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RESEARCH PAPER

Accelerated Stability Studies for Moisture-Induced Aggregation of Tetanus Toxoid

Nishant Kumar Jain • Ipsita Roy

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ABSTRACT

Purpose The study was carried out to evaluate the effect of exposing solid tetanus toxoid to moisture in two different ways on the structure and function of the toxoid.

Methods Tetanus toxoid was exposed to moisture by (i) the addition of an optimized amount of buffer and (ii) incubation under an environment provided by a saturated solution of K_2CrO_4. The changes in the conformational, structural and antigenic properties of tetanus toxoid were measured and compared.

Results Results show that even at a similar level of moisture-induced aggregation, the amounts of water absorbed by the two preparations of tetanus toxoid are different. Differences in antigenicity and changes in structure of the toxoid at primary, secondary and tertiary structure levels were seen.

Conclusion Although both conditions are used to mimic accelerated stability conditions in the laboratory, the final products are different in the two cases. Thus, conditions for 'accelerated stability studies' for therapeutic proteins need to be selected with care so that they resemble the fate of the actual product.

KEY WORDS moisture-induced aggregation · protein stability · solid-dose formulation · tetanus toxoid · vaccine stability

N. K. Jain · I. Roy (✉)
Department of Biotechnology
National Institute of Pharmaceutical Education & Research
Sector 67, S.A.S. Nagar, Punjab 160062, India
e-mail: ipsita@niper.ac.in

INTRODUCTION

Long-term stability is a critical factor that decides the suitability of any therapeutic protein formulation. In general, solid proteins are the preferred mode of storage, since in solution dosage forms, proteins and even plasmid DNA are relatively unstable (1,2). A minimum amount of water is required for maintaining the three-dimensionally folded native conformation of a protein in the solid state. Since water acts as a reactant in many of the degradation mechanisms associated with proteins (3,4), increasing the water content makes the protein structure flexible and prone to destabilization (2,5). Removal of water molecules from the protein formulation inhibits these unwanted side reactions. However, since water is essential for the maintenance of the three-dimensional architecture of the protein, osmolytes like polyols, etc. are often included in the protein solution before the drying process so that the native structure can be retained (6,7).

The mechanism of degradation/aggregation of solid-state proteins is slowly becoming clear with the elucidation of various routes of protein degradation (3,4,8). One of the stages at which moisture (or elevated humidity) induces aggregation in solid therapeutic proteins is during their storage (2). Moisture-induced aggregation of proteins is also encountered during delivery of therapeutic proteins using controlled-release pumps (9), due in part to the high amount of protein incorporated in such devices to facilitate sustained release. Solid/lyophilized formulations of such devices face the problem of protein aggregation during the exposure of vaccines to moisture during storage and rehydration at the time of release in the body, when it

comes in contact with body fluid. Moisture, or a small amount of added water, can have different effects on different proteins (3,10–13). In some cases, it can decrease the glass transition temperature (T_g) of the protein. In others, it can accelerate reaction rates by modifying the surface charge properties of a protein. In yet other cases, water can provide a medium in which protein molecules diffuse in and react with each other. In most cases, the effect seen is a mixture of all, with one outcome dominating over others depending on the nature of the protein. Most of these studies have been carried out in the laboratory by exposing the solid protein to what are referred to as "accelerated stability" conditions where moisture is deliberately introduced into the solid protein sample, and the aggregation/stability profile of the protein is monitored by various physical, chemical and biological parameters. There are usually two ways by which humidity level of a protein can be altered. The first one is by adding increasing amounts of water/buffer to the solid protein and incubating it for a defined length of time under controlled temperature conditions (6,12–15). The second is by placing the solid protein in a stability chamber with controlled humidity or in a desiccator containing saturated salt solutions (10,16,17). Both these conditions have been used quite extensively. Whether the two conditions result in similar patterns of aggregation and share any resemblance with the aggregates that are formed under real-time conditions has not been investigated so far. We have monitored the rate of aggregation as well as the nature of aggregates formed by tetanus toxoid following exposure to elevated humidity using two different approaches. To our knowledge, this is the first report of comparison of 'accelerated stability' conditions of any solid protein under two different conditions. Since results from accelerated stability testing are taken up further in developing formulations, it is essential that the aggregation pattern occurring in these samples be understood clearly.

MATERIALS AND METHODS

Materials

Tetanus toxoid (2,550 Lf/unit with antigenic purity of 1,572 Lf/mg of protein nitrogen) was obtained as a gift from Shantha Biotechnic, Hyderabad, India. Mouse anti-tetanus toxoid monoclonal antibody (HYB 278-01) was purchased from Santa Cruz Biotechnology, Inc., California, USA. Goat anti-mouse horseradish peroxidase-conjugated monoclonal antibody and tetramethyl benzidine/hydrogen peroxide substrate were obtained from Bangalore Genei, Banglore, India. 2,4,6-Trinitrobenzene sulphonic acid (TNBSA), 5-5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent), standard protein markers (29–200 kD) and thioflavin T were purchased from Sigma-Aldrich, Bangalore, India. All other reagents and chemicals used were of analytical grade or higher.

