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A peptide sequence—YSGVCHTDLHAWHGDWPLPVK [40–60]—in yeast alcohol dehydrogenase prevents the aggregation of denatured substrate proteins☆

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Abstract

The structural and functional characteristics of a yeast alcohol dehydrogenase (ADH) peptide (YSGVCHTDLHAWHGD WPLPVK, residues 40–60) have been studied in detail. The peptide is hydrophobic in nature, binds the hydrophobic probe bis-ANS, and is mostly present in a random coil conformation. It shows chaperone-like activity by preventing dithiothreitol (DTT)-induced aggregation of insulin at 27 °C, oxidation-induced aggregation of γ-crystallin at 37 °C, and aggregation of thermally denatured ADH and β_L-crystallins at 52 °C. However, the ADH peptide does not solubilize protein aggregates as do surfactants. Substitution of Pro for His in the ADH peptide leads to diminished anti-aggregation activity. Further, analysis of ADH incubated at 47 °C suggests that a significant portion of the enzyme remains as soluble inactive protein with negligible conformational change. Therefore, we propose that the residues 40–60 in native protein may be an intramolecular chaperone site of yeast ADH.

Keywords: Alcohol dehydrogenase; ADH peptide; Intramolecular chaperone

Anfinsen demonstrated that all the information necessary for folding of a protein resides in the amino-acid sequence of that protein [1]. His hypothesis—that the protein structure is uniquely determined by the specificity of the sequence—has been verified for many proteins [1]. The native state of a protein is generally believed to be global free energy minimum [2]. Since the three-dimensional structure of proteins determines their function, denaturation and aggregation of proteins poses a major problem in various fields of research and application. Protein aggregation generally results in total loss of functional activity [3]. It is therefore of utmost importance to devise ways to prevent protein aggregation.

Molecular chaperones are a class of proteins which recognize unstable conformations of substrate proteins and bind to them instantly, preventing their aggregation in vitro or in vivo [4–6]. Molecular chaperones assist the non-covalent folding and assembly of other proteins in vivo, but they are not permanent components of these structures when they are performing their normal biological functions. They perform their functions by preventing non-specific or non-productive inter- and intra-molecular interactions of polypeptide chains through binding and stabilizing otherwise unstable polypeptides and facilitating their delivery to the correct cellular destinations. This process is often ATP-dependent and in several cases requires the help of so-called co-chaperones, or helper proteins [4]. However, in the case of the small heat-shock protein α-crystallin, which functions like a molecular chaperone, a stable complex is formed between the denaturing protein and α-crystallin [7]. In some instances, a propeptide region in an enzyme, such as the subtilisin propeptide, promotes folding by direct stabilization of the rate-limiting folding intermediate and functions as an intra-molecular chaperone [8].

☆ *Abbreviations:* ADH, alcohol dehydrogenase; DTT, dithiothreitol; bis-ANS, 1,1′-bi[4-anilino]naphthalene-5,5′-disulfonic acid, sHSPs, small heat-shock proteins.

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Using a novel crosslinker, we have shown that the 40–60 region in thermally denaturing yeast alcohol dehydrogenase (ADH) binds to the molecular chaperone α-crystallin [9]. Here, we report the structural and functional properties of residues 40–60 (YSGVCHTDL HAWHGDWPLPVK) of ADH, and show that this peptide exhibits anti-aggregating properties against denaturing substrate proteins similar to the anti-aggregation activity of mini-αA-crystallin [10]. We propose that the 40–60 region in ADH may also be acting like an intra-molecular chaperone.

Materials and methods

Materials. 1,1-bi[4-anilino]naphthalenesulphonic acid [bis-ANS], yeast alcohol dehydrogenase [ADH], bovine insulin, and dithiothreitol [DTT] were all obtained from Sigma Chemical, USA. γ-Crystallin was prepared from calf lenses by standard protocol [7]. The ADH-peptide YSGVCHTDLHAWHGDWPLPVK and all other peptides used in our study were supplied by Research Genetics. Concentrations of the peptides used in assays were determined by the Biuret method. All other chemicals were of analytical grade.

