T_d$ and $\Delta_{den}C_p$ is the difference in the heat capacity of the protein solution before and after the denaturation transition. The entropy ($\Delta_{den}S$) and Gibbs energy ($\Delta_{den}G$) of denaturation are calculated from the

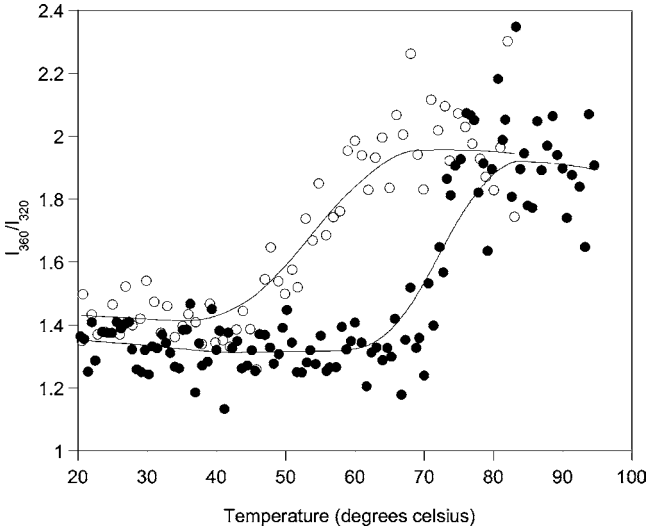



**Figure 4.** Fluorescence emission intensity at 360 nm divided by the fluorescence emission intensity at 320 nm on heating the samples for lysozyme (filled symbols) and succinylated lysozyme (open symbols).

following thermodynamic relations:

$$\Delta_{\text{den}}S(T) = \frac{\Delta_{\text{den}}H_{T_{\text{d}}}}{T_{\text{d}}} + \int_{T_{\text{d}}}^{T} \Delta_{\text{den}}C_{\text{p}}\, \text{dln}T \qquad (2)$$

and

$$\Delta_{\text{den}}G(T) = \Delta_{\text{den}}H(T) - T\Delta_{\text{den}}S(T) \qquad (3)$$

It is well-documented that unfolding of proteins in an aqueous environment leads to an increase in the heat capacity ($\Delta_{\text{den}}C_{\text{p}}$) of the system (*8*). Most DSC thermograms, like the ones shown in **Figure 5**, indeed show positive values for $\Delta_{\text{den}}C_{\text{p}}$. However, these $C_{\text{p}}$ shifts are too small to be recorded with satisfactory reproducibility. A more accurate estimate of $\Delta_{\text{den}}C_{\text{p}}$ is obtained from the slope of a plot of $\Delta_{\text{den}}H$ as a function of $T_{\text{d}}$, shown in **Figure 6**. For this plot, data points are used corresponding to the cases where $\Delta_{\text{den}}H/D_{\text{den}}H^{\text{vH}}$ deviates less than 10% from unity. Larger deviations may be due to less reliable enthalpy data or may reflect that the transition does not proceed as a two-state process.

From the plots in **Figure 6**, for lysozyme and succinylated lysozyme, values of $\Delta_{\text{den}}C_{\text{p}}$ are obtained, which are 7.5 ± 0.2 and 16.7 ± 0.9 kJ/mol/K, respectively. The value for lysozyme compares well with literature data (*8*, *12*). The value for succinylated lysozyme is much higher than usually found for globular proteins. It suggests a more progressed exposure of hydrophobic groups of the protein to water after unfolding. To test this suggestion, we measured the CD and fluorescence spectra of both proteins at a temperature above the denaturation temperature, i.e., 80 °C. The near-UV CD measurement gave no signal, indicating that the tertiary structure of both proteins was denatured (data not shown).With fluorescence, for both proteins, $I_{360}/I_{320}$ is 1.4 for the native state and 2.0 for the denatured state. The far-UV CD spectrum of lysozyme thermally denatured at 80 °C was the same as that below the denaturation temperature, i.e., 20 °C (**Figure 3**), but for succinylated lysozyme, a much smaller fraction of ordered secondary structure was found in the thermally denatured sample (**Figure 7**). Therefore, the high value of $\Delta_{\text{den}}C_{\text{p}}$ for succinylated lysozyme is ascribed to a more extensive breakdown of its