Moisture-Induced Aggregation of Tetanus Toxoid with Added Buffer

Tetanus toxoid was dialyzed against sodium phosphate buffer (10 mM, pH 7.4). Three-hundred-μl aliquots of the dialyzed solution containing 1.8 mg tetanus toxoid were lyophilized in 5 ml glass vials for 20 h. Native PAGE and FTIR spectra in amide I region (1,600–1,700 cm^{-1}) confirmed that there were no structural changes in tetanus toxoid following the mild conditions used for lyophilization (data not shown). Two μl (per mg protein) of sodium phosphate buffer (10 mM, pH 7.4) was added to the solid protein. The resultant sample was thoroughly mixed using a pipette tip (6,12). Each vial was sealed with parafilm, wrapped with aluminium foil, and placed in a controlled temperature incubator maintained at 37°C. After specified periods of time, the vials were opened, 1.5 ml of sodium phosphate buffer (10 mM, pH 7.4) was added and the resultant suspension was stirred at room temperature for 2 h. Undissolved solid was removed by centrifugation (16,200 g for 10 min). The amount of protein precipitated was determined by subtracting the amount of protein present in the supernatant from the initially added protein, by the dye binding method using bovine serum albumin as the standard protein (18). The aggregates obtained in this manner have been referred to as TTB throughout the text, and samples reconstituted in 1.5 ml of sodium phosphate buffer (10 mM, pH 7.4) were used for further analysis.

Moisture-Induced Aggregation of Tetanus Toxoid Under Elevated Humidity Levels Provided by Saturated Salt Solutions

Tetanus toxoid was dialyzed against sodium phosphate buffer (10 mM, pH 7.4). Three-hundred-μl aliquots of the dialyzed solution containing 1.8 mg tetanus toxoid were lyophilized in 5 ml glass vials for 20 h. These vials were placed in separate desiccators containing saturated solutions of different salts to provide different levels of relative humidity (LiCl, 11%; $MgCl_2$, 32%; $Mg(NO_3)_2$, 51%; NaCl, 75%; K_2CrO_4, 86%; K_2SO_4, 96%) (10) and in the presence of anhydrous P_2O_5, which acts as a desiccant. Desiccators were placed in a controlled temperature incubator maintained at 37°C. After incubation, for different time periods, the vials were removed from desiccators, 1.5 ml of sodium phosphate buffer (10 mM, pH 7.4) was added to each vial and the resultant suspensions were stirred at room temperature for 2 h. Undissolved solid was

removed by centrifugation (16,200 g for 10 min). The amount of protein precipitated was determined by subtracting the amount of protein present in the supernatant from the initially added protein, by the dye binding method using bovine serum albumin as the standard protein (18). The aggregates obtained in this manner have been referred to as TTS throughout the text, and samples reconstituted in 1.5 ml of sodium phosphate buffer (10 mM, pH 7.4) were used for further analysis.

Estimation of Water Content

Lyophilized samples in vials were incubated at 37°C under environments of saturated solutions of different salts and in anhydrous P_2O_5 to provide different levels of relative humidity for 3 days and with added buffer as a source of moisture for 2 days. The vials were opened after specified time periods, and each sample was reconstituted in 1 ml of anhydrous methanol. Known amounts of samples were transferred to a Karl Fisher titrator vessel (716 DMS, Metrohm), and the amount of water adsorbed by the samples was determined by Karl Fischer titration. The values obtained were corrected for the moisture content of anhydrous methanol, which was taken as the blank.

Polyacrylamide Gel Electrophoresis (PAGE)

Non-denaturing gel electrophoresis (native PAGE) was carried out on a 4–10% gradient polyacrylamide gel under reducing and non-reducing conditions (19) under conditions of constant voltage. Proteins were visualized by staining with 0.1% Coomassie brilliant blue R-250 and destaining with a destaining solution (10% acetic acid, 7.5% methanol in water).

Enzyme-Linked Immunosorbent Assay (ELISA)

Tetanus toxoid samples incubated for different time periods under different conditions of moisture were reconstituted in 1.5 ml phosphate buffer (10 mM, pH 7.4) by stirring for 2 h. ELISA was carried out following the protocol described earlier (20). The colour formed was measured at 450 nm.

Determination of Free Amine Groups (21)

An aliquot (150 µl) of reconstituted tetanus toxoid samples was taken and diluted with 250 µl of reaction buffer (0.2 M sodium bicarbonate, pH 8.5). Two-hundred-and-fifty µl of 2,4,6-trinitrobenzene sulphonic acid (TNBSA) solution (0.01%) was added and incubated at 37°C. After incubation for 2 h, the reaction was stopped by the addition of 250 µl of 10% SDS and 125 µl of 1 N HCl. The samples were centrifuged at 13,200 g for 5 min, and the absorbance of the supernatant was measured at 335 nm. The amount of free amine groups was calculated from the corresponding calibration curve plotted using L-alanine as the standard molecule.

Determination of Free Sulphydryl Groups (22)

Reconstituted tetanus toxoid samples (1.25 ml) was taken, and an equal volume of reaction buffer (0.2 M sodium phosphate, pH 8.0, containing 2 mM EDTA) was added. The stock solution of 5-5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) was prepared in the reaction buffer at 4 $mgml^{-1}$. Ellman's reagent (50 µl) was added to the tetanus toxoid samples and incubated for 15 min at 37° C. The colour generated by the samples was read at 412 nm. In case of aggregates, the samples were centrifuged at 13,200 g for 5 min after the incubation period before measuring the absorbance of the supernatant. Free thiols were calculated using the extinction coefficient of 14,150 $M^{-1}cm^{-1}$ for the TNB dianion (23).