Chaperone-like activity assay for ADH-peptides. Chaperone-like activity of ADH-peptide was measured at 27 °C against DTT-induced insulin aggregation [11,12]. Briefly, 0.4 mg/ml insulin was reduced with 20 mM DTT in 50 mM phosphate buffer, pH 7.2, containing 0.1 M NaCl, in the absence and presence of different concentrations of ADH-peptide. The scattering was monitored at 360 nm in a Shimadzu UV2401PC spectrophotometer fitted with a Peltier temperature regulator.

The anti-aggregating properties of the ADH peptide at 37 °C were measured in a H_2O_2 oxidation induced aggregation system for γ-crystallin described earlier [13]. Using previously published procedures, the ability of the ADH-peptide to suppress thermal aggregation of yeast ADH and β_L-crystallin was investigated [10]. The light scattering by the aggregating protein samples was measured at 360 nm in a Shimadzu UV2401PC spectrophotometer. In these assays, the observed suppression of light scattering is a measure of the anti-aggregation (chaperone-like) activity of the peptide or protein.

Bis-ANS fluorescence measurements. The interaction of bis-ANS with various peptides was measured in a Jasco FP750 spectrofluorimeter. A 20 μM bis-ANS solution was added to the peptide solution [0.1 mg/ml] in 50 mM phosphate buffer, pH 7.2, containing 0.1 M NaCl. The mixture was thoroughly mixed and incubated for 5 min at room temperature. Fluorescence emission was then noted between 400 and 600 nm using an excitation wavelength of 390 nm. The excitation and emission slits were set at 5 nm.

Circular dichroism measurements. Far UV CD spectra were recorded for the peptides in the region 200–250 nm in a AVIV 62DS spectropolarimeter. The path length of the cuvette was 0.1 cm.

Results and discussion

Light-scattering aggregates of insulin B chain are formed when native insulin is reduced. Molecular chaperones have the ability to suppress light scattering due to the aggregation of insulin B chain [11]. Fig. 1 shows plots of the DTT-induced aggregation kinetics of 0.4 mg/ml insulin in the absence and presence of two different concentrations of the ADH-peptide at 27 °C.

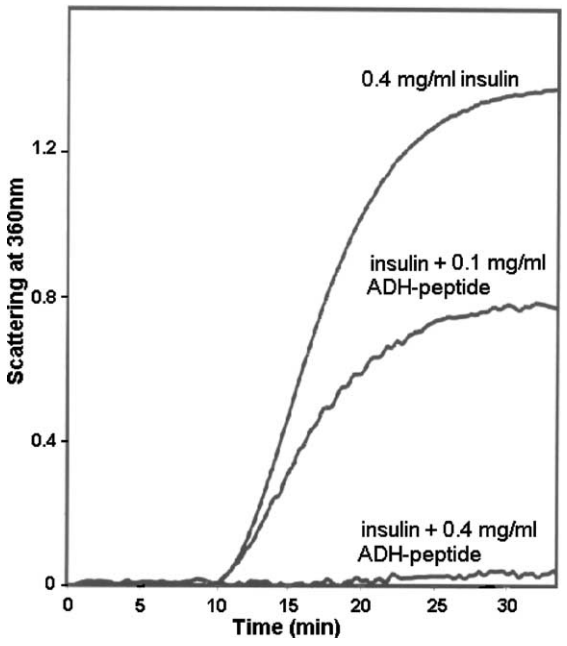

Fig. 1. DTT-induced aggregation of insulin in the absence and presence of ADH-peptide at 27 °C.

The graphs show that the anti-aggregation property of ADH-peptide is concentration dependent. At 1:0.6 mol/mol ratio of insulin:ADH-peptide, there is about 40% suppression in aggregation of reduced insulin, whereas at 1:2.4 mol/mol ratio (insulin:ADH-peptide) there is near complete suppression of reduced insulin aggregation. α-Crystallin, one of the major proteins in the eye lens and a peptide representing its chaperone-site, exhibits similar anti-aggregating activity against DTT-induced insulin aggregation [11,12,14]. We reported earlier that a synthetic peptide, DFVIFLDVKHFSPEDLTVK, corresponding to residues 70–88 in αA-crystallin (termed mini-αA-crystallin), displays anti-aggregation activity against denaturing insulin [14] and α-lactalbumin [15]. When the reaction mixture contained insulin and mini-αA-crystallin in 1:1.2 ratio (mol/mol) about 50% reduction in light scattering due to aggregation of reduced insulin was observed [14]. When the concentration of mini-αA-crystallin was increased by twofold a complete suppression of light scattering was observed. Therefore, the anti-aggregating activity of the ADH-peptide towards DTT-induced insulin aggregation appears to be quite similar to the anti-aggregation activity of mini-αA-crystallin [10,13–15].

