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Thermochimica Acta 253 (1995) 117–125

thermochimica acta

Thermal stability of alkylated and hydroxyalkylated lysozymes ☆

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Received 8 August 1994; accepted 23 August 1994

Abstract

Five derivatives of lysozyme which were modified in four of the original six lysine residues were prepared by reductive alkylation with three aliphatic aldehydes of different chain lengths and two hydroxyaldehydes of different hydroxyl-group contents. The thermal stability of these derivatives was investigated by DSC. The alkylated derivatives were less stable than unmodified lysozyme, depending on the size of the introduced alkyl groups. However, there was no significant difference in the thermal stability among unmodified and two hydroxylated lysozymes. The change in the thermal stability upon lysine modification of lysozyme is remarkably dependent on the qualitative factors of the entering substituents, and on other factors such as steric hindrance.

Keywords: Alkylation; Denaturation; DSC; Lysozyme; Stability

1. Introduction

Reductive alkylation is a convenient procedure for converting amino groups in proteins to their alkylamine derivatives through the reduction of the Schiff bases that form between the amino groups and added aldehydes. The reaction is extremely specific for the ε-amino groups of lysine residues and the α-amino groups

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☆ Presented at the International and III Sino-Japanese Symposium on Thermal Measurements, Xi'an, 4–6 June 1994.

SSDI 0040-6031(94)02083-3

of terminus [1,2]. Conformational changes induced in hen egg-white lysozyme on reductive alkylation with different aldehydes have been studied using circular dichroic (CD) spectroscopy [3]. CD spectra were affected only to a very limited extent by the modification. Retention of native conformation in the protein by methylation has also been shown by enzymatic activity measurements and proton nuclear magnetic resonance (NMR) observations [4].

Previous study [5] showed that ribonuclease A was thermally destabilized by reductive alkylation with aliphatic aldehydes of varying chain length, but that chymotrypsinogen A was slightly stabilized by the modification, depending on both the chain length of the entering alkyl groups and the degree of modification. These observations suggest that alkylation involves not only steric hindrance by the entering substituents but also such factors that thermally stabilize the protein. In the present study, we prepared five derivatives of lysozyme by reductive alkylation with three aliphatic aldehydes of different chain lengths and two hydroxyaldehydes of different hydroxyl-group contents, in which about four of the original six lysine residues were modified, and determined the effect of lysine modification with differently sized and characterized substituents on the thermal stability of the protein from the thermal denaturation measurements by DSC. We also investigated the temperature dependences of the enzymatic activities of these protein derivatives against ethylene glycol chitin.

2. Experimental

2.1. Materials

Hen egg-white lysozyme (salt-free, six-times recrystallized) and ethylene glycol chitin were purchased from Seikagaku Kogyo (Japan). Aliphatic aldehydes were freshly distilled prior to use. Hydroxyaldehydes were obtained from Aldrich Chemicals (USA). All other chemicals were of analytical reagent quality and used without further purification.

2.2. Modification of protein

Reductive alkylation of protein was carried out according to the previously described procedure [5]. Lysozyme at a lysine residue concentration of 10 mM in 0.1 M Hepes buffer (pH 7.5) was incubated with 50–150 mM aldehyde or hydroxyaldehyde in the presence of 30 mM sodium cyanoborohydride, as the reductant, at about 5°C for 3 h. After the reaction, 0.2 M HCl was slowly added to adjust the pH of the reaction mixture to below 4 in order to destroy the excess amount of reductant. The modified protein was exhaustively dialyzed against water and then lyophilized.

2.3. Analytical methods

Amino acid analysis was performed on a Hitachi L-8500 analyzer after hydrolysis of protein sample in 6 M HCl under vacuum at 110°C for 20 h. CD spectra were

recorded with a Jasco J-600 spectropolarimeter in 0.05 M glycine buffer (pH 3.0) at room temperature. Gel filtration chromatography was carried out using a Beckman System Gold liquid chromatograph equipped with a YMC-Pack Diol-120 column (300 × 8.0 mm i.d.). Protein was eluted with a 0.1 M sodium phosphate buffer (pH 7.0) containing 0.2 M NaCl. Capillary electrophoresis was performed on a Beckman P/ACE System 2100 and 5510 with a fused silica capillary of 50 cm effective length.