Amino Acid Analysis

Lyophilized samples were incubated at 37°C under 86% relative humidity (RH) for 3 days and with added buffer as a source of moisture for 2 days. After incubation, amino acid analysis of the samples was carried out (Alta Biosciences, University of Birmingham, Birmingham, United Kingdom). Samples were hydrolyzed by constant boiling with 5.8 N HCl for 24 h at 110°C under vacuum. The hydrolyzed samples were separated by ion exchange chromatography. Post column derivatization of samples was carried out for 5 min at 125°C with ninhydrin reagent, and the derivatized amino acids were detected at 460 and 660 nm. The amounts of various amino acid residues were calculated using norleucine as the internal calibrant.

Circular Dichroism (CD) Spectroscopy

Circular dichroism measurements were recorded in the far UV region (200–250 nm) for the reconstituted samples. Samples were diluted to 1.33 µM in phosphate buffer (10 mM, pH 7.4). Measurements were performed on a CD spectrometer (J-815, Jasco) with a quartz cell of 0.1 cm pathlength. The blank was comprised of a spectrum of phosphate buffer (10 mM, pH 7.4) alone and was subtracted from the scanned spectra. Three spectra were collected for each sample, and the average was taken for secondary structure calculation using K2D2 software (24).

Thioflavin T Binding Assay (25)

Tetanus toxoid samples were reconstituted in 1.5 ml phosphate buffer (10 mM, pH 7.4) by stirring for 2 h as

described earlier. The reconstituted tetanus toxoid was diluted to a final concentration of 5 μM in phosphate buffer (10 mM, pH 7.4). Fifty μM of ThT was added, and ThT fluorescence emission spectrum was recorded with an excitation wavelength of 440 nm (slit width 5 nm) and emission wavelength range of 450–650 nm (slit width 10 nm). The spectrum of sample with no protein (dye alone) was subtracted from the spectra of the corresponding samples containing ThT.

Scanning Electron Microscopy

Different samples incubated under different moisture conditions as mentioned above were reconstituted in 1.5 ml sodium phosphate buffer (10 mM, pH 7.4). Two μl of samples were deposited over broken cover slip and dried under air. Dried samples were gold-coated and viewed under scanning electron microscope (S-3400N, Hitachi High-Technologies Corporation).

Intrinsic Fluorescence Spectroscopy

Different reconstituted samples were diluted to a final concentration of 1.06 μM in phosphate buffer (10 mM, pH 7.4). Resultant solutions were excited at 280 or 295 nm, and emission fluorescence spectra were recorded in 290–450 nm and 305–450 nm ranges, respectively. The excitation and emission slit widths were kept at 5 nm each. Spectra obtained were corrected by subtraction of spectrum of solution contacting no protein.

RESULTS AND DISCUSSION

Water acts as a plasticizer and introduces crystallinity in the protein structure. Because of structural polymorphism, different conformations of a protein can be formed on moisture exposure, leading to protein inactivation or aggregation. When the solid protein is incubated in the presence of a saturated salt solution, the protein is expected to interact with water in the vapour state. This situation probably mimics the one where storage stability of the protein is monitored (16). On the other hand, when water is added to solid protein, the interaction can be visualized to be that between solid and liquid. This situation probably resembles the status of a solid protein encapsulated in a controlled release polymer (26,27). Any difference in the structure of the protein seen is thus due to the difference between solid-gas (vapour) vis-à-vis solid-liquid interaction. Most of the water sorption studies involve exposing the solid protein to water vapour, as in the environment provided by saturated salt solutions. The isotherm in such cases is divided into three distinct regions (28). The first region includes binding of water molecules to ionized groups on the protein surface. This is usually less than a monolayer. The initial layer causes the water molecules to become immobile and increases the flexibility of the protein molecule (29). Proteins which have more water molecules associated with them per unit mass are thus likely to be more reactive and are therefore less stable than those which have less water molecules associated with them (29). The middle region denotes the transition from monolayer to multilayer coverage. The third region denotes the clustering of water molecules around weak binding sites and marks the water layer held loosely around the protein molecule.

We have chosen to carry out studies with tetanus toxoid, since the toxoid is prone to moisture-induced aggregation, especially in tropical climates; hence, a number of studies have been carried out to decipher the mechanism of aggregation and suggest suitable stabilization strategies (16,26,27). Such studies, however, use both the conditions listed above for accelerated stability testing without determining if similar types of final formulations result after exposure to different conditions. Tetanus toxoid was exposed to moisture in two different ways in this study: by incubation in a humid atmosphere provided by saturated salt solution (TTS) and by incubation with added buffer as a source of moisture (TTB). As shown in Fig. 1, there was an increase in the moisture content of the lyophilized tetanus toxoid with increase in RH. Lyophilized tetanus toxoid, incubated in an environment provided by a saturated solution of K_2SO_4 (96% RH), absorbed the maximum amount of moisture (Fig. 1a). The maximum amount of aggregation (80%) occurred in the case of the sample incubated in an environment due to a saturated solution of K_2CrO_4 (86% RH) (Fig. 1b). This kind of bell-shaped curve for amount of protein aggregated v/s amount of moisture has been observed for tetanus toxoid and other proteins earlier (10,16). At sufficiently low water content, the mobility of the protein is hindered; thus, aggregation is not favoured. At intermediate levels of water, water acts as a lubricant, mediating interaction between neighbouring protein molecules and causing aggregation. As the water content continues to increase, it starts acting as a solvent and dilutes the protein sufficiently to inhibit proximity-induced interactions. In addition, water molecules act as competing nucleophiles along with amine groups of proteins for reaction with Schiff bases (formed after reaction of amine groups of proteins with formaldehyde present in the toxoid). Thus, further crosslinking of the protein molecules is prevented, and the rate of aggregation of the protein is slowed down. Therefore, tetanus toxoid incubated at 86% RH was used for further studies. The control sample incubated in the presence of anhydrous P_2O_5 and subjected to the same treatment