After discovering the anti-aggregation property of the ADH-peptide at room temperature (27 °C), we investigated whether the same peptide displays chaperone-like activity at physiological temperatures, as has been demonstrated with αA-crystallin-derived peptide [10,13–15]. Fig. 2 shows a plot of the OH radical mediated oxidation-induced aggregation kinetics of γ-crystallin at 37 °C in the absence and presence of the

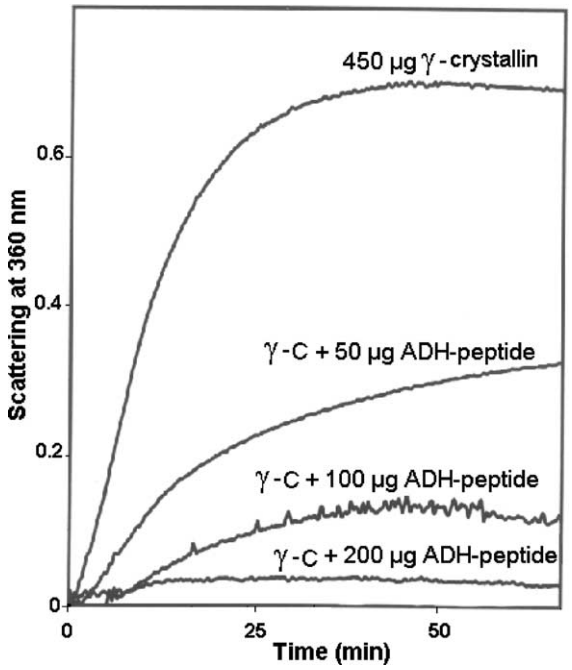

Fig. 2. Oxidation-induced aggregation of γ-crystallin at 37 °C in the absence and presence of ADH-peptide. γC-, γ-crystallin.

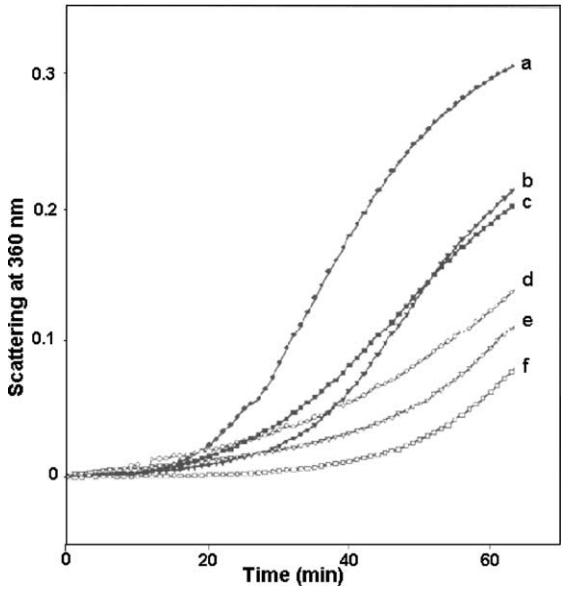

Fig. 3. Thermal aggregation of ADH and β_L-crystallin in the absence and presence of ADH-peptide at 52 °C. (a) 0.25 mg ADH; (b) 0.25 mg β_L-crystallin; (c) 0.25 mg ADH +0.125 mg ADH-peptide; (d) 0.25 mg ADH + 0.25 mg ADH-peptide; (e) 0.25 mg β_L-crystallin + 0.125 mg ADH-peptide; and (f) 0.25 mg β_L-crystallin + 0.25 mg ADH-peptide.

