EFFECT OF CHARGE NEUTRALIZATION AT LYSINE RESIDUES ON THE FREE ENERGY OF STABILIZATION OF HEN EGG WHITE LYSOZYME

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Positive charge at lysine residues of hen egg white lysozyme (HEWL) was neutralized through carbamylation reaction and its effect on the conformational stability of the enzyme was studied. Additionally, guanidination of HEWL was also performed, in which the modified derivative retained the positive charge at lysine residues, but with the addition of guanidino group. About 88% and 85% of lysine residues of HEWL were modified in carbamylated (C88) and guanidinated (G85) preparations, respectively. Polyacrylamide gel electrophoresis and Sephadex G-75 gel chromatographic results confirmed the charge and size homogeneity, respectively, of the modified derivatives. A comparison of Stokes radius and frictional ratio values of native (1.31 nm; 0.83), C88 (1.39 nm; 0.87) and G85 (1.20 nm; 0.75) HEWLs suggested an increase in the hydrodynamic volume of C88 HEWL and a more compact conformation for G85 HEWL. Decrease in free energy of stabilization ($\Delta G^\text{D}_{\text{H2O}}$) from 8258 and 8918 cal/mol (for native HEWL) to 6362 and 6137 cal/mol (for C88 HEWL) as obtained from GdnHCl denaturation studies using fluorescence intensity and emission maximum probes, respectively, clearly indicated destabilization of the enzyme upon carbamylation. This destabilization can be attributed to the increase in electrostatic free energy in C88 HEWL. These findings were further supported by $\Delta G^\text{D}_{\text{H2O}}$ values obtained for G85 HEWL (8092 and 8386 cal/mol), in which retention of positive charge on HEWL was responsible in maintaining the stability of the enzyme. All these results revealed significant contribution of positively charged lysine residues in the conformational stability of HEWL through salt bridges.

Key words: carbamylation, conformational stability, denaturation, guanidination, lysozyme.

INTRODUCTION

Amino acid substitution through site-directed mutagenesis is one of the several strategies employed for increasing enzymes’ stability in order to withstand severe reaction conditions used in industries (1). However, characteristics of

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genetically modified enzymes remain ambiguous as the mutant can be either stable (2) or unstable (3, 4). This can be attributed to the lack of details regarding contribution of a particular amino acid type towards the stability of an enzyme and selection of an appropriate candidate for its substitution.

The three-dimensional structure of a protein is stabilized by various intramolecular noncovalent interactions such as hydrogen bonds, hydrophobic, electrostatic and \textit{van der Waals} interactions between different amino acid side chains (5, 6). Although hydrophobic interactions and hydrogen bonds are believed to be the main stabilizing forces of native protein conformation, which has low conformational stability, the role of other forces becomes important to keep the equilibrium towards the native state. Being present at the molecular surface, charged residues offer possible sites in mutagenesis studies of various proteins (7). The role of charged residues in protein stability through electrostatic interactions has been discussed in detail (7–9) and charge reversal mutations of several proteins have been shown to either increase or decrease protein stability (10, 11). Chemical modification offers a complementary approach to site-directed mutagenesis in providing information about the importance of a particular amino acid type in protein’s stability and its suitability for substitution (12). In many cases, altered activity and stability have been noticed upon chemical modification (12–14 and other references cited therein).

Hen egg white lysozyme (HEWL) (EC 3.2.1.17) has been used as a preferred protein model due to its smaller size, single polypeptide chain with known amino acid sequence and three-dimensional structure (15–17). Moreover, the use of HEWL in food, dairy, poultry (18–20) and pharmaceutical (21) industries has classified it as an industrial enzyme. The remarkable stability of the enzyme can be ascribed to the presence of four disulfide bridges along with other non-covalent interactions (22). Being charged and surface-exposed (17), lysine residues of HEWL have been selected as suitable target for a variety of modification reactions, and their role in structure-activity/stability relationship of the enzyme has been described (23–28). However, none of these studies has shown any quantitative data on the stability of modified derivatives against chemical denaturants as well as free energy of stabilization.

Recently, we have shown the effect of succinylation of lysine residues on the conformational stability of HEWL using GdnHCl denaturation (27). Since charge imbalance caused by succinylation of lysine residues was more severe due to the conversion of positively charged amino group into negatively charged succinyl group (29), we thought of using a mild carbamylation reaction – where positive charge on amino group is neutralized (30) – to study the effect of charge neutralization on the conformational stability of HEWL. In order to compare the relative strength of these modification reactions towards electrostatic destabilization of HEWL, guanidination – where positive charge on lysine residue is retained (31) –
of HEWL was also performed. Here, we describe the effect of carbamylation and guanidination of lysine residues of HEWL on its conformational stability using GdnHCl denaturation.

