JACOBS PUBLISHERS

OPEN ACCESS

Jacobs Journal of Cell and Molecular Biology

Research Article

Influence of Thymoquinone on the Unfolding Transition of Human Serum Albumin

Zeyad J. Yasseen*[1], Jehad H. Hammad[1], and Hussein A. AL Talla[1]

[1]*Gaza -Islamic University of Gaza / Faculty of Science/ Chemistry Department.*

**Corresponding author:* *Dr. Zeyad J. Yasseen, Gaza -Islamic University of Gaza / Faculty of Science/ Chemistry Department, P.O. Box 108, Gaza, Tel: 00972599928024; Email: zyasseen@iugaza.edu.ps*

Received: 12-18-2014

Accepted: 04-21-2015

Published: 04-28-2015

Abstract

The effect of thymoquinone (TQ) on the conformational stability of human serum albumin (HSA) at different temperatures in the range of 25 – 50 °C has been investigated by means of fluorescence spectroscopy. Urea induced unfolding transition of free and TQ-bounded HSA were characterized by fluorescence spectra and the results were analyzed using the linear extrapolation method (LEM), to characterize the effect of TQ binding on the conformational stability of HSA during the denaturation by urea. The unfolding profiles were shifted towards the higher urea concentration in presence of TQ., at lower temperatures.

Our results provide evidence that TQ alters the urea-induced unfolding pathway of HSA. It is found that TQ acts as a stabilizing agent and causing a substantial impact against denaturation by urea in the temperature range studied.

The data were analyzed on the assumption of a two-state approximation to obtain the apparent equilibrium constant (K_u), and the conformational stability parameters (ΔG_u^w , and m) in addition to the solvent-exposed surface area (ΔASA) during the urea-induced unfolding process. It is found that, both proteins showed different values of the exposed surface area under urea denaturation, being more in case of free protein.

Keywords: Thymoquiunone; Nigella Sativa ; Human Serum Albumin; Fluorescence Spectroscopy; Linear Extrapolation Method; Solvent-Exposed Surface Area.

Introduction

There has been increased interest in the use of chemical denaturants in the study of protein folding processes because of their the high denaturing potential. Denaturation [1,2]. Denaturation by urea is one of the primary ways used to measure and to compare the conformational stabilities of proteins.

Proteins can be unfolded in aqueous urea solution in which one molecule of urea can bind two peptide residues on the protein and hence it acts as a bifunctional agent, and ea enhances the solubility of non-polar compounds in water, it has an influence on the intermolecular hydrogen bonded structure of liquid water [3-7].

Equilibrium denaturation studies provide a reasonable method for estimation the relatively small free energies involved in stabilizing the three-dimensional structure of a folded protein. It is well known that folding of some small proteins presents highly cooperative two-state behavior, while folding of multidomain proteins with population [8].

The mechanism by which proteins fold from a structure-free denatured state to their unique biologically active state is an intricate process. This process is even more complex in multidomain proteins where each domain may be able to refold independently and interdomain interactions may affect the overall folding process.

Thymoquinone (TQ) is a phytochemical compound present in of a plant called Nigella Sativa (*Kalonji* or simply *Nigella*) which is an annual flowering plant in the family Ranunculaceae. TQ possesses important properties such as analgestic and anti-flammatory protection of organs against oxidative damage induced by a variety of free radical generating agents. It is a potent anti tumor agent against human colorectal cancer cells [9-11]. Moreover, TQ is the predominant bioactive constituent present in black seed oil (*Nigella sativa*) and has been tested for its efficacy against cancer. TQ has anti-inflammatory effects, and it inhibits tumor cell proliferation through modulation of apoptosis signaling, inhibition of angiogenesis, and cell cycle arrest.

Human serum albumin (HSA), which is found in tissues and secretions throughout the body homological with the serum albumin of human and other mammalian, has been intensively studied. It is a single polypeptide chain with 585 residues arranged in a three homologous domains (I, II, III). Each domain is known to consist of two separate sub-domains (named A and B), connected by a random coil as shown in Fig. 1. Each domain contains two sub-domains. Terminal regions of sequential domains contribute to the formation of flexible interdomain helices linking domain I to II, and II to III, respectively [12–19].