ADH-peptide. The results show that the aggregation of oxidized γ-crystallin is suppressed by the ADH-peptide in a concentration-dependent way. While use of 1:1 mol/mol ratio of γ-crystallin:ADH-peptide resulted in about 50% suppression of light scattering during the assay, increasing the ADH-peptide concentration by fourfold resulted in complete suppression of light scattering by oxidized γ-crystallin aggregates. Earlier, it was reported that 1:0.46 and 1:2.3 ratios of γ-crystallin:mini-αA-crystallin also prevented oxidation-induced aggregation of γ-crystallin 40% and 100%, respectively, at 37 °C [13]. Therefore, the ADH-peptide appears to be possess about 50% of the activity displayed by mini-αA-crystallin against oxidized γ-crystallin [13].

Fig. 3 shows the thermal aggregation profile of 0.25 mg/ml yeast ADH and 0.25 mg/ml β_L-crystallin at a temperature higher than physiological (52 °C), in the absence and presence of different concentrations of ADH-peptide. From the figure it is evident that, when the peptide concentration in the assay is equal to the denaturing protein (W/W), there is at least a 50% suppression of the aggregation of denaturing ADH or β_L-crystallin, and the suppression of aggregation is concentration-dependent as well. The above data show that the anti-aggregation property of the ADH-peptide against denatured substrate proteins is retained at elevated temperatures. This property is similar to that of mini-αA-crystallin peptide, which shows anti-aggregation activity over a wide range of temperatures, 25–48 °C [13–15].

To determine whether the peptide acts like a surfactant by solubilizing the aggregated or aggregating substrate proteins, as seen with casein [16], we allowed 0.4 mg/ml of insulin to aggregate by reduction with 20 mM DTT at room temperature. After 35 min of reaction, we added 0.2 mg of the ADH-peptide to the cuvette in 20 μl of buffer using a Hamilton syringe, and allowed the reaction to continue (Fig. 4). Unlike casein or surfactants which solubilize the protein aggregates, the addition of ADH-peptide did not result in solubilization of insulin aggregates. However, as shown in Fig. 4 the ADH-peptide was found to halt further aggregation of DTT-reduced insulin. This property of ADH-peptide is similar to that of mini-αA-crystallin, which also did not solubilize protein aggregates once they were formed [13].

An association between the exposed hydrophobic sites and the chaperone-like activity of α-crystallin and other heat shock proteins has been observed by a number of investigators [17–20]. Those studies lead to the hypothesis that hydrophobic sites in molecular chaperones interact with denaturing proteins. The function of hydrophobic sites as chaperone sites in sHSPs was confirmed by us [20,21] and others [19] by mapping the chaperone sites. Additionally, we have shown that mini-αA-crystallin, which shows chaperone-like activity, is also hydrophobic in nature [10]. Bis-ANS has been widely used as a fluorescent dye to measure the amount of surface hydrophobicity in proteins and peptides [17,20]. The binding of the probe to the proteins or peptides results in a shift in the fluorescence emission

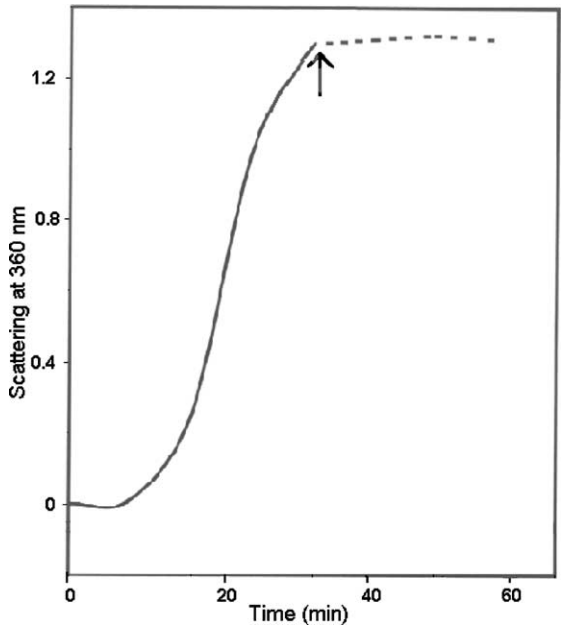

Fig. 4. DTT-induced aggregation of insulin at 27 °C, showing the effect of addition of ADH-peptide after initiation of aggregation. Arrow mark shows the time point when 0.2 mg of ADH-peptide was injected to 0.4 mg of denaturing insulin. Note that addition of ADH-peptide prevented further aggregation of insulin.