MATERIALS AND METHODS

Hen egg white lysozyme (HEWL) (Lot 096K1237), Sephadex G-75 (Lot 018K1469), guanidine hydrochloride (GdnHCl) (purity \( \geq 99\% \)) (Lot 027K5419) and different marker proteins, such as bovine serum albumin (BSA) (Lot 015K0591), carbonic anhydrase (Lot 99H0669), \( \alpha \)-chymotrypsinogen A, type II (Lot 16H7075), myoglobin (Lot 064K7006) and cytochrome c (Lot 27H7065) were all products of Sigma Chemical Co., USA. Potassium cyanate and O-methylisourea hydrogen sulfate were purchased from Fluka, Germany. Trinitrobenzenesulfonic acid (TNBS) was procured from Pierce Chemical Co., USA. All other reagents used were of analytical grade purity. All the experiments were performed at 28°C, unless otherwise stated.

Analytical methods. Protein concentration was determined using the method of Lowry et al. (32), with BSA as the standard. The concentration of GdnHCl stock solution was routinely checked using the data of Nozaki (33), as recommended by Pace et al. (34).

Modification of amino groups of HEWL. Carbamylation of HEWL was carried out according to the method of Strosberg and Kanarek (35) with slight modification. Solid potassium cyanate equivalent to 0.95 M was added successively to a continuously stirred protein solution (500 mg HEWL dissolved in 80 mL of 0.5 M Tris-HCl buffer, pH 8.0) placed in a water bath at 40°C over a period of 30 min. The pH of the reaction mixture was maintained at pH 8.0 by simultaneous addition of 1.0 M acetic acid throughout the reaction time. The reaction was allowed to proceed at 40°C for 4 h. Upon completion of the reaction, the protein solution was extensively dialyzed against eight liters of 0.1 M sodium acetate buffer, pH 4.5 with successive changes for 48 h at room temperature and finally stored at 6°C in the same buffer.

Guanidination of HEWL was performed in the same way as described by Masuda et al. (25). Twenty five milliliters of 1.0 M O-methylisourea hydrogen sulfate solution in water were added in aliquots to a continuously stirred protein solution (400 mg HEWL dissolved in 25 mL solution mixture, which was prepared by adding 2.5 mL of 0.1 M Tris-HCl buffer, pH 8.4, containing 1.0 M NaCl and 22.5 mL water) at 4°C for a total period of 30 min. The final concentration of O-methylisourea hydrogen sulfate was 0.5 M. Appropriate volumes of 5.0 M NaOH solution were used to adjust the pH of the reaction mixture at pH 10.5. The reaction
was continued at 4°C for 4 days with stirring. Finally, the protein solution was extensively dialyzed against 0.06 M sodium phosphate buffer, pH 7.0 for 48 h at 6°C and stored in refrigerator in the same buffer.

Modified amino groups (both α- and ε-amino groups) in carbamylated and guanidinated HEWL preparations were quantified by the TNBS reaction method (36) as described in the Pierce Technical Bulletin (37).

Polyacrylamide gel electrophoresis (PAGE). The method of Reisfeld et al. (38) was used to determine the charge homogeneity of native and modified HEWL preparations using 15% (w/v) polyacrylamide gel under acidic condition. The gel was stained with coomassie brilliant blue R-250, as recommended elsewhere (39).

Analytical gel chromatography. A Sephadex G-75 column (1.66 × 56 cm) equilibrated with 0.06 M sodium phosphate buffer, pH 7.0 containing 0.02% sodium azide was used to determine hydrodynamic properties, such as Stokes radius and frictional ratio of native and modified HEWL preparations following the procedure described elsewhere (40). Different marker proteins used for column calibration with their Stokes radii given in parentheses were: BSA (3.63 nm) (41), carbonic anhydrase (2.30 nm) (42), α-chymotrypsinogen A (2.20 nm) (43), myoglobin (1.90 nm) (44) and cytochrome c (1.70 nm) (45). Values of elution volume of different proteins including native and modified HEWLs were transformed into distribution coefficient, $K_d$ and available distribution coefficient, $K_{av}$ as described earlier (40). Linear plots of $(-\log K_{av})^{1/2}$ versus Stokes radius (46) and Stokes radius versus $\text{erfc}^{-1}K_d$ (47) were used to determine the Stokes radii of native and modified HEWLs. Frictional ratio ($f/f_o$) of native and modified preparations was calculated using the following formula, where different terms have their usual significance (40):

$$\frac{f}{f_o} = \frac{r}{3\nu_2 M/4\pi N}^{1/3}$$

(1)

A value of 0.703 mL/g for partial specific volume ($\nu_2$) was used for both native and modified HEWLs (48). A molecular weight value of 14,307 dalton was used for native HEWL (15), whereas molecular weight contributions of carbamyl and guanidino groups were taken into consideration for the molecular weight calculations of carbamylated and guanidinated HEWLs, respectively.