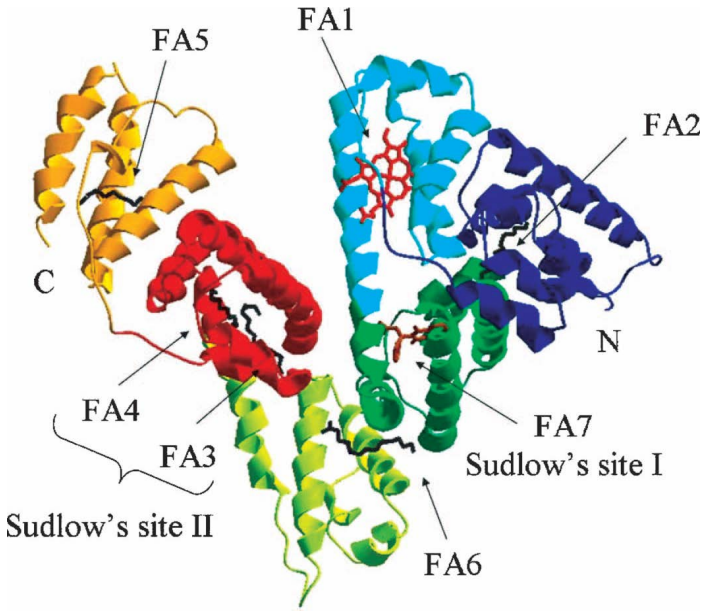

Figure 1. Native structure of HSA.showing the six subdomains of HSA colored as follows: subdomain IA: blue; subdomain IB: cyan; subdomain IIA: dark green; subdomain IIB: light green; subdomain IIIA: red; subdomain IIIB: orange. The heme (red) fits the primary cleft in subdomain IB.
Sudlow's site I (in subdomain IIA) is occupied by warfarin (brown).
Sudlow's site II (in subdomain IIIA) is occupied by two myristate anions.

The main objective of this study is to understand the effect of TQ binding on the folding pathway and conformational stability parameters for HSA under urea denaturation at different temperatures has been investigated based on fluorescence spectra.

The quantitative conformational parameters accompanying the chemical unfolding of HSA in presence and absence of TQ have been studied, and correlated with the exposed surface areas for both free and bounded proteins .

Experimental part

Chemicals

Human serum albumin (HSA, fatty acid free < 0.05 %) and Urea were purchased from Sigma-Aldrich company. Thymoquinone, was ultrapure grade, purchased from Sigma-Aldrich and its stock solution was prepared in 5% ethanolic solution. The other substances were of reagent grade, and were used without further purification. phosphate buffer (20 mM, pH = 7.0) was used through the study.

All solutions were prepared using doubly distilled water.

Protein and urea solutions

Protein samples (free and TQ-bounded) and urea solutions were prepared in 20 mM phosphate buffer (pH 7.0) for denaturation studies.

The concentration of protein samples were determined pectrophotometrically, using a molar extinction coefficient of 35,219 M^{-1} cm^{-1} [20]. Thymoquinone was dissolved in the same buffer solution containing 5% ethanol, and urea solutions were prepared immediately before use and their concentrations were determined by refractive index measurements as described elsewhere [21].

Different volumes of denaturant urea solution (8 M) were added to obtain the desired concentration of denaturant. The fluorescence spectra of HSA were measured at the desired temperature in the range of 25 ºC up to 50 ºC.

protein (free and TQ-bonded) samples were incubated in 20 mM phosphate buffer, pH 7.0, containing different concentrations of Urea for at least 1 h, at the desired temperature (20-50 °C). No changes were apparent at longer incubation times (up to 24 h).

Fluorescence measurements

Unfolding study for both free and TQ-bounded HSA (1.0 µM concentration in phosphate buffer solution (pH 7.0) were performed spectrofluorimeterically on Perkin Elmer luminescence (series no. 70412) interfaced to a computer for data collection and analysis.