2.4. DSC measurements

Thermal denaturation was measured on a Seiko SSC-560U differential scanning calorimeter at a scanning rate of 1 K min^{-1} and at protein concentrations of 8–12 mg ml^{-1} in 0.05 M glycine buffer at pH 3.0. Some experiments were carried out in the same buffer at different pH values in the range of 1.8–4.2. Protein solution was subjected to dialysis against the solvent prior to making the DSC measurements. Sample solution (0.07 ml) was hermetically packed in a silver calorimetric vessel. Protein concentration was determined spectrophotometrically by using an extinction coefficient of 2.69 cm^2 mg^{-1} at 280 nm [6], which was assumed not to be affected by modification. The calorimetric enthalpy of denaturation ΔH_{cal} was evaluated from the DSC recordings in accordance with the previously described method [7]. The temperature of maximal excess heat flow was assumed as the denaturation temperature T_d at which the transition is half complete, because of the symmetrical nature of the peak.

2.5. Activity measurements

Enzymatic activities at various temperatures were determined using ethylene glycol chitin as a substrate in 0.1 M acetate buffer at pH 4.5. The reducing power produced after 30 min reaction was measured in accordance with the procedure of Imoto and Yagishita [8].

3. Results and discussion

Lysozyme was chemically modified with three aliphatic aldehydes of varying chain length, acetaldehyde, *n*-butylaldehyde and *n*-hexylaldehyde, and two hydroxyaldehydes of varying hydroxy-group content, glycolaldehyde and glyceraldehyde. Amino acid analyses showed that all the chemical modifications used in this study were directed towards the amino groups of the protein and were specific for this group under the conditions used. These modified preparations gave a single sharp peak in the gel filtration chromatographic and capillary electrophoretic patterns, indicating that the derivatives were homogeneous with respect to size and charge or, at most, that there was a very narrow distribution of the number of modified lysine residues in each preparation. Furthermore, the near- and far-ultraviolet CD spectra of all the modified derivatives were almost similar to those of unmodified lysozyme,

Table 1
Amino acid analyses of the lysine residues of unmodified and modified lysozymes [a]

Lysozymes	ε-*N*-modified lysine	Lysine	Total
Unmodified		6.13	6.13 [b]
Ethylated	3.89 [c]	1.94	5.83
n-Butylated	3.93	1.95	5.88
n-Hexylated	3.79	2.13	5.92
2-Hydroxyethylated	4.07	1.81	5.88
2,3-Dihydroxypropylated	4.06	1.97	6.03

[a] The values are expressed as molar ratios normalized to 6.00 for valine. [b] The amino acid sequence of lysozyme contains 6 lysine residues. [c] The value contains 0.15 of ε,ε-*N*,*N*-diethyllysine.

indicating that the modifications had little effect on the secondary structure of the protein and on the asymmetry of the environment and degree of rotational constraint of the aromatic residues.

As shown in Table 1, approximately four of the original six lysine residues of lysozyme were modified in each preparation. The single NH_2-terminal and six lysine ε-amino groups in hen egg-white lysozyme have been demonstrated to be in different protein environments by X-ray crystallography [9–11]. The ε-amino groups of lysine 1 and 13 which interact with the carboxyl groups of glutamic acid 7 and the COOH-terminal leucine 129 to form ion pairs, respectively, would be extremely resistant to the modification. Lysine 97 is only located on the outside of the protein and the side chain projects into the surrounding solvent. This amino group probably possesses high reactivity to the modifying reagents. Among the remaining four amino groups which are involved in hydrogen bonds to other protein residues, the NH_2-terminal α-amino group would be partially shielded from the solvent compared with the ε-amino groups of lysine 33, 96 and 116, as expected from the amino acid analytical result. There is no significant modification of the NH_2-terminal α-amino group with the formation of a product which does not react with ninhydrin and hence is not monitored by the amino acid analyzer.