GdnHCl denaturation. Denaturation studies were performed in the same way as described by Ong et al. (27). Different volumes of stock (7.0 M) GdnHCl solution were added to 0.5 mL stock (10 µM) protein (native or modified HEWLs) solution in order to get the desired concentration of denaturant in a total volume of 5.0 mL which was made with 0.06 M sodium phosphate buffer, pH 7.0. The contents of the reaction mixture were mixed well and incubated for 12 h at 25°C. All these samples were subjected to intrinsic fluorescence measurements on a Hitachi fluorescence spectrophotometer, model F-2500, using a 1 cm pathlength.
Conformational stability of carbamylated lysozyme

As unfolding transition of native HEWL in GdnHCl is known to follow a two-state mechanism (27), denaturation data were normalized into apparent fraction of denatured form, \( F_D \), using equation (2),

\[
F_D = \frac{Y - Y_N}{Y_D - Y_N}
\]

where different terms have their standard meaning (49). Values of \( Y_N \) and \( Y_D \) were obtained by the linear extrapolation method as described earlier (49). Values of \( F_D \) ranging from 0.25 to 0.75 (0.20 to 0.80 in some cases) were used to calculate apparent equilibrium constant, \( K_D \) and subsequently, free energy change, \( \Delta G_D \) using equations (3) and (4), respectively.

\[
K_D = \frac{F_D}{1 - F_D}
\]

\[
\Delta G_D = -RT \ln K_D
\]

The free energy of stabilization, \( \Delta G_D^{H_2O} \) value was obtained from the Y-axis intercept of the linear plot between \( \Delta G_D \) and denaturant concentration, \([D]\) (equation (5)).

\[
\Delta G_D = \Delta G_D^{H_2O} - m[D]
\]

RESULTS AND DISCUSSION

ANALYTICAL PROPERTIES OF MODIFIED HEWL DERIVATIVES

Positive charge at lysine residues of HEWL was neutralized and converted into guanidino group with the retention of positive charge through carbamylation and guanidination reactions, respectively. Figure 1 shows the results of carbamylated and guanidinated reactions performed on HEWL as monitored by TNBS color reaction. A marked decrease in the slope value of the linear plots between absorbance at 335 nm and amount of protein, observed with carbamylated, ‘C’ (0.33) and guanidinated, ‘G’ (0.42) preparations compared to native, ‘N’ (2.73) HEWL, suggested the modification of amino groups (both \( \alpha \)- and \( \varepsilon \)-amino groups) of HEWL. Substitution of these slope values into the formula, \% modification = \( 1 - m/m_0 \times 100 \), where \( m_0 \) and \( m \) are the slope values obtained with native and modified proteins, respectively, yielded the values of 88% and 85% modification for carbamylated and guanidinated HEWLs, respectively (Table 1). Number of amino groups modified in both preparations was calculated to be six (Table 1) after taking the total number of amino groups in HEWL as seven (1 \( \alpha \)-amino and 6 \( \varepsilon \)-amino groups) (15). In view of the presence of all six lysine residues at the...
surface of the protein (17), such a high degree of modification seems justifiable. Under the experimental conditions used in this study (reaction pH of 8.0 and extensive dialysis against 0.1 M sodium acetate buffer, pH 4.5), carbamylation reaction seem to have occurred primarily at amino groups (30, 35).

![Graph](image)

**Fig. 1.** – TNBS reaction to determine the extent of modification in modified HEWL preparations. Native, ‘N’ (■), carbamylated, ‘C’ (▲) and guanidinated, ‘G’ (●) HEWL preparations. Straight lines were drawn using least squares analysis.

<table>
<thead>
<tr>
<th>Analytical property</th>
<th>HEWL preparations</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Native</td>
<td>Carbamylated</td>
</tr>
<tr>
<td>Extent of modification (%)</td>
<td>0</td>
<td>88</td>
</tr>
<tr>
<td>No. of amino groups modified</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Relative cathodic mobility, Rm</td>
<td>0.56</td>
<td>0.30</td>
</tr>
</tbody>
</table>

**Table 1**

Analytical properties of native and modified HEWL preparations

*Note:* a Total number of amino groups in HEWL was taken as 7 (6 ε-amino + 1 α-amino) (15). b According to the method of Reisfeld et al. (38).