The excitation and emission slits were fixed at 10 nm each and the path length of the sample cuvette was 1 cm. Intrinsic fluorescence and tryptophan fluorescence spectra were recorded in the range 250–500 nm after exciting the sample at 278 nm, and the fluorescence emission was monitored at 340 nm. The temperature of the sample was controlled well at the desired temperature range (25-50 °C) using a thermostatted cuvette holder a refrigerated circulating water bath. Fluorescence intensity was corrected for background, adjusted for dilution by the titrant, and corrected for innerfilter effects according to the Eq. (1) [22, 23].

$$F_{corr} = F \cdot 10^{A/2} \quad \ldots\ldots \quad (1)$$

where F_{corr} and F are the corrected and observed fluorescence intensities, respectively, and A is the absorbance at 295 nm.

Free and TQ-bonded human serum albumin were incubated in the phosphate buffer (20 mM, pH 7) containing urea solution for 1 h at the desired temperature in the range of 25 °C up to 50 °C. The spectrofluometric titrations were performed by sequentially adding aliquots of the HSA solutions containing fixed amounts of HSA in different denaturant concentrations ranged from 0 up to 8 M Urea.

Data analysis

From the data of fluorescence spectra, the fraction of unfolded HSA can be estimated using the following equation:

$$Y = Y_a + f_u (Y_u - Y_n) \quad \ldots\ldots \quad (2)$$
$$f_u = (Y-Y_n)/(Y_d-Y_n) \quad \ldots\ldots \quad (3)$$

Where *Y* is the observed parameter at a given urea concentration and Y_n, and Y_u, are the values of fluorescence intensity at the folded and unfolded states extrapolated from the pre and post-transition baselines to the urea concentration under consideration [24–26].

The values Y_n, and Y_u, in the transition region were (determined by least-squares analysis of the linear portions of the pretransition and posttransition regions andextrapolated them to the transition region.

The denaturation data obtained were converted into the fraction denatured, f_u, using the equations:

Denaturation curves were evaluated according to the linear extrapolation method LEM), and were analyzed by amethod described by Pace [3- 6] and Morjana et. al. [22]**.** The equilibrium constant , *K*u, corresponding to the denaturation pathway of HSA, was calculated at each point in the transition region of the denaturation curve by the equation:

$$Ku = \frac{[unfolded]^2}{Folded} = 2P_t \frac{f_U^2}{1 - f_U} \quad (3)$$

Where, P_t being the total protein concentration and f_U the fraction of unfolded protein.

Assuming that this model may provide a reasonable thermodynamic description of the denaturation reaction of free and TQ-bounded HSA, then one should calculate the value of the (ΔG_u) (calculated as -RT ln K_u).

A linear dependence of the Gibbs free energy of unfolding on the denaturant is assumed [26]: $\Delta G_u = \Delta G_u^w$ - m × [Urea] --(4)

Where: ΔG_u^w is the difference in Gibbs free energy between the unfolded and folded protein in the absence of denaturant

Both (ΔG_u^w) and (m) represent the conformational stability parameters.

Results and Discussion

Binding study

Fluorescence quenching measurements of single tryptophan residue in free HSA (Trp 214) was used to monitor the TQ/HSA interaction and to measure the binding affinity of the interaction occurred. The Fluorescence measurements performed in the presence of TQ showed a decrease in the intrinsic fluorescence emission intensity of the protein upon excitation at 278 nm (Fig. 2).

From Fig. 2 it is evident that addition of TQ quenches the fluorescence intensity of HSA and was accompanied by a small red shift in maximum fluorescence intensity of emission. This behavior could be attributed to an increase in the environmental polarity. The binding curves at different temperatures in the range of 25–50 °C, depecting the change in fluorescence of HSA as a function of molar ratio [HSA]/[TQ] are shown in Fig. 3. Here, it is clear to see that the binding curve displayed saturation at certain values of the molar ratio at each studied temperature, clearly indicating that the binding occurred at specific binding sites on the HSA.

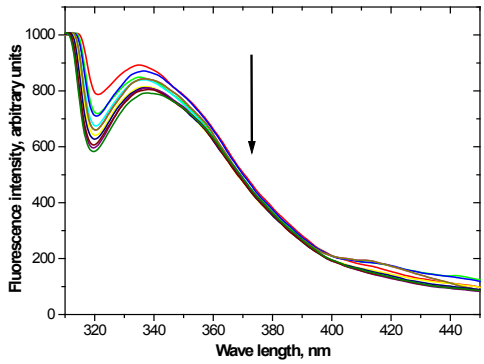

Figure 2. Emission spectra of HSA (1 μM) in 20 mM phosphate buffer (pH = 7.0) and 25 °C. Excitation carried out at 278 nm. The arrow (with its direction) shows that the increasing TQ concentration is accompanied with fluorescence intensity quenching for HSA.