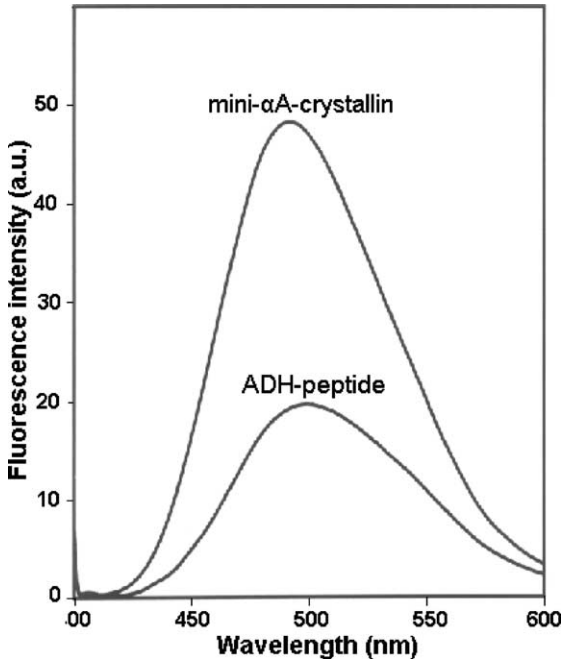

Fig. 5. Bis-ANS interaction with ADH-peptide and mini-αA-crystallin. Fluorescence spectra were recorded as described under Materials and methods, using 10 μM bis-ANS and 0.05 mg/ml of ADH-peptide or mini-αA-crystallin.

maximum of the dye to a lower wavelength, with a simultaneous increase in the fluorescence as well. When the chaperone-like ADH-peptide was allowed to interact with bis-ANS, as shown in Fig. 5, we observed a significant increase in the fluorescence of bis-ANS. The emission maximum of the dye also shifted to a lower wavelength of 499 nm, compared to the emission maximum of 520 nm for the dye alone. The blue shift in the emission maximum for the dye was, however, less pronounced than the blue shift with mini-αA-crystallin, a synthetic mini-chaperone which also binds bis-ANS with an emission maximum at 492 nm (Fig. 5).

We have proposed that both hydrophobicity and β-sheet structure are responsible for the anti-aggregation activity of the peptide chaperone mini-αA-crystallin [14]. Since the ADH-peptide possesses both hydrophobicity and anti-aggregation properties like mini-αA-crystallin [10], we subjected the ADH-peptide to CD spectroscopy to determine whether it also displays β-sheet structure. When 0.2 mg/ml of the peptide was scanned in the far-UV region (200–250 nm), the spectrum shown in Fig. 6 was obtained. It appears that the peptide is mostly in a random-coil conformation. This is in contrast to our earlier study of mini-αA-crystallin which showed significant β-sheet structure under similar conditions [10,14]. It has been reported that α_s-casein prevents aggregation and precipitation of denaturing proteins and therefore exhibits chaperone-like properties [16]. α_s-Casein is a random-coil protein [22]. Therefore, it appears that the mechanism of interaction of ADH-peptide with denaturing proteins may be similar to the interaction between α_s-casein and aggregation-prone proteins. Further, although there is a β-sheet forming sequence