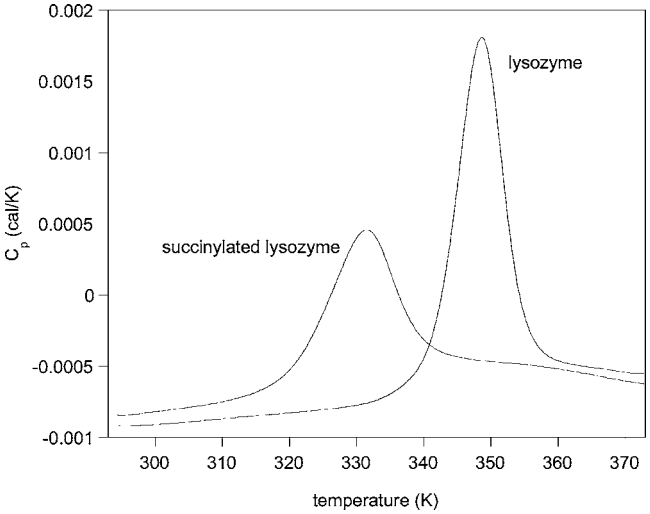



**Figure 5.** DSC thermogram of the denaturation of lysoyzme and succinylated lysozyme at pH 5.

**Table 1.** Denaturation Temperature and Enthalpy of Lysozyme (lsz) and Succinylated Lysozyme (succ lsz) at Different pH Values

| pH | $T_{\text{d}}$ (K) | $\Delta_{\text{den}}H$ (kJ/mol) | $\Delta_{\text{den}}H^{\text{vH}}$ (kJ/mol) | $\Delta_{\text{den}}H/\Delta_{\text{den}}H^{\text{vH}}$ |
|---|---|---|---|---|
| | | succ lsz | | |
| 3.5 | 330.5 | 290 | 563 | 0.8 |
| 4 | 333.6 | 425 | 253 | 1.68 |
| 5 | 330.4 | 320 | 323 | 0.99 |
| 7 | 329.8 | 309 | 310 | 1.00 |
| 8 | 327.1 | 268 | 256 | 1.05 |
| 9 | 326.8 | 257 | 252 | 1.02 |
| 11 | 325.6 | 239 | 243 | 0.99 |
| | | lsz | | |
| 3.5 | 348.8 | 500 | 457 | 1.10 |
| 4 | 349.0 | 500 | 456 | 1.10 |
| 5 | 347.6 | 491 | 485 | 1.01 |
| 6 | 346.7 | 494 | 494 | 1.19 |
| 7 | 345.3 | 459 | 434 | 1.06 |
| 8 | 345.2 | 477 | 477 | 0.97 |
| 9 | 345.6 | 493 | 493 | 0.97 |
| 11 | 342.7 | 451 | 451 | 1.21 |

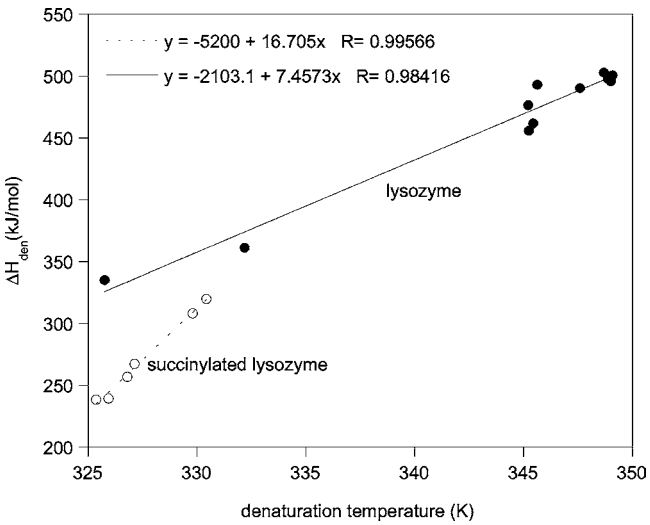



**Figure 6.** Denaturation enthalpy of lysozyme (closed symbols) and succinylated lysozyme (open symbols) plotted against the denaturation temperature.

secondary structure. Further exposition to the aqueous environment of the apolar parts of the succinyl residues may also additionally contribute to the unusual high value of $\Delta_{\text{den}}C_{\text{p}}$.