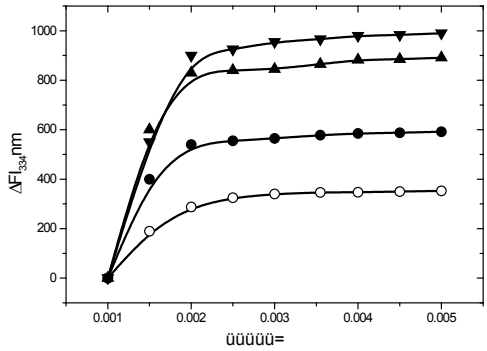

Figure 3. Binding curves for spectrofluorimetric titration of TQ with HSA at four different temperatures 25 (○), 30 (●), 40(▲) and 50 (▼) °C in phosphate buffer solution (pH = 7.0). Change in fluorescence intensity (ΔFI) was plotted as a function of molar ratio [HSA]/[TQ].

3.2. Urea-Induced Unfolding Study

Individual samples of free and TQ-bonded HSA at a final concentration of 1 μM each, were kept incubated in urea solutions (0–8 M) for 1 hr in 20 mM phosphate buffer at pH 7.

Typical urea denaturation profiles of free and TQ-bounded HSA are shown in Figure 4. The data have been plotted in terms of f_u,, to facilitate the comparison between different samples (free and TQ-bounded) of HSA. The most noteable aspect is the occurrence of rapid denaturation transition of free HSA samples.

It may be noted that a sharp denaturation induced at relatively low urea concentration was observed in case of free HSA. Whereas, in case, of the TQ-bounded HSA the denaturation occurs in another fashion, i.e nearly linear from the beginning up to about 3.25 M, and then steeply changed with increasing urea concentration, and the two modes of denaturation being comparatively equal at high urea concentrations.
This compartment revealed the vital role induced by TQ ligand that may increase the stability of the bounded protein and suggesting that TQ acts as a stabilizing factor induced in the TQ-HSA conjugate complex conformation.

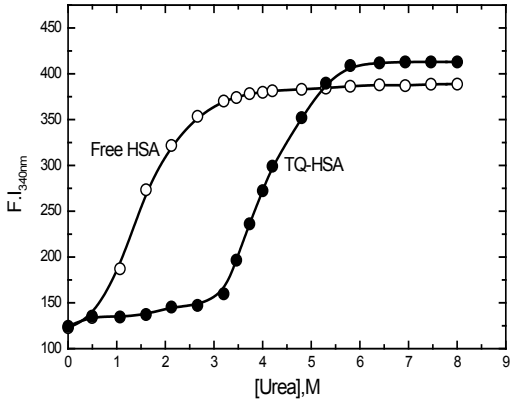

Figure 4. Urea-induced denaturation transitions of free HSA (○) and TQ-bounded HSA (●). The protein concentration was 1 μM, in each case. All solutions were prepared in 20 mM sodium phosphate, pH 7.0.

The determination of free energy (ΔG_u^w) and m as criteria of conformational stabilities of a globular "module" (e.g. protein, domain, etc), is based on the assumption that the native module can be directly converted to the denatured state via two state model.

Unfolding Study at different temperatures

The unfolding fraction Profiles (f_u, versus urea concentration) were obtained at several temperatures within the range of 25–50 °C (Fig. 5). These profiles were analyzed assuming the two-state denaturation model.
The fitting curves allowed us to estimate the conformational stability parameters (ΔG_u^W), and *m*-value, at different temperatures for both HSA samples (free and TQ-bounded).

It is known that the size (or the exposed surface area to solvent) of the protein, upon unfolding, is the major structural determinant factor for the *m*-value [19]. The *m*-values accompanying the chemical denaturation of free HSA was kept nearly constant at 5.89 kJ mol^{-1} M^{-1} with increasing temperature, which indicates that the area of unbounded HSA, exposed to solvent during unfolding, is practically unaltered with temperature in the studied temperature range.