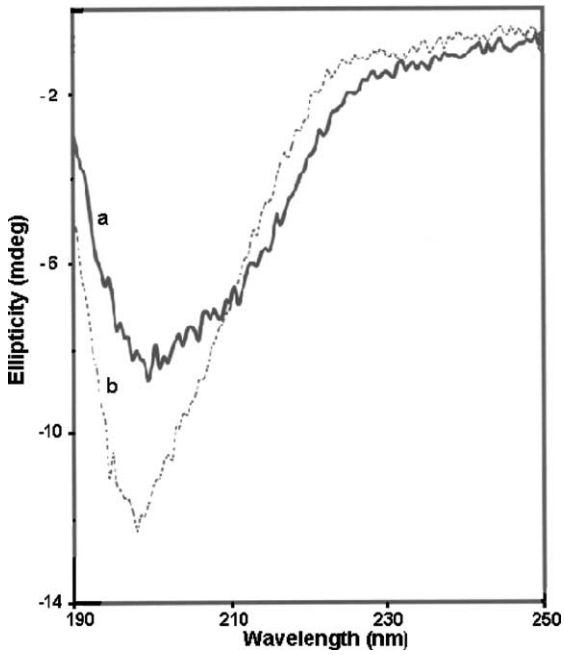

Fig. 6. Far-UV (190–250 nm) CD spectra of ADH-peptides. (a) 0.1 mg/ml ADH-peptide (residues 40–60) and (b) 0.1 mg/ml of ADH-peptide 6 (one of the proline substituted peptides in Table 1) in 50 mM phosphate buffer, pH. 7.0.

(residues 41–45) in the 40–60 region of the native ADH molecule [23], it appears that the 40–60 region, when removed from the protein, loses its propensity to form β-sheet. Therefore, it can be argued that the adjacent residues or other regions in the protein molecule may have a role in imparting a β-sheet conformation to the 41–45 region in native protein. Again, this property of ADH-peptide is distinctive when compared with the mini-αA-crystallin peptide, which shows β-sheet structure by itself, even when removed from the native protein [10].

To verify whether amino-acid sequence 40–60 is a unique region in ADH, we investigated the anti-aggregating properties of peptides representing other regions of the protein. We measured the anti-aggregation profiles of three other peptides, each a 20-mer, one close to the amino-terminus, a second from the middle region, and a third close to the carboxyl-terminus of the protein (Table 1). Fig. 7 shows the light scattering, measured at 360 nm, during DTT-induced aggregation of insulin at 37 °C in the absence and presence of three peptides (ADH-peptide 1, ADH-peptide 2, and ADH-peptide 3), representing different regions of ADH. From the results, it is evident that only the peptide representing the 40–60 region in ADH exhibits anti-aggregating property against insulin B chain. When a twofold higher concentration of the peptides was used only the 40–60 region peptide showed a twofold increase in the chaperone-like activity, whereas the peptides 1, 2, and 3 showed negligible increase in activity (data not shown). These data suggest that the 40–60 sequence is unique to the protein.

The sequence of the active ADH-peptide, YSGVCHTDLHAWHGDWPLPVK, is found only in yeast ADH, and a BLAST search analysis showed that several amino-acid residues in this region are also highly conserved within the family of dehydrogenases. Earlier, we reported that the substitution of highly conserved His residue in a chaperone peptide (DRFSVNLDVKHFSPEELKVK) derived from sHSP αB-crystallin abolishes its chaperone-like activity [23]. Therefore, we investigated whether substitution of highly conserved His in the 40–60 sequence would result in a loss of its anti-aggregating property. We synthesized

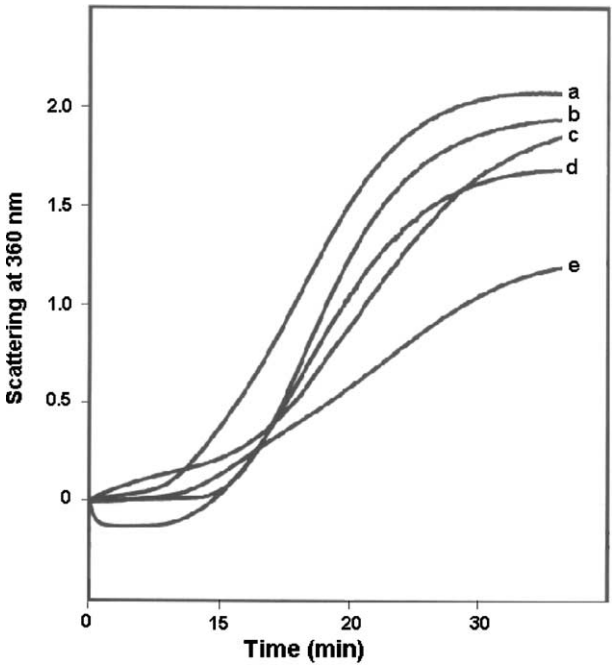

Fig. 7. DTT-induced aggregation of insulin at 37 °C in the absence and presence of various ADH-peptides. (a) Insulin + 0.2 mg/ml ADH-peptide 3; (b) insulin + 0.2 mg/ml ADH-peptide 1; (c) 0.4 mg/ml insulin alone; (d) insulin + 0.2 mg/ml ADH-peptide 2; and (e) insulin + 0.2 mg/ml ADH-peptide (residues 40–60).

