International Journal of Biological Macromolecules 60 (2013) 301–308

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Refolding of urea denatured ovalbumin with three phase partitioning generates many conformational variants

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ARTICLE INFO

Article history:
Received 18 April 2013
Received in revised form 6 June 2013
Accepted 10 June 2013
Available online 15 June 2013

Keywords:
Protein aggregation
Ovalbumin
Protein refolding
Three phase partitioning
Amyloid fibrils

ABSTRACT

Three phase partitioning is a process in which mixing *t*-butanol with ammonium sulphate with a protein solution leads to the formation of three phases. Generally, the interfacial protein precipitate (formed between upper *t*-butanol rich and lower aqueous phase) can be easily dissolved back in aqueous buffers. In case of ovalbumin, this led to a precipitate which was insoluble in aqueous buffers. This precipitate when solubilized with 8 M urea and subjected to three phase partitioning under various conditions led to many refolded soluble conformational variants of ovalbumin. One of these showed trypsin inhibitory activity, had marginally higher β-sheet content and had higher surface hydrophobicity (both with respect to native ovalbumin). Scanning electron microscopy and Atomic force microscopy of this preparation showed a thread like structure characteristic of amyloid fibrils. The behaviour of ovalbumin during three phase partitioning makes it a valuable system for gaining further understanding of protein aggregation.

1. Introduction

The correlation between structure and activity has been the basis for understanding biological phenomena. In the case of proteins, the belief has been that primary sequence dictates a unique conformation and this in turn dictates a unique function. The work carried out earlier in this laboratory had shown that subjecting the protein to three phase partitioning (TPP) could lead to new conformation which would in fact show much higher activity even under low water conditions [1]. TPP generally consists of mixing ammonium sulphate and *t*-butanol to an aqueous solution of a protein. Under optimized conditions, greater than 90% protein can be obtained as an interfacial layer in the form of a precipitate in between lower aqueous phase and upper *t*-butanol rich phase. Interesting enough, TPP of urea denatured proteins [2] and even inclusion bodies solubilized in urea [3] led to refolding of many proteins to their active forms. However, in the present work we show that urea denatured ovalbumin by TPP under various conditions produced several conformers which differed widely in several structural details. One of these was found to specifically inhibit trypsin (and not other proteases). There have been several reports of BSA catalyzing C—C bond formation [4]. Such behaviour by proteins and enzymes has been called catalytic promiscuity [5–7]. This report is first in showing that a precipitation can lead to a protein acquiring a biological activity, – a kind of promiscuous behaviour.

Our interest in refolding of urea denatured ovalbumin originated from the unusual behaviour of ovalbumin after being subjected to TPP. The precipitate of ovalbumin obtained after TPP did not dissolve in aqueous buffer(s). Based upon this, we decided to treat this precipitate like we dealt with inclusion bodies [3], that is refold the protein after solubilizing in the urea.

During both folding and refolding, the various well defined states of protein are: denatured state, molten globule and refolded/folded protein. On the other hand, TPP treatment of ovalbumin under different conditions seems to generate various structural variants of ovalbumin which are similar in several respects and differ in few other respects. Thus, TPP of ovalbumin and its products offer a somewhat unique opportunity of further understanding of structural aspects of not merely folding/refolding of proteins but possibly structure–activity correlation paradigm of proteins.

2. Materials and methods

2.1. Materials

Ovalbumin (chicken egg white, Cat. No. A5503) was obtained from Sigma–Aldrich, St. Louis, USA. The ovalbumin was found to contain about 20% protein as aggregates. The aggregates were removed by gel filtration on Sephacryl S-100 HR column (Fig. S1 Supplementary Material). The solution of the non aggregate-ovalbumin (major peak) only was used for studies

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http://dx.doi.org/10.1016/j.ijbiomac.2013.06.008

described in this work. Lyophilization was avoided as it was found to lead to formation of insoluble protein mass. Trypsin (bovine pancreas), α-chymotrypsin (bovine pancreas), N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA), benzoyl-L-tyrosine ethyl ester (BTEE), N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, and casein were obtained from Sigma–Aldrich, St. Louis, USA. Urea, ammonium sulphate, *t*-butanol were purchased from the Merck India Limited (Mumbai). Dithiothretiol was purchased from SRL India limited (Mumbai). All other reagents were of analytical grade.

2.2. Three phase partitioning (TPP) of ovalbumin

Ovalbumin (2 ml; 0.38 mg/ml, pH 7.0) solution was mixed with varying concentrations of ammonium sulphate (w/v). The varying amounts of *t*-butanol were added to this solution, vortexed, and incubated at the 25 °C. After 1 h the mixture was centrifuged at 2000 × *g* for 10 min for three-phase formation. The upper *t*-butanol layer was pipetted out. Thereafter, the interfacial precipitate (of the ovalbumin) was pierced to collect the lower aqueous layer [8]. The amount of ovalbumin precipitated was calculated by measuring the amount of protein in aqueous phase with the starting amount taken as 100%.