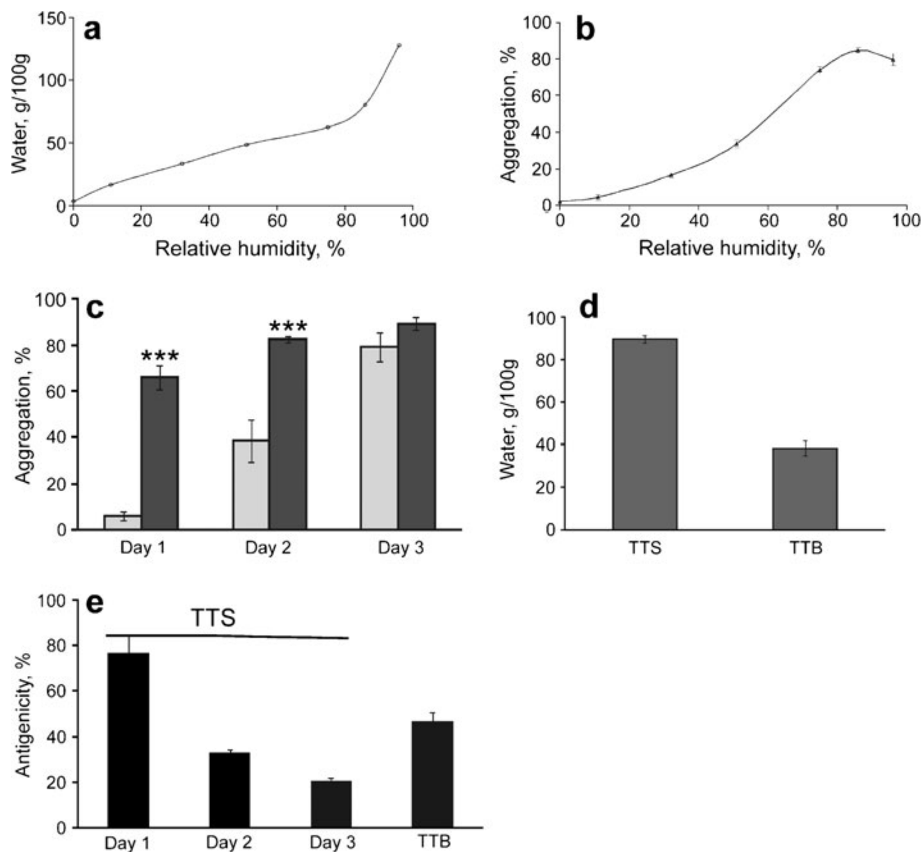

Fig. 1 **a** Water content in the lyophilized tetanus toxoid after incubation in saturated solutions of different salts at 37°C for 3 days. The moisture content was estimated by carrying out Karl Fisher's titration. Values represent mean of three independent experiments; error bars represent the standard errors of mean. **b** Moisture induced aggregation of tetanus toxoid at different moisture levels. Values represent mean of three independent experiments; error bars represent the standard error of mean. 100% aggregation assumes that all of the initially added protein has aggregated. **c** Moisture induced aggregation of tetanus toxoid by adding phosphate buffer (10 mM, pH 7.4). Control samples (*grey bars*) were incubated for the same period as samples but without the addition of moisture. Values represent mean of three independent experiments; error bars represent the standard error of mean. *** represents $p < 0.001$ *vs*. control. 100% aggregation assumes that all of the initially added protein has aggregated. **d** Water content of tetanus toxoid samples incubated with added buffer as a source of moisture (TTB) after 2 days and in the presence 86% RH (TTS) after 3 days, at similar levels of aggregation. Measurements were carried out as described in (**a**). 100% aggregation assumes that all of the initially added protein has aggregated. **e** Measurement of antigenicity of tetanus toxoid samples incubated under different conditions. In TTS, lyophilized tetanus toxoid was incubated at 37°C in an environment of saturated solution of K_2CrO_4 (86% RH) for 1, 2 and 3 days. In TTB, samples were incubated with added buffer as a source of moisture for 2 days. ELISA was carried out for the reconstituted samples after centrifugation. Values are represented as percentage change as compared to native tetanus toxoid whose antigenicity was taken as 100%. Values represent mean of three independent samples and error bars are standard errors of mean.

resulted in complete solubilisation of the toxoid showing that aggregation occurred due to exposure to moisture and not because of any subsequent processing step.