Charge homogeneity of modified preparations was checked by native PAGE carried out at pH 4.5 (38), since HEWL is a basic protein with an isoelectric point of 11.5 (25). Both 88% carbamylated (C88) and 85% guanidinated (G85) HEWLs were found to be homogeneous with respect to charge due to their migration in the form of a single band as shown in the electrophoretogram (Inset of Fig. 2). However, the relative cathodic mobility (Rm) of C88 HEWL decreased significantly to a value of 0.30 compared to 0.56 observed with both native and G85 HEWLs (Table 1).
Fig. 2. − Elution profiles of native, N ( ), 88% carbamylated, ‘C88’ ( ) and 85% guanidinated, ‘G85’ ( ) HEWLs on Sephadex G-75 column (1.66 × 56 cm) equilibrated with 0.06 M sodium phosphate buffer, pH 7.0 containing 0.02% sodium azide. Elution volumes of native (N) and modified (C88 and G85) HEWLs are marked by arrows. Inset: Electrophoretic pattern of native (N) and modified (C88 and G85) HEWLs on 15% polyacrylamide gel following the method of Reisfeld et al. (38). Approximately 10 µL of the sample containing 10 µg of protein was loaded in each well and electrophoresis was run in β alanine-acetic acid buffer, pH 4.5, for about 2 h. The gel was stained with 0.2% (w/v) coomassie brilliant blue R-250 and destained in 5% methanol, 7% acetic acid solution according to the method of Laemmli (39). Position of the tracking dye, methylene blue is shown by an arrow.

Since carbamylation neutralizes the positive charge at lysine residues (30) and thus leads to a decrease in the net positive charge on the protein, decrease in relative cathodic mobility observed with C88 HEWL seems understandable. In an earlier study, decrease in relative cathodic mobility has also been shown upon acetylation of HEWL (25) which also neutralizes the positive charge at lysine residues (50). Acetylation of HEWL caused the reduction in the isoelectric point from 11.1−11.5 to 9.5−10.0 (25, 51) suggesting a decrease in the net positive charge in the modified protein. In view of the similar type of neutralization of positive charge in carbamylation reaction, a similar decrease in the net positive charge in C88 HEWL preparation is expected which might be responsible for the decrease in relative cathodic mobility observed in the carbamylated preparation. Our results on G85 HEWL showing similar relative cathodic mobility (0.56) to that of native HEWL (0.56) (Table 1), agreed well with a previous report (25) and seem justifiable due to the retention of positive charge on lysine residues upon guanidination (31).
EFFECTS OF CARBAMYLATION AND GUANIDINATION ON THE CONFORMATION OF HEWL

Size homogeneity of both C\textsubscript{88} and G\textsubscript{85} HEWL preparations was evident from their elution profiles on Sephadex G-75 column (1.66 × 56 cm) showing a single symmetrical peak (Fig. 2). However, significant differences were noted in the values of elution volume. Whereas C\textsubscript{88} HEWL eluted a little earlier with an elution volume of 93 mL compared to 95 mL obtained with native HEWL, G\textsubscript{85} HEWL took longer in eluting out from the column with an elution volume of 98 mL (Fig. 2, Table 2).

<table>
<thead>
<tr>
<th>Gel chromatographic parameter</th>
<th>HEWL preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
</tr>
<tr>
<td>Elution volume, V\textsubscript{e} (mL)</td>
<td>95</td>
</tr>
<tr>
<td>Distribution coefficient, K\textsubscript{d}</td>
<td>0.767</td>
</tr>
<tr>
<td>Available distribution coefficient, K\textsubscript{av}</td>
<td>0.667</td>
</tr>
<tr>
<td>(−log K\textsubscript{av})\textsuperscript{1/2}</td>
<td>0.420</td>
</tr>
<tr>
<td>erfc\textsuperscript{−1}K\textsubscript{d}</td>
<td>0.209</td>
</tr>
</tbody>
</table>

Since elution volume relates inversely to the molecular size of the protein, decrease in elution volume observed with C\textsubscript{88} HEWL was indicative of molecular expansion in this modified preparation. Such increase in hydrodynamic volume of C\textsubscript{88} HEWL is expected due to the increase in electrostatic free energy induced by charge neutralization in carbamylated preparation. In contrast, increase in elution volume observed with G\textsubscript{85} HEWL suggested a more compact conformation in the modified preparation compared to native HEWL. This was in agreement with previous reports, in which guanidination has been shown to produce compact protein structures (52, 53). In order to strengthen these findings, hydrodynamic properties such as Stokes radius and frictional ratio of these modified preparations were determined using analytical gel chromatographic data. Table 2 shows values of K\textsubscript{av}, (−log K\textsubscript{av})\textsuperscript{1/2} and erfc\textsuperscript{−1}K\textsubscript{d} of native and modified HEWL preparations obtained after transforming elution volumes of these proteins according to the standard procedure (40). Treatment of gel chromatographic data of marker proteins according to Laurent and Killander (46) and Ackers (47) yielded straight line plots (Fig. 3) which fitted the following straight line equations, respectively:

\[
(−\log K\textsubscript{av})^{1/2} = 0.233 \text{ Stokes radius, nm} + 0.119 \quad (6)
\]

\[
\text{Stokes radius, nm} = 2.709 \text{ erfc}^{-1}K\textsubscript{d} + 0.766 \quad (7)
\]