three other 21-amino-acid peptides (ADH-peptides 4–6 in Table 1) by replacing the histidine residues with proline—a structure-disrupting amino acid—one at a time, and then investigated the ability of those mutant peptides to suppress the aggregation of reduced insulin. Fig. 8 shows the light scattering at 360 nm during DTT-induced aggregation of insulin at 37 °C in the absence and presence of proline-substituted ADH-peptides. From the figure, it is evident that substitution of any one of the histidine residues with proline results in complete loss of the anti-aggregation property of the native ADH-peptide. None of the proline-substituted ADH-peptides aggregated themselves at 37 °C under the experimental conditions (data not shown). Furthermore, all ADH-peptides showed a greater extent of random coil structure compared to the native ADH-peptide (in Fig. 6—far-UV CD spectra for one of the His substituted

Table 1
ADH-peptides used in the study

ADH-peptides	Sequence
ADH-peptide (residues 40–60)	YSGVCHTDLHAWHGDWPLPVK
ADH-peptide 1 (residues 11–30)	IFYESHGKLEYKDIPVPKPK
ADH-peptide 2 (residues 181–200)	GGLGSLAVQYAKAMGYRVLG
ADH-peptide 3 (residues 301–320)	DTREALDFFARGLVKSPIKV
ADH-peptide 4	YSGVCPTDLHAWHGDWPLPVK
ADH-peptide 5	YSGVCHTDLPAWHGDWPLPVK
ADH-peptide 6	YSGVCHTDLHAWPGDWPLPVK

In case of ADH-peptides 4–6, the histidine residue replaced by proline is underlined.

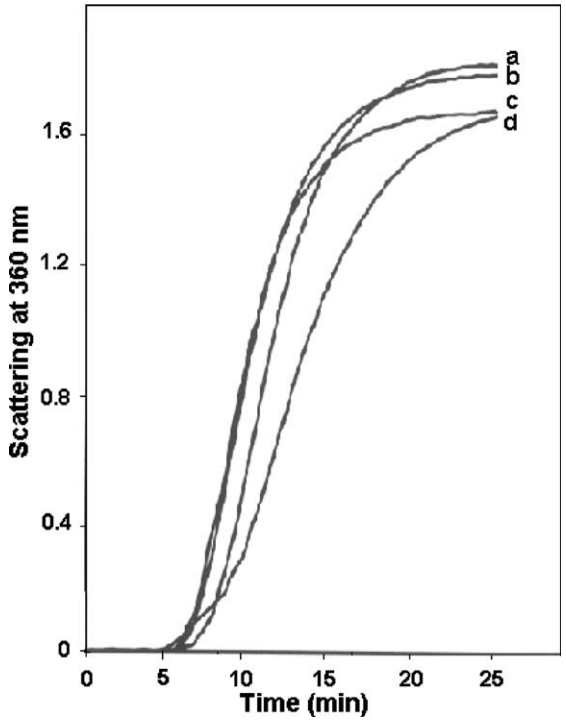

Fig. 8. DTT-induced aggregation of insulin at 37 °C in the absence and presence of proline-substituted ADH-peptides. (a) Insulin + 0.2 mg/ml ADH-peptide 4; (b) insulin + 0.2 mg/ml ADH-peptide 5; (c) insulin + 0.2 mg/ml ADH-peptide 6; and (d) 0.4 mg/ml insulin alone.

ADH-peptides, peptide-6 is shown for comparison). Taken together, these data suggest that all three His in the ADH-peptide are necessary for the anti-aggregation activity of the peptide. Therefore, we propose that the three histidines—His 45, 49, and 52 which are located near the active site and on the surface of the native protein [24]—play a role in suppressing the aggregation and precipitation of denaturing ADH as well.