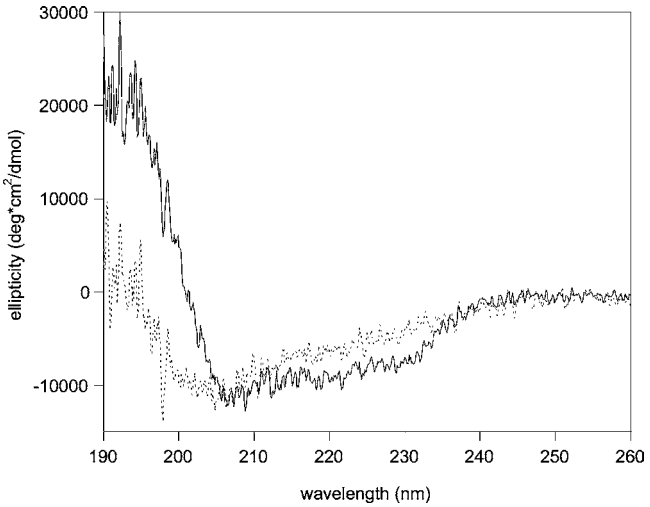



**Figure 7.** CD spectrum of succinylated lysozyme at 20 (thin line) and 80 (thick line) °C.

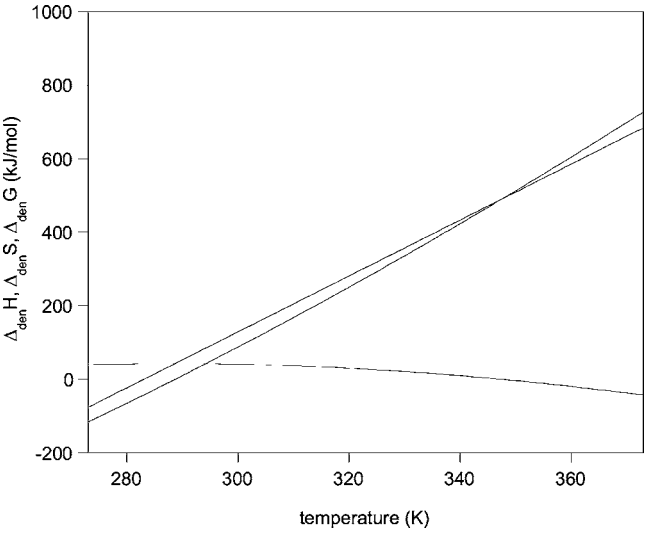



**Figure 8.** Enthalpy, entropy, and Gibbs energy of denaturation of lyszoyme plotted against temperature.

Now, using the experimental data of $\Delta_{den}H_{T_d}$, $T_d$, and $\Delta_{den}C_p$, curves for $\Delta_{den}H$, $T\Delta_{den}S$, and $\Delta_{den}G$ are plotted as a function of temperature in **Figures 8** and **9**. The entropy and enthalpy effects largely compensate each other so that the variations in the Gibbs energy are small and hardly visible in these figures. Therefore, the Gibbs energy of denaturation is replotted on an enlarged scale in **Figure 10**. It reveals that the Gibbs energy of denaturation for succinylated lysozyme is at all temperatures less positive (or more negative) than in the case of lysozyme. In other words, over the whole temperature range, succinylation lowers the thermodynamic stability of the compact protein structure.