The thermal stability of the unmodified and modified lysozymes was investigated with thermal denaturation experiments by DSC in 0.05 M glycine buffer at different pH values. Repeating scans exhibited good reversibility in the thermal denaturation of modified lysozymes, as well as the unmodified protein, when the sample was rapidly cooled after the preceding scan. Therefore, the equilibrium thermodynamic analysis was used to obtain parameters characterizing the thermal denaturation of these proteins. A two-state process for the thermal denaturation of unmodified lysozyme has been well established [12–14]. The temperature T_d and calorimetric enthalpy ΔH_{cal} of denaturation obtained at pH 3.0 are listed in Table 2, in which each value is the average from five or more experiments. There is an apparent difference between alkylation and hydroxyalkylation in the observed effect on the T_d and ΔH_{cal} of lysozyme. Alkylation resulted in a substantial reduction in the T_d and ΔH_{cal} of the protein, depending on the size of the introduced alkyl groups.

Table 2
Thermodynamic parameters characterizing the thermal denaturation of unmodified and modified lysozymes in 0.05 M glycine buffer at pH 3.0

Lysozymes	T_d [a]/ °C	ΔH_{cal}/ (kJ mol^{-1})	$\Delta_d C_p$ [b]/ (kJ K^{-1} mol^{-1})	$\Delta G(25°C)$ [c]/ (kJ mol^{-1})
Unmodified	72.5	490 ± 9	6.21	46
Ethylated	70.9	436 ± 8	6.14	38
n-Butylated	69.4	424 ± 11	5.96	37
n-Hexylated	63.5	358 ± 13	5.82	28
2-Hydroxyethylated	72.5	485 ± 10	6.22	45
2,3-Dihydroxypropylated	72.6	469 ± 14	6.18	43

[a] Temperature has experimental errors of ±0.1°C. [b] Heat capacity change on denaturation was determined from the temperature dependence of the calorimetric denaturation enthalpy measured at different pH values. [c] Gibbs energy of denaturation at 25°C, $\Delta G(25°C)$, was calculated using Eq. 1.

Hydroxyalkylation, however, had little effect on the T_d and ΔH_{cal} of lysozyme, which was not affected by the size or hydroxyl-group content of the entering hydroxyalkyl groups.

The ΔH_{cal} of unmodified, dihydroxypropylated, butylated and hexylated lysozymes obtained at various pH values in the range of 1.8–4.2 are plotted against the corresponding T_d in Fig. 1. Calorimetric denaturation enthalpy is known to be a linear function of temperature and the slope corresponds to the heat capacity change, $\Delta_d C_p (= d\Delta H_{cal}/dT)$, upon denaturation [15,16]. The $\Delta_d C_p$ values obtained from such plots are listed in Table 2. The $\Delta_d C_p$ for alkylated lysozymes appears to decrease with an increase in the size or hydrophobicity of the entering alkyl groups, but the difference is within the experimental error. Becktel and Schellman [16] have stated that the actual error in determining $\Delta_d C_p$ on measuring T_d and ΔH_{cal} as a function of pH is about ±10% in favorable cases. In addition, there is no significant difference in the $\Delta_d C_p$ among unmodified and two hydroxyalkylated lysozymes. The results suggest that the enthalpies of both the native and the denaturated states may be affected in the same manner by alkylation or hydroxyalkylation. The heat capacity change observed on protein unfolding is thought to result mainly from the exposure of buried non-polar side chains to solvent [17]. As indicated by the CD spectroscopic results, the modification used in the present study had little effect on the native conformation of lysozyme. These observations suggest that there may be no significant difference in the denatured forms between unmodified and modified lysozymes.