Earlier, we reported that the anti-aggregation activity of mini-αA-crystallin (a 19-amino-acid chaperone peptide of α A-crystallin) is associated with its hydrophobicity and β-sheet structure [14]. From our results we find that the ADH-peptide, which is mostly in the random-coil conformation, can bind significant amount of bis-ANS and also display anti-aggregating properties towards denaturing substrate proteins in spite of the absence of β-sheet structure. The mutant form of ADH-peptide (Fig. 6) is more randomly coiled compared to the native ADH-peptide and binds less bis-ANS (data not shown). Therefore, it appears that at least a rudimentary structural organization super-imposed with hydrophobicity is required for the ADH-peptide to exhibit its anti-aggregation property against denaturing substrate proteins.

In spite of the presence of the 40–60 region, one can observe aggregation of ADH when it is subjected to heat treatment (Fig. 1). However, analysis of the precipitate showed that only about 20–25% of the denaturing ADH forms large aggregates and precipitate. A gel filtration analysis of the ADH that remained in solution after a heat denaturation assay at 48 °C showed that a significant portion (70–75%) of the remaining ADH was in its soluble form and eluted as 140 kDa protein, with an elution profile similar to that of native ADH. The amount of high-molecular-weight soluble aggregate is usually less than 5%. Further, the analysis of heat-denatured ADH that did not precipitate showed that there was little or no change in the Trp emission spectra when the protein was excited at 295 nm (Fig. 9). The Trp emission spectra of ADH remaining in solution after 60 min of heat treatment showed only a minor difference with respect to the emission maximum of the native ADH. This is similar to the fluorescence shift observed with denaturing rhodanese or γ-crystallin bound to α-crystallin chaperone lacking tryptophan [25] or the α-lactalbumin captured by mini-αA-crystallin [15]. Therefore, we propose that interactions involving residues 40–60 in one of the ADH subunits with other regions of the same subunit, or with another subunit, may be responsible for the formation of soluble aggregates. In view of these data, we propose that residues 40–60 in yeast ADH probably function like an intramolecular chaperone, thereby preventing complete unfolding of yeast ADH during thermal stress.

In conclusion, the mechanism of interaction between ADH peptide and denaturing proteins may be different

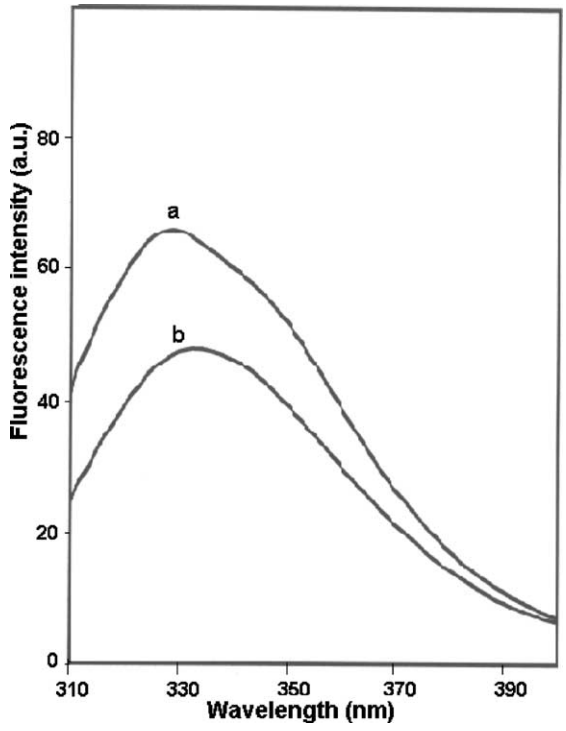

Fig. 9. Tryptophan fluorescence spectra of native and heat-denatured ADH. (a) Native ADH, 200 μg and (b) heat-denatured ADH, 200 μg. The sample in 50 mM PO_4 buffer, pH 7 was exited at 295 nm.

than the interactions observed between casein and target proteins [16] or between mini-αA-crystallin and target proteins [10,13–15]. The 40–60 sequence in ADH may be functioning like an intramolecular chaperone as well as contributing to the active site domain of the enzyme. The β-sheet-forming residues 41–45 in ADH—which have a propensity to interact with the other β-sheet forming sequences 68–71 and 369–374 in native protein—may be interacting with other unfolding regions of the denaturing protein, resulting in the suppression of aggregate formation.

Acknowledgments

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