The temperatures where $\Delta_{den}G = 0$ are the denaturation temperatures. It appears that the $\Delta_{den}G$ curve of succinylated lysozyme has two such temperatures. This would imply that in addition to a heat-induced denaturation, succinylated lysozyme should undergo a cold-induced denaturation as well. Cold denaturation of proteins has been reported more often in the literature (*8*, *13*). The cold denaturation of most proteins takes place at temperatures well below the freezing temperature of water. By increasing the pressure or by adding denaturants, like urea, the temperature of cold denaturation can be adjusted to above the freezing point (*14*).

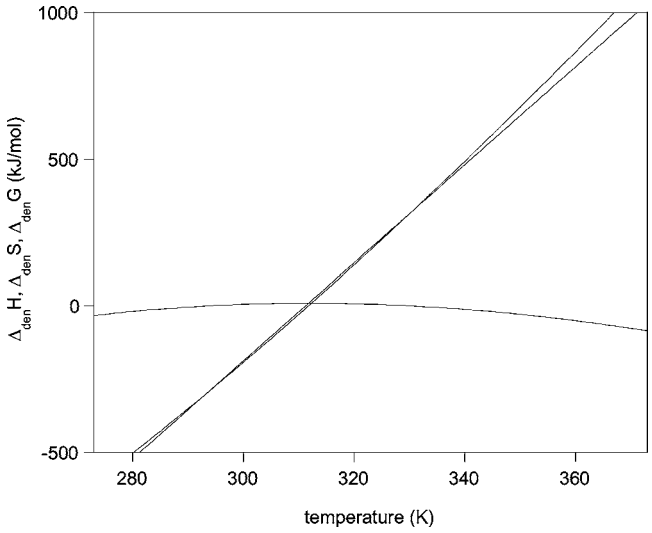



**Figure 9.** Enthalpy, entropy, and Gibbs energy of denaturation of succinylated lysozyme plotted against temperature.

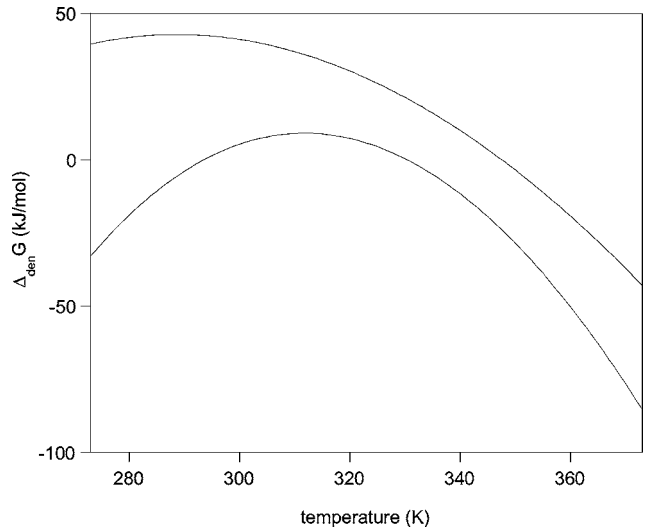



**Figure 10.** Gibbs energy of denaturation of lyszoyme and succinylated lysozyme.

**Figure 10** predicts cold denaturation of succinylated lysozyme at a temperature of 293 K (20 °C). However, at this temperature, the protein presents itself in a globular, nativelike state, suggesting that the onset of cold denaturation is (well) below 20 °C. We tried to detect the cold denaturation transition of succinylated lysozyme by calorimetry. In a DSC experiment, the protein was cooled from 20 to 5 °C at a scan rate of 0.5 K/min (*8*, *13*). The measurement did not produce a visible denaturation peak. We also tried to trace cold denatured protein by fluorescence and CD after keeping the protein overnight in a refrigerator. The fluorescence and the near-UV CD spectra of the cooled protein were measured at 5 °C, and the spectra were the same as that of the native protein. Hence, the expected denaturation could not be detected in the temperature range of 5−20 °C.