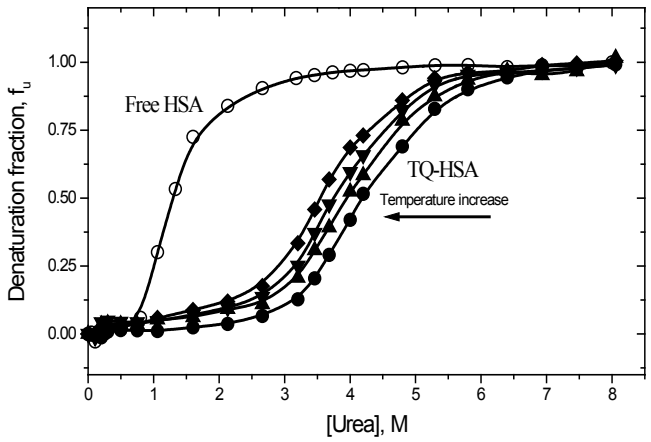

Figure 5. Urea-unfolding equilibrium profiles for HSA in presence (filled symbols) and in absence of thymoquinone (○). unfolded fraction, f_u, is plotted vs. urea concentration at different temperatures 25 °C (●), 30 °C (▲), 40 °C (▼) and 50 °C (♦) in 20 mM sodium phosphate, at pH 7.0 . The solid lines represent the best fits of the experimental data to a two-state model.

Calculations based on (m) values and solvent-accessible surface areas

The *(m)* value is an experimental measure of the dependence of ΔG on denaturant concentration. Consequently, for proteins that unfold by a two-state mechanism, the fractions of buried groups can be estimated by comparing the calculated *m* values which is related to the protein accessible area (ΔASA) by the equation suggested by Myeres et, al., [26]: $m = 1563.32 + 0.46 \times (\Delta ASA)$

Table 1. Unfolding data for HSA at pH 7.0 and at different temperatures.

T (°C)	$m_{(TQ\text{-}bounded\ HSA)}$ (kJ mol^{-1} M^{-1})	ΔG_u^W ($kJ\,mol^{-1}$) (*Free*)	(*TQ – bounded*)	(ΔASA)[a] (Å^2) (Free)	(TQ- bounded)
25	3.10 ± 0.22	24.36 ± 0.54	25.48± 0.97	9405.83 ± 233	3340.61 ± 312
30	3.77 ± 0.11	24.04 ± 0.63	24.26 ± 0.74		4797.13 ± 251
40	4.23 ± 0.18	23.52 ± 0.32	23.80± 0.51		5797.13 ± 315
50	5.08 ± 0.15	22.98 ± 0.72	22.65 ± 0.62		7644.95 ± 297

A different behavior was found in case of the TQ-bounded protein, where the *m*-values decrease with increasing temperature, reducing therefore the area exposed to solvent.

Presumably, the result indicate that the free protein is less sensible to temperature change than the TQ-bounded protein (in the studied range). The different behaviors found between free and bounded HSA might be indicative of some structural differences in both proteins, with which one would expect a different behavior in the conformational properties of both samples.

Conclusion

In summary, the influence of thymoquinone binding on the folding pathway and conformational stability of human serum albumin (HSA) have been studied, at different temperatures in the range of 25 °C – 50 °C. It is found that occupancy of HSA specific binding site by TQ strongly hinders the unfolding of HSA to an appreciated level up to about 3.25 M urea concentration (at low temperature).

References

1. R. Gupta, S. Yadav and F. Ahmad. Protein stability: urea induced versus guanidine-induced unfolding of metmyoglobin. Biochemistry. 1996, 35(36): 11925-11930.

2. M. Roseman, W. Jencks. Interactions of urea and other polar compounds in water. J. Am. Chem. Soc. 1975, 97(3): 631- 640.

3. C. Pace. The stability of globular proteins. Crit. Rev. Biochem. Mol. Biol. 1975, 3(1): 1-43.

4. C. Pace. Determination and analysis of urea and guanidine hydrochloride denaturation curves. Methods Enzymol. 1986, 131: 266–280.

5. C. Pace, B. Shirley, J. Thomson. Protein Structure: a practical approach measuring conformational stability of a protein. Second edition. Oxford, UK: IRL press at Oxford University Press: 311, 1989.