Substitution of (−log K\textsubscript{av})\textsuperscript{1/2} and erfc\textsuperscript{−1}K\textsubscript{d} values obtained for native, C\textsubscript{88} and G\textsubscript{85} HEWLs into equations (6) and (7) yielded values of Stokes radii of these proteins,
Fig. 3. – Stokes radius determination for native (N) and modified (C88 and G55) HEWLs according to Laurent and Killander (46) and Ackers (47). Different marker proteins are shown by numbers 1–5: 1. BSA; 2. carbonic anhydrase; 3. α-chymotrypsinogen A; 4. myoglobin and 5. cytochrome c. Positions of N, C88 and G55 HEWLs are marked by arrows. Straight lines were drawn using least squares analysis.

Table 3
Hydrodynamic properties of native and modified HEWL preparations as obtained from analytical gel chromatographic data

<table>
<thead>
<tr>
<th>Hydrodynamic property</th>
<th>Native</th>
<th>88% Carbamylated</th>
<th>85% Guanidinated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stokes radius, r (nm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>From Eq. (6)</td>
<td>1.29</td>
<td>1.37</td>
<td>1.17</td>
</tr>
<tr>
<td>From Eq. (7)</td>
<td>1.33</td>
<td>1.40</td>
<td>1.23</td>
</tr>
<tr>
<td>Mean</td>
<td>1.31</td>
<td>1.39</td>
<td>1.20</td>
</tr>
<tr>
<td>Frictional ratio, f/f₀</td>
<td>0.83</td>
<td>0.87</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Note: Eq. = Equation

which along with the mean values are given in Table 3. Smaller value of Stokes radius (1.31 nm) obtained for native HEWL compared to the one (1.90 nm) reported in the literature (48) can be ascribed to the anomalous elution behaviour of native HEWL on Sephadex G-75 column due to possible interaction of HEWL with Sephadex gel (54). Since all the experimental points of native and modified derivatives of HEWL had fallen outside the range of standard proteins used in the ‘calibration curve’ (Fig. 3) due to their interaction with the gel, such experimentally
determined Stokes radii of these proteins may not be taken as the real Stokes radii rather said to be the ‘available Stokes radii’ of these preparations. In most of the earlier studies involving column calibration of Sephadex G-75, cytochrome c has been used as the standard protein with the lowest molecular weight (12,400 dalton) and Stokes radius (1.70 nm) (55–57). Had lysozyme shown its normal elution behaviour on this column, it should have eluted closer to myoglobin which has similar Stokes radius (1.90 nm) (44). In spite of getting the anomalous behaviour of lysozyme and its modified derivatives, ‘available Stokes radii’ can be used to investigate the conformational alteration (if any) in lysozyme upon modification. In view of the above, a comparison of Stokes radii of native and modified HEWLs obtained with analytical gel chromatography seems appropriate. For example, C<sub>88</sub> HEWL showed a slight increase in Stokes radius from 1.31 to 1.39 nm, whereas a decrease in Stokes radius from 1.31 to 1.20 nm was observed with G<sub>85</sub> HEWL, suggesting a smaller expansion in C<sub>88</sub> HEWL and a compact structure for G<sub>85</sub> HEWL. Using a molecular weight value of 14,565 and 14,571 dalton for C<sub>88</sub> and G<sub>85</sub> HEWLs, respectively, frictional ratio (f/f<sub>o</sub>) of these preparations was calculated following the procedure described in ‘MATERIALS AND METHODS’ and values are given in Table 3. Emergence of a smaller f/f<sub>o</sub> value (0.83) observed for native HEWL compared to a value of 1.01 reported earlier (58) was the result of fitting the smaller value of Stokes radius obtained from gel chromatographic data in the given formula (equation (1)). Unequivocally, a comparison of f/f<sub>o</sub> values obtained for native and modified HEWLs suggested an increase in size of C<sub>88</sub> HEWL and a more compact conformation for G<sub>85</sub> HEWL. Taken together, both hydrodynamic parameters (Stokes radius and f/f<sub>o</sub>) indicated expansion in HEWL conformation upon carbamylation. This seems justifiable in view of the increase in the net negative charge on the protein by 1 unit per lysine residue modified (30) leading to electrostatic repulsion and subsequent destabilization of native protein conformation. Similar destabilization effect has been shown earlier for a number of proteins upon acetylation and carbamylation of their lysine residues (52, 53, 59). In contrast, G<sub>85</sub> preparation was found to be more compact compared to native protein as evident from the decrease in Stokes radius and f/f<sub>o</sub> values. These results were also in line with earlier reports on guanidination of proteins (52, 53).