A more definite estimation of the effect of lysine modification on the protein stability is given by the comparison of the Gibbs energy ΔG of denaturation at identical temperatures. Assuming that the $\Delta_d C_p$ is independent of temperature, the value of ΔG at any temperature T, $\Delta G(T)$, can be calculated from T_d, ΔH_{cal} and $\Delta_d C_p$ with the equation [16,18]

$$\Delta G(T) = \Delta H_{cal}(1 - T/T_d) - \Delta_d C_p[(T_d - T) + T \ln(T/T_d)] \quad (1)$$

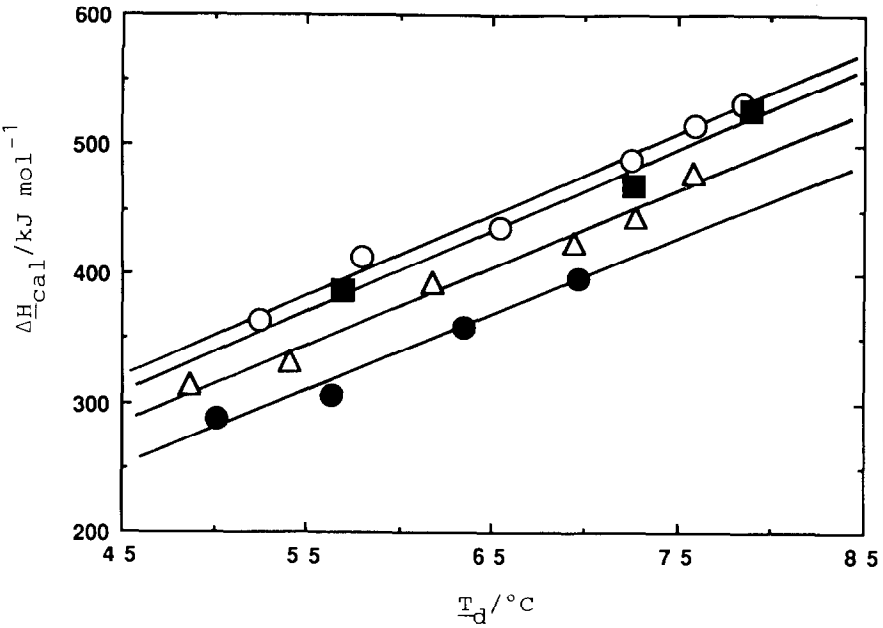


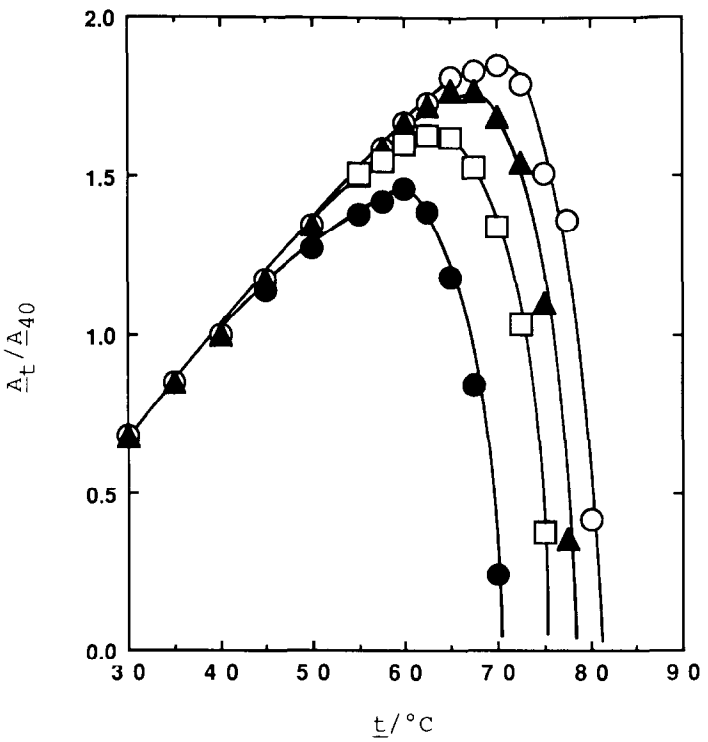
Fig. 1. Dependence of the denaturation enthalpy of unmodified (○), 2,3-dihydroxypropylated (■), *n*-butylated (△), and *n*-hexylated (●) lysozymes on the denaturation temperature. The values of unmodified and *n*-butylated lysozymes were obtained in 0.05 M glycine buffer at pH 1.8, 2.2, 2.6, 3.0, 3.3 and 4.2; the values of the *n*-hexylated derivative were obtained at pH 2.2, 2.6, 3.0 and 4.2; and the values of the 2,3-dihydroxypropylated derivative were obtained at pH 2.0, 3.0 and 4.0. Ordinate, ΔH_{cal}/(kJ mol^{-1}); abscissa, T_d/°C.