As the value of $\Delta_{den}C_p$ is subject to some statistical error, we checked whether this may affect our thermodynamic analysis and invalidate the conclusion that for succinylated lysozyme $\Delta_{den}G = 0$ somewhere between 5 and 20 °C. This is not the case; the uncertainty in $T_d$ is less than 2 °C.

Taking into account the standard deviation of the $\Delta_{den}C_p$ value did not significantly influence the curves in **Figures 8**−**10**. We

therefore conclude that the compact structure of succinylated lysozyme is metastable at temperatures below 20 °C: The compact structure is trapped in a local Gibbs energy minimum from which transition to the thermodynamically more favorable unfolded structure is (kinetically) hampered.

## ACKNOWLEDGMENT

We thank Anton Korteweg for skillfully performing the calorimetry experiments.

## LITERATURE CITED

(1) Song, K. B.; Damodaran, S. Influence of electrostatic forces on the adsorption of succinylated $\beta$-lactoglobulin at the air−water interface. *Langmuir* **1991**, *7*, 2737−2742.

(2) Krause, J. P.; Kragel, J.; Schwenke, K. D. Properties of interfacial films formed by succinylated legumin from faba beans (*Vicia faba* L.). *Colloids Surf., B* **1997**, *8*, 279−286.

(3) Wierenga, P. A.; Meinders, M. B. J.; Egmond, M. R.; Voragen, F. A. G. J.; de Jongh, H. J. Protein exposed hydrophobicity reduces the kinetic barrier for adsorption of ovalbumin to the air−water interface. *Langmuir* **2003**, *19*, 8964−8970.

(4) Van der Veen, M.; Norde, W.; Cohen Stuart, M. Electrostatic interactions in protein adsorption probed by comparing lysozyme and succinylated lysozyme. *Colloids Surf., B* **2004**, *35*, 33−40.

(5) Tian, M.; Lee, W.; Bothwell, M. K.; McGuire, J. Structurqal stability effects on adsorption of bacteriophage T4 lysozyme to colloidal silica. *J. Colloid Interface Sci.* **1998**, *200*, 146−154.

(6) Kosters, H. A.; Broersen, K.; de Groot, J.; Simons, J.; Wierenga, P.; de Jongh, H. H. J. Chemical processing as a tool to generate ovalbumin variants with changed stability. *Biotechnol. Bioeng.* **2003**, *84*, 61−70.

(7) Lakkis, J.; Villota, R. Effect of acylation on substructural properties of proteins: A study using fluorescence and circular dichroism. *J. Agric. Food Chem.* **1992**, *40*, 553−560.

(8) Privalov, P. L.; Gill, S. J. Stability of protein structure and hydrophobic interaction. *Adv. Protein Chem.* **1988**, *39*, 191−234.

(9) Kelly, S. M.; Price, N. C. The application of circular dichroism to studies of protein folding and unfolding. *Biochim. Biophys. Acta* **1997**, *1338*, 161−185.

(10) Larsericsdotter, H.; Oscarsson, S.; Buijs, J. Thermodynamic analysis of proteins adsorbed on silica particles: Electrostatic effects. *J. Colloid Interface Sci.* **2001**, *237*, 98−103.

(11) Privalov, P. L.; Potehkin, S. A. Scanning microcalorimetry in studying temperature-induced changes in proteins. *Methods Enzymol.* **1986**, *131L*, 4−51.

(12) Haynes, C. A.; Norde, W. Structures and stabilities of adsorbed proteins. *J. Colloid Interface Sci.* **1995**, 313−328.

(13) Griko, Y. V.; Privalov, P. L.; Sturtevant, J. M.; Venyaminov, S. Y. Cold denatruation of staphylococcal nuclease. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 3343−3347.

(14) Kunugi, S.; Tanaka, N. Cold denaturation of proteins under high pressure. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **2002**, *1595*, 329−344.

**Received for review January 20, 2005. Revised manuscript received May 3, 2005. Accepted May 6, 2005. This work was carried out as part of the Innovation Oriented Research Program on Industrial Proteins (IOP-IE 98004) sponsored by The Netherlands Ministry of Economic Affairs.**

JF058029F