CONFORMATIONAL STABILITIES OF CARBAMYLATED AND GUANIDINATED HEWLS

GdnHCl denaturation studies of native, C<sub>88</sub> and G<sub>85</sub> HEWL preparations were made in order to determine the effect of carbamylation and guanidination on the conformational stability of HEWL. Figure 4 shows fluorescence spectra of native (A), C<sub>88</sub> (B) and G<sub>85</sub> (C) HEWLS both in the absence and presence of increasing GdnHCl concentrations in 0.06 M sodium phosphate buffer, pH 7.0 at 25°C upon excitation at 295 nm (to excite tryptophan (Trp) residues only). Fluorescence
spectra obtained for native and modified HEWLs under similar conditions but upon excitation at 280 nm (to excite both tyrosine (Tyr) and tryptophan (Trp) residues) have been omitted for brevity. GdnHCl denaturation results of native HEWL (upon excitation at 295 and 280 nm) were adapted from our recently published paper (27). Native HEWL showed an emission maximum at 334 nm when excited at 295 nm (Fig. 4A) and 340 nm upon excitation at 280 nm (data not shown). These values remained unchanged in both modified (C88 and G85) HEWLs. As can be seen from Fig. 4, fluorescence intensity of modified HEWLs increased with increasing GdnHCl concentrations in the same way as shown by native HEWL. This can be ascribed to the release of quenching of tryptophan fluorescence upon denaturation which was otherwise quenched by the closest disulfide bonds (60). Furthermore, red shift in emission maximum was also observed upon denaturation due to exposure of more tryptophan residues in HEWL to polar solvent (60, 61).

Fig. 4. – Fluorescence spectra of native, ‘N’ (A), carbamylated, ‘C88’ (B) and guanidinated, ‘G85’ (C) HEWLs obtained in 0.06 M sodium phosphate buffer, pH 7.0, at 25°C both in the absence and presence of increasing GdnHCl concentrations [(A) 0 – 5.3 M; (B) 0 – 4.3 M and (C) 0 – 5.5 M] upon excitation at 295 nm.
Values of fluorescence intensity at 360 nm obtained from fluorescence spectra (Fig. 4) and emission maximum (taken from selected fluorescence spectra of Fig. 4) were normalized into $F_D$ values using equation (2) as described in ‘MATERIALS AND METHODS’ and plotted against GdnHCl concentration for native, $C_{88}$ and $G_{85}$ HEWLs (Fig. 5A and C respectively). It is apparent from Fig. 5A and C that denaturation of both native and modified HEWL preparations obeyed a two-state, single-step transition model. Several earlier reports also described the denaturation of HEWL as a single-step, two-state transition (61, 62). Values of start-, mid- and end-points of the denaturation transitions of native and modified ($C_{88}$ and $G_{85}$) HEWLs obtained from these plots (Fig. 5A and C) are listed in Table 4. According to Fig. 5A and C (fluorescence intensity and emission maximum as probes), the transition for native HEWL started around 3.5 and 3.3 M GdnHCl and ended around 4.7 and 4.9 M GdnHCl, respectively with a mid-point occurring around 4.1 M GdnHCl (Table 4). These results on native HEWL denaturation (in terms of start- and end-points of transition) obtained with fluorescence intensity and emission maximum as probes agreed well to those resulted from UV difference spectroscopy (63). On the other hand, the transition curve obtained with $C_{88}$ HEWL shifted towards a lower GdnHCl concentration range (Fig. 5A and C). More specifically, transition of $C_{88}$ HEWL started earlier around 2.4/2.3 M GdnHCl and tailed off around 3.8 M GdnHCl with a mid-point at 3.0 M GdnHCl (Table 4) based on fluorescence intensity (Fig. 5A) and emission maximum (Fig. 5C) curves. Such a significant shift in the whole transition curve of $C_{88}$ HEWL towards lower GdnHCl concentration was indicative of reduced conformational stability of the modified derivative. $G_{85}$ HEWL denaturation profiles showed more or less similar transition features to those obtained with native protein (Fig. 5A and C) suggesting guanidination caused neither destabilization nor stabilization of HEWL. Transition of $G_{85}$ HEWL was found to start around 3.1 and 3.3 M GdnHCl and end around 4.9 M GdnHCl with a mid-point falling at 3.9 M GdnHCl (Table 4) when probed by fluorescence intensity (Fig. 5A) and emission maximum (Fig. 5C), respectively. Transition data in terms of start-, mid- and end-point values for different HEWL preparations obtained with fluorescence intensity and emission maximum probes upon excitation at 280 nm (figures omitted for brevity) were found similar to the values obtained upon excitation at 295 nm (Table 4).