In case of TTB also, the conditions were optimized to obtain the same level of aggregation of tetanus toxoid as that of TTS. For this, lyophilized tetanus toxoid was incubated with different amounts of sodium phosphate buffer (10 mM, pH 7.4) (1–4 μl/mg lyophilized protein) at 37°C up to 10 days. Lyophilized protein incubated without buffer was taken as the control. All the test samples showed increased aggregation of protein on exposure to added buffer as a source of moisture (data not shown). Maximum aggregation was observed in case of samples incubated with 2 μl buffer (per mg protein). However, the corresponding controls also showed similar aggregation patterns, and the difference between the aggregation profiles of control and test samples decreased gradually with increase in the time of incubation. Therefore, the time of incubation was reduced, and the samples were incubated up to 3 days. After day 1 and day 2, a significant difference in the aggregation patterns of the control and test samples was observed, whereas after day 3, the control sample also exhibited substantial aggregation, and only a marginal difference between controls and samples could be observed (Fig. 1c). After incubation at 37°C for 2 days with 2 μl (per mg protein) of sodium phosphate buffer (10 mM, pH

7.4), tetanus toxoid showed 80% aggregation of the protein. This level of aggregation was comparable to that obtained when tetanus toxoid was exposed to 86% RH (TTS). However, the amount of water absorbed by the protein under the two conditions was different (Fig. 1d), even though the extent of aggregation was the same. In the case of TTS, the amount of water present in the protein was 0.89 g/g (89.6%) after 3 days, whereas it was 0.38 g/g (38.3%) in the case of TTB after 2 days, at a similar level of aggregation. Thus, the nature of the aggregates, or more importantly, the mechanism by which moisture induces aggregation in tetanus toxoid, is different in the two cases. The level of water in protein formulations has been used as a means of comparing the storage stability of different solid preparations. Calorimetric measurements have shown that there is a threshold level (10%, w w^{-1}) beyond which water cannot interact with a protein (30). In contrast, gravimetric data have suggested that the enthalpy of sorption reaches a plateau at higher water content, i.e. 20% (w w^{-1}) (31). There are a number of studies which have reported the sorption enthalpy reaching equilibrium values at different amounts of water content (32,33). With increase in % RH, no significant change in ^{1}H relaxation times had been observed for a number of proteins, showing that the mobility or molecular motion of the protein was not considerably affected by the number of water molecules on the protein surface up to 98% RH (29). Also, as discussed earlier, the mechanism of water sorption by the two approaches (solid-gas in case of TTS vis-a-vis solid-liquid in case of TTB) is different. Since there is a lot of ambiguity in the data available for protein topology as a function of its hydration level, we decided to use similar aggregation levels as the normalization parameter for the two cases, although its use is less common. Moreover, since a change in the toxoid structure would be expected to change its antigenicity, we wanted to investigate whether similar levels of aggregation in the two cases would result in different antigenicities. Hence, these two conditions, with equal extents of protein aggregation, were taken for further studies.

Antigenicity of toxoids is a key parameter by which the integrity of the protein, following exposure to any stress condition, can be evaluated. The antigenicity of tetanus toxoid was measured by ELISA using mouse anti-tetanus toxoid monoclonal antibody as the primary antibody. It was observed that the toxoid exhibited 80% loss in antigenicity on being exposed to TTS after 3 days (Fig. 1e). In case of TTB, this loss was 50%. Thus, even though the extent of aggregation was similar in both the cases, the loss in antigenicity after aggregation was more in the case of TTS than TTB. This could be because of the lower amount of water absorbed in the case of TTB than in TTS. Therefore, it was essential to investigate the mechanism involved in aggregation of the tetanus toxoid under these two different conditions. For this purpose, studies were carried out to monitor changes in primary, secondary and tertiary structures of tetanus toxoid upon aggregation under the above conditions.

Changes in the Primary Structure of Tetanus Toxoid Upon Moisture Induced Aggregation

Analysis of Free Amine Groups

The change in the number of free amine groups of tetanus toxoid upon incubation under different conditions was monitored by TNBSA assay (21). In the case of TTS, the number of free amine groups decreased to 8.4 nmol mg^{-1} protein with increase in the period of exposure of tetanus toxoid to 86% RH (TTS) (Fig. 2a). At a similar level of aggregation, TTB also showed fewer numbers of free amine groups (16.98 nmol mg^{-1} protein) than the control sample (27.5 nmol mg^{-1} protein) after 2 days of incubation. The extent of decrease in free amine groups was significantly lower ($p<0.001$) in the case of TTS (after 3 days) than TTB (after 2 days) at the same level of aggregation. The decrease in the number of free amine groups indicated that covalent bonds were formed between amino acid residues which resulted in aggregation of tetanus toxoid under both conditions. The contribution of covalent linkages was higher in the case of TTS than TTB, since more amino acids were involved in the formation of aggregates.

Since the extent of aggregation was the same, while the residual antigenicity was higher in the case of TTB, it may be speculated that the amino acid residues that form the crosslinked structure are different in the two cases. In the case of TTS, more amine groups from the epitope-defining region of the toxoid are probably involved in forming the crosslinked structure. Amino acid analysis showed that there was a decrease in histidine content in both types of aggregates along with an increase in tyrosine content as compared to the control sample (data not shown). The loss of histidine residues was more in the case of TTS than TTB, while the increase in the number of tyrosine residues was more in the case of TTS than TTB. Aggregation of tetanus toxoid has been shown to involve the modification in the amino acid content of the protein (16). Several studies have shown that formaldehyde present in the preparation reacts with amino acids like lysine, histidine, tyrosine, cysteine and tryptophan, and forms Schiff bases which are electrophilic in nature (14,16,34). These Schiff bases can form cross links with other amino acid residues and result in a toxoid preparation which is nontoxic but immunogenic. Some of these Schiff bases remain stored in the dormant form after detoxification of the toxin. Upon exposure to moisture, at intermediate water levels, these Schiff bases become active and attack neighbouring residues, forming crosslinks. In many cases, these crosslinks