The ΔG values at 25°C, ΔG(25°C), obtained from the temperature dependence of ΔG at pH 3.0 are listed in the last column in Table 2. The ethylated, *n*-butylated and *n*-hexylated derivatives were 8, 9 and 18 kJ mol^{-1}, respectively, less stable than unmodified lysozyme at pH 3.0 and 25°C, depending on the size or hydrophobicity of the entering alkyl groups. However, 2-hydroxyethylation and 2,3-dihydroxypropylation had little effect on the stability of lysozyme under the conditions employed.

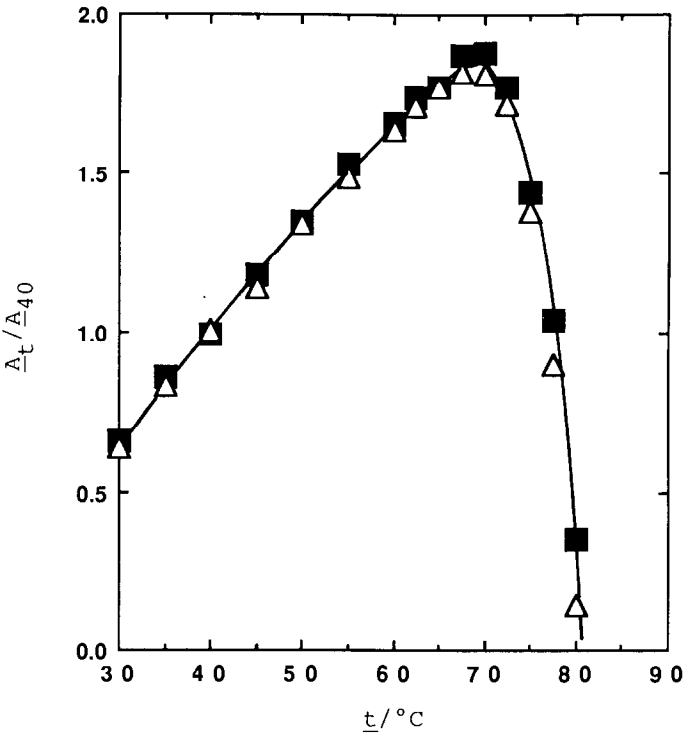
The temperature dependence of the enzymatic activities of unmodified and modified lysozymes were determined using ethylene glycol chitin as a substrate in 0.1 M acetate buffer at pH 4.5. The activities were expressed as values relative to the activity of unmodified lysozyme at 40°C and are plotted as a function of temperature in Fig. 2. As the temperature increased, the activity of every lysozyme first increased, reached a maximum, then decreased, and vanished at above 73, 77, 80, 82, 82 and 82°C for the hexylated, butylated, ethylated, hydroxyethylated, dihydroxypropylated derivatives and the unmodified protein, respectively. Below 45°C, the activities of the modified enzymes were similar to that of the unmodified enzyme. The activities of unmodified, hydroxyethylated and dihydroxypropylated

lysozymes reached their maxima at about 70°C. The temperature of the maximal activity for the alkylated derivatives was 67, 63 and 60°C for the ethylated, butylated and hexylated derivatives, respectively, decreasing with an increase in the size or hydrophobicity of the entering alkyl groups. This order was exactly the same as that of the thermal stability. For the maximum activity, the order of the magnitude was: unmodified = 2-hydroxyethylated = 2,3-dihydroxypropylated > ethylated > *n*-butylated > *n*-hexylated lysozymes. This order is exactly the same as that of the thermal stability.