Use of emission maximum data for the determination of free energy of stabilization ($\Delta G_{D}^{\text{H2O}}$) has been questioned in several earlier reports (64, 65), as it may lead to erroneous and significantly varied results compared to those derived from fluorescence intensity data. However, in the present study, transition characteristics obtained from emission maximum data were found to be similar to those obtained from fluorescence intensity data (Table 4). Therefore, to evaluate further, normalized data of both fluorescence intensity and emission maximum upon excitation at 295 nm (Fig. 5A and C) were transformed into $K_D$ and $\Delta G_D$ values using
Table 4

GdnHCl denaturation data of native and modified HEWL preparations at pH 7.0 as monitored by intrinsic fluorescence

<table>
<thead>
<tr>
<th>HEWL preparations</th>
<th>Probe</th>
<th>Transition</th>
<th>$\Delta G^\text{H}_2\text{O}$ (cal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>Excitation (295 nm)</td>
<td>F.I. at 360 nm</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Emission maximum</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>Excitation (280 nm)</td>
<td>F.I. at 360 nm</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Emission maximum</td>
<td>3.0</td>
</tr>
<tr>
<td>88% Carbamylated</td>
<td>Excitation (295 nm)</td>
<td>F.I. at 360 nm</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Emission maximum</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>Excitation (280 nm)</td>
<td>F.I. at 360 nm</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Emission maximum</td>
<td>2.4</td>
</tr>
<tr>
<td>85% Guanidinated</td>
<td>Excitation (295 nm)</td>
<td>F.I. at 360 nm</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Emission maximum</td>
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<tr>
<td></td>
<td>Excitation (280 nm)</td>
<td>F.I. at 360 nm</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Emission maximum</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Note: F.I. = Fluorescence intensity

Equations (3) and (4), respectively, as described in ‘MATERIALS AND METHODS’ for native, C88 and G85 HEWLs. Linear plots of $\Delta G^\text{D}$ against GdnHCl concentration for native and modified HEWLs obtained from fluorescence intensity and emission maximum data upon excitation at 295 nm are shown in Fig. 5B and D, respectively. Free energy of stabilization ($\Delta G^\text{D}^\text{H}_2\text{O}$) values for different HEWL preparations were computed from Y-intercepts of these plots and are shown in Table 4. The value of $\Delta G^\text{D}^\text{H}_2\text{O}$ obtained for native HEWL (Table 4) was similar to earlier reports (62, 63). Fluorescence data (fluorescence intensity and emission maximum) obtained upon excitation at 280 nm were treated in the same way and values of $\Delta G^\text{D}^\text{H}_2\text{O}$ thus obtained, for native and modified HEWLs, are also included in Table 4. These values were in agreement with those obtained from fluorescence data upon excitation at 295 nm (Table 4). Interestingly, $\Delta G^\text{D}^\text{H}_2\text{O}$ values derived from emission maximum data were also found to be comparable with those obtained from fluorescence intensity data (Table 4) for each HEWL preparation. Therefore, these values were also included in our results. A comparison of $\Delta G^\text{D}^\text{H}_2\text{O}$ values obtained for native and modified HEWLs suggested a decrease in $\Delta G^\text{D}^\text{H}_2\text{O}$ value from 7956 and 8559 cal/mol (upon excitation at 295 and 280 nm, respectively, with fluorescence
Fig. 5. – Normalized transition curves of GdnHCl denaturation of native, ‘N’ (■), carbamylated, ‘C88’ (▲) and guanidinated, ‘G85’ (●) HEWLs. Values of $F_0$ were derived from fluorescence intensity data at 360 nm (A) and emission maximum (C) obtained from fluorescence spectra shown in Fig. 4. Plots of $\Delta G_D$ against GdnHCl concentration for the transition of native (N), carbamylated (C88) and guanidinated (G85) HEWLs as obtained from fluorescence intensity (B) and emission maximum (D) data.