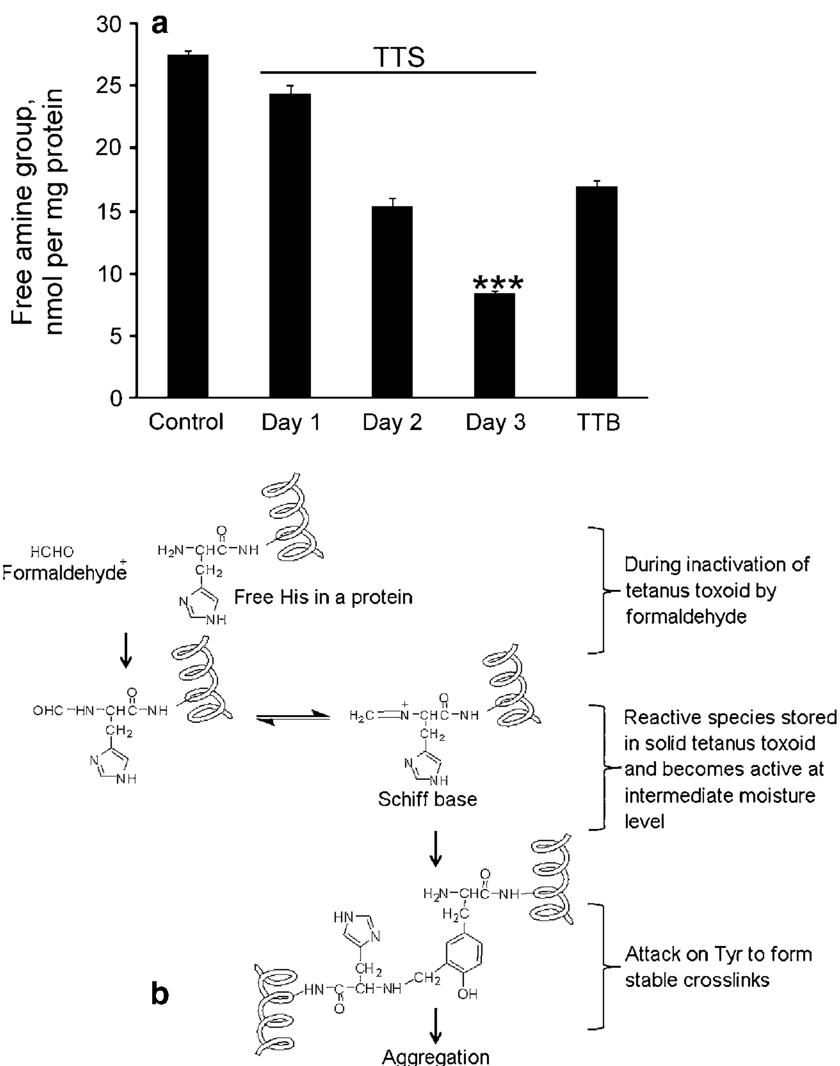

Fig. 2 **a** Estimation of free amine groups of tetanus toxoid incubated under different conditions. Value represents mean of three independent samples and error bar are standard errors of mean. *** represents $p<0.001$ *vs*. TTB (2 days). **b** Scheme for proposed histidine-mediated crosslinking of tetanus toxoid during moisture induced aggregation.

are resistant to acid hydrolysis. The decrease in histidine content indicates that this amino acid may be involved in Schiff base-mediated crosslinking and aggregation of tetanus toxoid under elevated humidity. Previous reports of moisture-induced aggregation of tetanus toxoid have shown a marked decrease in lysine content (16,27). However, in the current study, lysine content of tetanus toxoid after aggregation was found to remain unchanged (data not shown). Since histidine has been reported to act in a manner similar to lysine during moisture-induced aggregation of tetanus toxoid (16), a similar mechanism is probably working here (Fig. 2b).

Analysis of Free Thiol Groups

Native tetanus toxin is reported to contain six free thiols and two disulphide bonds (16). The role of free thiols in the aggregation of proteins has been well documented in the literature (2,4). In order to verify the involvement of free thiols in moisture-induced aggregation of tetanus toxoid under both conditions, Ellman's assay was carried out (22). As shown in Fig. 3a, there was a decrease in the free thiol content of tetanus toxoid upon incubation of the protein under 86% RH for different periods. Similar decrease was also seen in the case of tetanus toxoid exposed to added buffer as a source of moisture (TTB). Free thiol group content of native protein was 6.8 μMmg^{-1} protein. The concentration of the residual free thiol groups in the case of TTS (4.6 μMmg^{-1} protein) was significantly lower ($p=0.023$) than in the case of TTB (5.6 μMmg^{-1} protein).