2.3. Denaturation and refolding of ovalbumin

Denatured disulfide-reduced ovalbumin was prepared by incubating the native ovalbumin at 1.0 mg/ml, 25 °C for 3 h in buffer (50 mM Tris–HCl, pH 7.0) containing 8 M urea and 0.1 M dithiothretiol. Refolding of the denatured disulfide-reduced ovalbumin was initiated at 25 °C by two-step TPP. First step of TPP was started by mixing the 2 ml ovalbumin (fixed concentration) with 5% (w/v) ammonium sulphate. The fixed amount of *t*-butanol (1:1, aqueous to *t*-butanol ratio) was added to this solution, vortexed, and incubated at the 25 °C. After 1 h, the mixture was centrifuged at 2000 × *g* for 10 min for the three-phase formation. The upper *t*-butanol layer was pipetted out. Thereafter, the interfacial precipitate (of the ovalbumin) was pierced to collect the lower aqueous layer. This aqueous solution is used for the second step of TPP by mixing with 35% (w/v) ammonium sulphate. The fixed amount of *t*-butanol (1:1, aqueous to *t*-butanol ratio) was added to this solution, vortexed, and incubated at the 25 °C. After 1 h the mixture was centrifuged at 2000 × *g* for 10 min for the three-phase formation. The upper *t*-butanol layer was pipetted out. Thereafter, the interfacial precipitate (of the ovalbumin) was pierced to collect the lower aqueous layer. The interfacial precipitate could be completely dissolved in the buffer (50 mM Tris–HCl, pH 7.0) by adding 3 M NaOH solution drop wise until the pH increased to 11.0. After the precipitate had dissolved completely, the pH of the solution was decreased to pH 8.0 by adding 3 M HCl [3]. The dissolved precipitate was extensively dialyzed against the distilled water and the protein (T-ovalbumin) was then analysed for refolding by comparing the spectra with the native ovalbumin. The protein was also analysed for the serine protease inhibitory activity.

Just like native ovalbumin, the refolded ovalbumin (i.e. T-ovalbumin and other structural variants) was completely soluble in aqueous buffers. Hence, enzyme inhibition experiments, spectroscopic characterization, etc. could be carried out with these soluble protein samples.

2.4. Enzyme inhibition experiments

The equilibrium dissociation constant K_i of the trypsin–T-ovalbumin complex was determined by reacting constant concentration of trypsin (0.66 μM) with increasing concentration of T-ovalbumin in 50 mM Tris–HCl containing 20 mM $CaCl_2$, pH 8.2 at 25 °C for 1 h. The residual activities were measured at 410 nm using N-α-benzoyl-DL-arginine-p-nitroanilide as a substrate for trypsin [9].

The trypsin–T-ovalbumin complex was also analysed by reacting the constant concentration of trypsin (0.66 μM) with increasing concentration of T-ovalbumin in 50 mM Tris–HCl containing 20 mM $CaCl_2$, pH 7.8 at 25 °C for 1 h. The residual activities were measured at 578 nm using casein as a substrate for trypsin. Similar residual activities were measured for α-chymotrypsin using the same substrate [10].

The α-chymotrypsin–T-ovalbumin complex was also analysed by reacting the constant concentration of α-chymotrypsin (0.1 μM) with increasing concentration of T-ovalbumin in 80 mM Tris–HCl containing 100 mM $CaCl_2$, pH 7.8 at 25 °C for 1 h. The residual activities were kinetically measured at 256 nm using benzoyl-L-tyrosine ethyl ester as a substrate for α-chymotrypsin [11].

The α-chymotrypsin–T-ovalbumin complex was also analysed by reacting the constant concentration of α-chymotrypsin (0.1 μM) with increasing concentration of T-ovalbumin in 80 mM Tris–HCl containing 100 mM $CaCl_2$, pH 7.8 at 25 °C for 1 h. The residual activities were measured at 410 nm using N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide as a substrate for α-chymotrypsin [12].

2.5. Determination of the trypsin:T-ovalbumin binding stoichiometry

The trypsin:T-ovalbumin binding stoichiometry was determined by reacting increasing amounts of T-ovalbumin with constant amounts of trypsin (0.66 μM) and measuring the residual enzyme activity with a substrate (N-α-benzoyl-DL-arginine-p-nitroanilide) [9].

2.6. Temperature dependence of trypsin–T-ovalbumin complex

The temperature dependence of trypsin–T-ovalbumin complex was analysed by incubating trypsin (0.66 μM) with T-ovalbumin (1.65 μM) according to binding stoichiometry and incubate the complexes at various temperatures for two different time periods. The residual activities of trypsin were measured at 410 nm using N-α-benzoyl-DL-arginine-p-nitroanilide as a substrate.

2.7. Circular dichroism measurements

The Far-UV circular dichroism (CD) spectrum of various species of ovalbumin was recorded with a spectropolarimeter (JASCO 815) with protein samples in 20 mM sodium phosphate, pH 7.0 at 25 °C. Spectrum was recorded using a 2 nm band width, 8 s response rate, scan rate of 50 nm min^{-1} and a 0.1 nm pitch. Four accumulations were recorded and averaged for spectrum using a 0.1 cm path length cell. Near-UV CD spectra were recorded with protein concentration of 10 μM, using a 1 cm path length cell. The blank spectrum of the aqueous buffer was used to correct the observed spectrum of the sample. The CD data were expressed as mean residual ellipticity in deg cm^2 $dmol^{-1}$. The Far-UV CD spectrum was subjected to secondary structure analysis using k2d2 online software.

The melting temperatures of native ovalbumin and T-ovalbumin were also determined by recording the spectra over different temperatures at different wavelengths. The mean residual ellipticity values were plotted against temperature. The T_m values were also confirmed from the first derivative [$d\theta/dT$ vs T] plot [13].

2.8. Fluorescence measurements

Fluorescence spectra were recorded at 25 °C with a Varian Cary Eclipse spectrofluorimeter using a 1 cm cuvette. The emission spectra were obtained using the excitation at 295 nm. The excitation