intensity probe); 9119 and 8717 cal/mol (upon excitation at 295 and 280 nm, respectively with emission maximum probe), obtained for native HEWL to 6379 and 6345 cal/mol (upon excitation at 295 and 280 nm, respectively with fluorescence intensity probe); 6315 and 5958 cal/mol (upon excitation at 295 and 280 nm, respectively with emission maximum probe) upon 88% carbamylation of amino groups of HEWL (Table 4). Such a decrease in $\Delta G_D^{H_2O}$ value was indicative of conformational destabilization in C88 HEWL. Our results on hydrodynamic properties of C88 HEWL supported this theory since both Stokes radius and $f/f_0$ values of C88 HEWL (Table 3) showed an increase in hydrodynamic volume of the modified derivative. This seems justifiable as abolishment of positive charge at lysine residues upon carbamylation (30) might have disrupted some of the salt bridges in the protein, and thus contributed towards increase in electrostatic
free energy. In contrast, G85 HEWL yielded more or less similar $\Delta G^0_{D_2O}$ values (7997 and 8186 cal/mol upon excitation at 295 and 280 nm, respectively with fluorescence intensity probe; 8856 and 7916 cal/mol upon excitation at 295 and 280 nm, respectively with emission maximum probe) compared to those obtained with native HEWL (Table 4). In view of the retention of positive charge at lysine residues upon guanidination (31) of HEWL, similar stability of G85 HEWL was expected. Our results on G85 HEWL stability are in line with previous reports based on protease digestion (23, 66), thermal transition (66) and tritium-hydrogen isotope exchange (24) studies, in which the stability of HEWL has been shown to remain unaffected upon guanidination.

Taken together, all these results suggested significant conformational destabilization as evident by conformational change with concurrent decrease in $\Delta G^0_{D_2O}$ in HEWL upon 88% carbamylation of its lysine residues. However, this destabilization was less pronounced when compared to the one reported for succinylated HEWL (27). This seems logical as carboxylation of amino groups results in the increase in net negative charge on the protein by 1 unit whereas succinylion increased the net negative charge on the protein by 2 units per lysine residue modified (29, 30). Furthermore, our results are in accordance with earlier reports on acetylation (which abolishes the positive charge on lysine residues, similar to carboxylation) of HEWL where small but significant conformational changes in the protein have been reported as reflected from small perturbation of tryptophan residues (monitored by absorption (67) and near-UV CD spectroscopy (68)), increased rate of proteolytic digestion of the modified enzyme (51, 66, 67), a small but significant shift of thermal transition to the left (66), decreased final folding yield of reduced modified HEWL in renaturation experiment (69) and increased reducibility of disulfide bonds (23, 51) in acetylated HEWL. In a recent report on acetylation of HEWL, modified preparation has been shown to possess similar conformation to that of native protein (28). In view of the modification of tyrosine residues along with lysine residues in acetylation reaction (50) and neighbouring locations of various tryptophan, tyrosine and lysine residues in three-dimensional structure of HEWL (17, 22, 70), results presented in the recent paper (28) suggesting absence of conformational change in acetylated preparation against several earlier reports, remain inconclusive.

Both positively (arginine, histidine and lysine) and negatively (aspartic acid and glutamic acid) charged residues of proteins interact with each other through coulombic interactions and contribute towards protein stability (7, 9). About 21% of the total amino acids in HEWL are charged which are distributed over the molecular surface (17, 22). Three (Lys 13, 33 and 97) of the six positively charged lysine residues of HEWL, located at the C-termini of three helices (5–15, 25–35 and 88–99) in the primary sequence of the enzyme have been suggested to stabilize the helix dipole, as affirmed by mutagenesis study (71). X-ray crystallographic
studies have shown the involvement of lysine residues 1, 13 and 97 in electrostatic interactions through salt bridges with Glu 7, carboxyl terminal of Leu 129 and Asp 101, respectively (17, 22, 70). In addition, interaction between Lys 13 and Asp 18 through salt bridge has also been anticipated due to their close proximity in the three-dimensional structure (71). Based on our results, neutralization of positive charge on these lysine residues in carbamylated HEWL might have broken the above mentioned salt bridges between lysine and other negatively charged residues and thus decreased its conformational stability [reduction in $\Delta G_{D}^{310}$ from 8258 and 8918 cal/mol (for native HEWL) to 6362 and 6137 cal/mol (for C$_{88}$ HEWL) on average, as derived from fluorescence intensity and emission maximum data, respectively]. This seems conceivable as guanidinated preparation, where positive charge was retained on lysine residues, showed similar stability (average values of 8092 and 8386 cal/mol as determined from fluorescence intensity and emission maximum data, respectively) as that obtained with native HEWL. All these results affirmed significant contribution of positively charged lysine residues in the conformational stability of HEWL through salt bridges.

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