The decrease in the free thiol content can be attributed to two reasons. The formation of new disulphide bridges is one of the most common causes of the solid state aggregation of a protein. The second possibility is the chemical modification of cysteine. Cysteine residue can also form Schiff bases and hence may be involved in crosslinking tetanus toxoid (16). Cysteine modification cannot be observed with amino acid analysis as the modified product

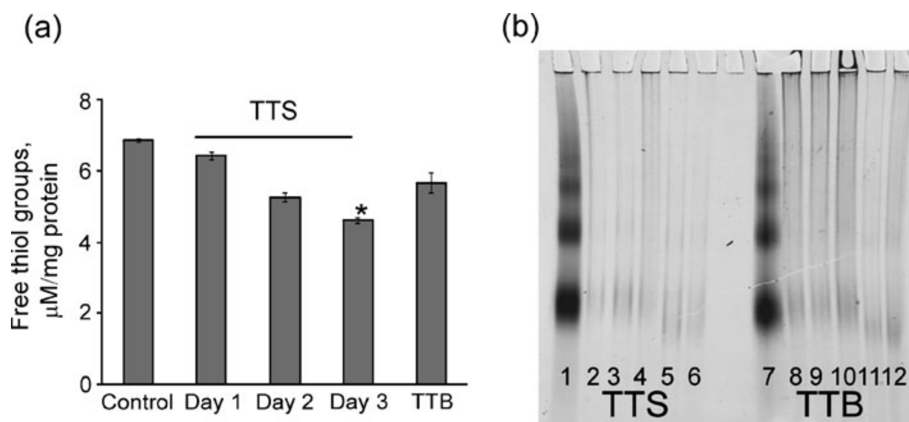

Fig. 3 **a** Estimation of free thiol groups of tetanus toxoid incubated under different conditions. Values represent mean of three independent samples and error bars are standard errors of mean. * represents $p < 0.05$ *vs*. TTB (2 days). **b** Native PAGE for tetanus toxoid incubated under different conditions and reconstituted in different reagents. Lanes 1–6: Moisture induced aggregation of tetanus toxoid in TTS. Lane 7–12: Moisture induced aggregation of tetanus toxoid in TTB. Lanes 1 and 7: Native tetanus toxoid; Lanes 2 and 8: Aggregated tetanus toxoid resuspended in phosphate buffer (10 mM, pH 7.4); Lanes 3 and 9: Aggregated tetanus toxoid resuspended in 0.001 M EDTA; Lanes 4 and 10: Aggregated tetanus toxoid resuspended in 0.001 M EDTA and 0.01 M DTT; Lanes 5 and 11: Aggregated tetanus toxoid resuspended in 8 M urea; Lanes 6 and 12: aggregated tetanus toxoid resuspended in a mixture of 0.001 M EDTA, 0.01 M DTT and 8 M urea.

degrades during acid hydrolysis. In order to confirm whether the decrease in free sulphydryl groups was due to the formation of disulphide linkages or modification of cysteine residues, native gel electrophoresis of the aggregated samples was carried out under reducing and non-reducing conditions. The traditional way to detect the presence of covalent/non-covalent interactions is by dissolution of a protein aggregate in different reagents capable of breaking these cross-links. These reagents include dithiothreitol, which reduces disulphide bonds, and denaturants, such as urea, which disrupt the non-covalent interactions among protein molecules. The aggregated samples were incubated with urea and dithiothreitol. The reconstituted samples were run on a 4–10% gradient polyacrylamide gel.

As can be seen (Fig. 3b), the bands for the samples obtained after moisture incubation appeared to be faint as compared to those of the native toxoid, indicating the inability of the aggregates (TTS as well as TTB) to enter the crosslinked polyacrylamide gel. Aggregates reconstituted in 0.01 M DTT were also not able to enter the gel, indicating the absence of thiol-mediated crosslinking. This also confirmed that the decrease in concentration of free thiol groups in Ellman's assay was due to chemical modification of cysteine residues and their involvement in crosslinking leading to the formation of aggregates. The inability of the aggregates to dissolve in 8 M urea and enter the crosslinked polyacrylamide gel indicated the absence of non-covalent forces in the formation of moisture-induced aggregates of tetanus toxoid. This was also confirmed by analyzing the samples on denaturing SDS-PAGE (data not shown). In none of the cases were the intensities of the bands stronger than those observed when the aggregates were suspended in 0.01 M phosphate buffer, pH 7.4, indicating that the aggregates are formed via non-disulphide-mediated covalent linkages, without the involvement of any non-covalent forces.

Changes in the Secondary Structure of Tetanus Toxoid Upon Moisture Induced Aggregation

Conformational changes in the secondary structure of tetanus toxoid upon incubation under different conditions were monitored by far UV circular dichroism (CD) spectroscopy. The far UV-CD spectra of samples incubated for more than one day exhibited a high degree of scattering and hence are not reported here. Lyophilized tetanus toxoid was incubated, at 37°C at 86% RH (TTS) and with added buffer as a source of moisture (TTB), for shorter periods of time to monitor the early events of aggregation. Samples were withdrawn at different time intervals and reconstituted in phosphate buffer (10 mM, pH 7.4) as before and analyzed by far UV-CD spectroscopy. Estimation of components of secondary structure (24) showed considerable α-helical content for the native protein, which is similar to what has been reported for tetanus toxoid (35). Upon moisture-induced aggregation, the protein exhibited an increase in β-sheet content over time (Fig. 4a). The lag time in the case of TTB was longer than with TTS, and, thus, the rate-determining step of nucleation in the case of TTB was much slower than in TTS.