Capillary electrophoresis demonstrated that all the modified derivatives were eluted a little later than the unmodified protein in 25 mM sodium phosphate buffer at pH 2.5; this may be ascribed to slight changes in the molecular size resulting from the modification and to changes in the pK value caused by the conversion of the primary amino groups into secondary amino groups. Either alkylation or hydroxyalkylation preserves the positive charges of the protein amino groups and has little or no effect on the charge distribution of the protein [1,4]. Alkylation causes an increase in the steric interference and hydrophobicity at the points of the modification. Hydroxyalkylation causes an increase in the steric interference, but does not cause an increase in the hydrophobicity at the points of the modification. All the alkylated lysozymes obtained here were found to be less



(a)



(b)

Fig. 2. (a) and (b). Temperature dependence of the activities of unmodified (a), alkylated (a) and hydroxyalkylated (b) lysozymes against ethylene glycol chitin in 0.1 M acetate buffer at pH 4.5. A_{40} and A_t are the enzymatic activity of unmodified lysozyme at 40°C and those of the unmodified and modified proteins at each temperature, respectively. The symbols are (○) unmodified, (▲) ethylated, (□) *n*-butylated, (●) *n*-hexylated, (■) 2-hydroxyethylated and (△) 2,3-dihydroxypropylated lysozymes. Ordinate, A_t/A_{40}; abscissa, t/°C.

thermally stable than unmodified lysozyme, but the thermal stabilities of hydroxyethylated and dihydroxypropylated lysozymes were almost similar to that of the unmodified protein. These observations suggest that the change in the thermal stability upon lysine modification of lysozyme is significantly dependent on qualitative factors such as the hydrophobicity or hydrophilicity of the entering substituents, as well as on other factors such as the steric hindrance induced by the entering substituents.

References

[1] G.E. Means and R.E. Feeney, Biochemistry, 7 (1968) 2192.
[2] N. Jenotoft and D.G. Dearborn, J. Biol. Chem., 254 (1979) 4359.

[3] K. Fretheim, B. Egclandsdal and O. Harbitz, Int. J. Pept. Protein Res., 25 (1985) 601.
[4] J.H. Bradbury and L.R. Brown, Eur. J. Biochem., 40 (1973) 565.
[5] Y. Fujita and Y. Noda, Int. J. Pept. Protein Res., 38 (1991) 445.
[6] K. Hamaguchi and H. Sakai, J. Biochem. (Tokyo), 57 (1965) 721.
[7] Y. Fujita and Y. Noda, Bull. Chem. Soc. Jpn., 57 (1984) 2177.
[8] T. Imoto and K. Yagishita, Agr. Biol. Chem., 35 (1971) 1154.
[9] W.J. Browne, A.C.T. North, D.C. Phillips, K. Brew, T.C. Vanaman and R.L. Hill, J. Mol. Biol., 42 (1969) 65.
[10] T. Imoto, L.N. North, A.C.T. North, D.C. Phillips and J.A. Rupley, in P.D. Boyer (Ed.), The Enzymes, Academic Press, New York, 3rd edn., Vol. 7, 1972, p. 665.
[11] R.J. Feldmann, Atlas of Macromolecular Structure on Microfiche, Tracor Jitco Inc., Rockville, MD, 1st edn., 1976, p. 491.
[12] A.J. Sophianopoulos and B.J. Weiss, Biochemistry, 3 (1964) 1920.
[13] C.C. McDonald, W.D. Philips and J.D. Glickson, J. Am. Chem. Soc., 93 (1971) 235.
[14] P.L. Privalov and N.N. Khechinashvili, J. Mol. Biol., 86 (1974) 665.
[15] P.L. Privalov, Adv. Protein Chem., 33 (1979) 167.
[16] W.J. Becktel and J.A. Schellman, Biopolymers, 26 (1987) 1859.
[17] R.L. Baldwin, Proc. Natl. Acad. Sci. USA, 83 (1986) 8069.
[18] G. Velicelebi and J.M. Sturtevant, Biochemistry, 18 (1979) 1180.