6. A. Caballero-Herrera, K. Nordstrand, K. Berndt, L. Nilsson.

Effect of urea on peptide conformation in water: molecular dynamics and experimental characterization. J. Biophys. 2005, 89(2): 842-857.

7. L.Masino, S. Martin, and P. Bayley. Ligand binding and thermodynamic stability of a multidomain protein, calmodulin. Protein Sci. 2000, 9(8): 1519-1529.

8. V. Daggett, A. Fersht. Is there a unifying mechanism for protein folding? Trends Biochem. Sci. TRENDS in Biochemical Sciences. 2003, 28(1): 18–25.

9. M. El Gazzar, R. El Mezayen, J. Marecki, M. Nicolls, A. Canastar, and S. Dreskin. Anti-inflammatory effect of thymoquinone in a mouse model of allergic lung inflammation. Int Immunopharmacol. 2006, 6: 1135–1142.

10. H. Gali-Muhtasib, M. Diab-Assaf, C. Boltze, J. Al-Hmaira, R. Hartig, A. Roessner, and R. Schneider-Stock. Thymoquinone extracted from black seed triggers apoptotic cell death in human colorectal cancer cells via a p53-dependent mechanism. Int. J. Oncol. 2004, 25: 857–866.

11. H.Gali-Muhtasib, A. Roessner, R. Schneider-Stock. Thymoquinone: a promising anti-cancer drug from natural sources. Int. J. Biochem. Cell Biol. 2006, 38: 1249–1253.

12. X. He, D. Carter. Atomic structure and chemistry of human serum albumin. Nature. 1992, 358: 209–215.

13. D. Carter, J. Ho. Structure of serum albumin. Adv. Protein Chem. 1994, 45: 153–203.

14. T. Peters. All about Albumin. Biochemistry, Genetics and Medical Applications, Academic Press, Orlando, FL, 1996.

15. S. Sugio, A. Kashima, S. Mochizuki, M. Noda, and K. Kobayashi. Crystal structure of human serum albumin at 2.5 A resolution. Protein Eng. 1999, 12: 439-446.

16. S. Curry. Beyond expansion: structural studies on the transport roles of human serum albumin. Vox Sang. 2002, 83 (Suppl. 1): 315–319.

17. M. Fasano, S. Curry, E. Terreno, M.Galliano,G. Fanali, P.Narciso, S.Notari, P. Ascenzi. The extraordinary ligand binding properties of human serum Albumin. IUBMB Life. 2005, 57: 787–796.

18. P. Ascenzi, A. Bocedi, S. Notari, G. Fanali, R. Fesce, and M. Fasano. Allosteric modulation of drug binding to human serum albumin. Mini Rev. Med. Chem. 2006, 6: 483–489.

19. K. Ulrich, C. Vivtor, T. Giam and O. Masaki. Practical Aspects of the Ligand-Binding and Enzymatic Properties of Human Serum Albumin. Biol. Pharm. Bull. 2002, 25(6), 695-704.

20. N. Stãnciuc, S. Dima, G. Rãpeanu.Effect of Calcium Addition on the Thermal Denaturation of Bovine Apo-α-lactalbumin -A Preliminary Study. Innovative Romanian Food Biotechnology. 2011, 9(9): 45-51.

21. S. Roy. Fluorescence quenching methods to study protein-nucleic acid interactions. Methods Enzymol. 2004, 379: 175–187.

22. N. Morjana,, B. McKeone, and H. Gilbert.Guanidine hydrochloride stabilization of a partially unfolded intermediate during the reversible denaturation of protein disulfide isomerase. Proc. Natl. Acad. Sci. U.S.A. 1993, 90:2107–2111.

23. F. Chiti, N.A.J. van Nuland, N. Taddei, F. Magherini, M. Stefani, G. Ramponi, C.M. Dobson, Biochemistry. 1998, 37: 1447–1455.

24. J.A. Schellman. Biopolymers. 1975, 14(5): 1305–1322.

25. I.H.M. van Stokkum, H. Linsdell, J.M. Hadden P.I, Haris D. Chapman M. Bloemendal, Biochemistry. 1995, 34: 10508–10518.

26. J. Myers, C. Pace, and J. Schultz,.Denaturant *m* values and heat capacity changes : relation to changes in accessible surface areas of protein unfolding. Protein Sci. 1995, 4: 2138 -2148.