The trend observed in CD data represents the classical pattern of aggregation kinetics where aggregation starts with a lag phase followed by an exponential phase and then the log (or stationary) phase (Fig. 4b). The samples

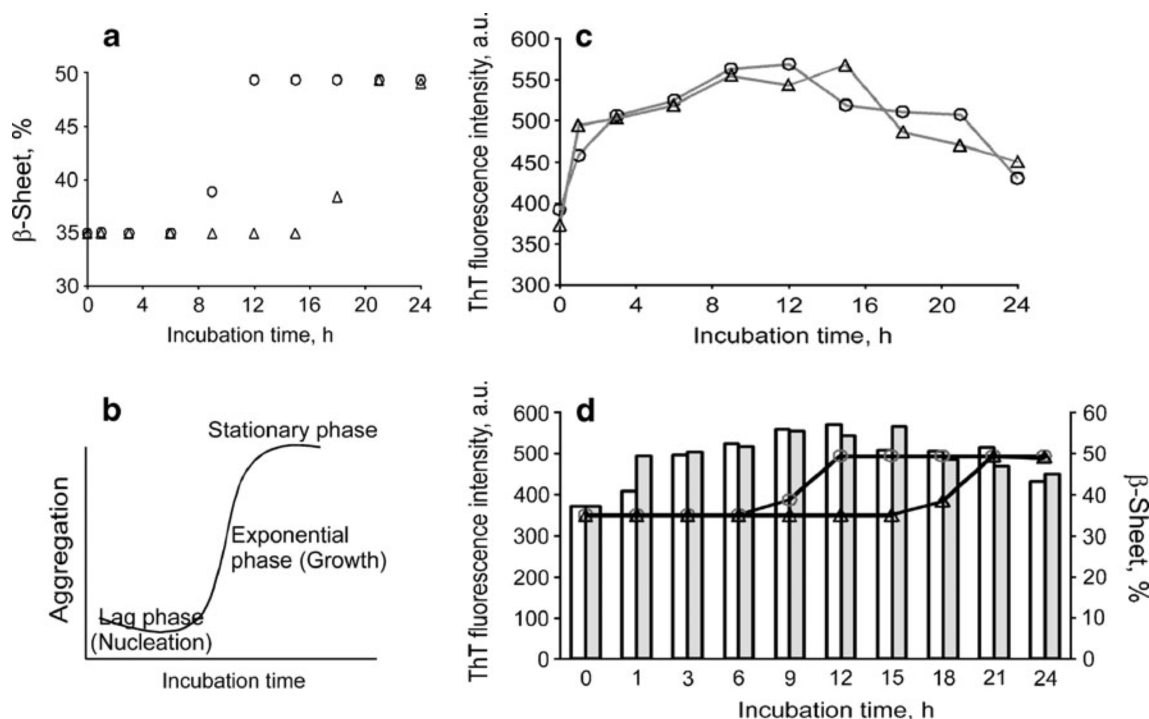

Fig. 4 **a** Comparative analysis of β-sheet content of tetanus toxoid incubated under TTS (*circle*) and TTB (*triangle*) for moisture induced aggregation. **b** Schematic diagram for aggregation kinetics of tetanus toxoid. **c** Thioflavin T (ThT) fluorescence enhancement spectra for tetanus toxoid incubated under TTS (*circle*) and TTB (*triangle*) for moisture-induced aggregation. **d** Correlation of change in β−sheet content with ThT fluorescence intensity during moisture-induced aggregation of tetanus toxoid for TTS (*empty bar, circle*) and TTB (*filled bar, triangle*). Bar plot represents ThT fluorescence intensity whereas line plot represents β-sheet content obtained by far UV-CD spectroscopy.

incubated under salt-saturated environment showed a faster onset of conformational changes. The pattern of aggregation, with a nucleation phase followed by a growth phase, is usually characteristic of amyloid type of aggregation (36). In order to find out the nature of aggregates formed in the two cases, the fluorescence emission intensity of Thioflavin T (ThT) was monitored in the presence of both types of aggregates. ThT is a cationic benzothiazole dye which shows a drastic enhancement in its fluorescence emission intensity upon interaction with amyloid-type aggregates (6,25). As shown in Fig. 4c, fluorescence intensity of ThT increased in the presence of tetanus toxoid aggregates. The fluorescence intensity reached a maximum value around the same time in both cases. In the case of TTS, this correlated with the increase in β-sheet content (Fig. 4d). In the case of TTB, however, the increase in β-sheet content trailed the increase in ThT fluorescence intensity. Although the fluorescence intensity of ThT increased in the same manner in both cases, the secondary structures of the aggregates at this stage (maximal ThT fluorescence) were different. In the case of aggregation in the presence of a saturated solution of K_2CrO_4, the β-sheet content and ThT-positive structures were formed simultaneously. With aggregation in the presence of added buffer as the source of moisture, on the other hand, the two steps were not simultaneous. The increase in the emission intensity of ThT preceded the increase in the β-sheet content of the protein, as determined by far UV-CD spectroscopy. This is similar to the pH-induced fibrillation of barstar where the alteration in the conformation of the protein has been shown to either lead or trail amyloid fibrillation (37,38). In a study with glucagon, different fibril types have been reported under different experimental conditions (36). These fibrils have been classified into four types depending upon their secondary structure and ThT binding properties. These include (1) type A, which is formed under high protein concentration and exhibits high ThT emission with β sheet like CD spectra, (2) type B, which is formed at low protein concentration and low salt concentration and shows low ThT emission with β turn like CD spectra, (3) type D, which is formed under intermediate salt concentration and shows low ThT emission and classic β sheet like CD spectra, and (4) sulphate type structure, which is formed under high sulphate ion concentration and low pH conditions and exhibits high ThT emission with α helix like CD spectra. A slight change in the experimental condition can result in a different type of molecular packing of the agglomerate with different ThT binding properties. This is true in the current study as well and explains the patterns observed in Figs. 4c and d. The aggregates formed under TTS resemble the 'type A'

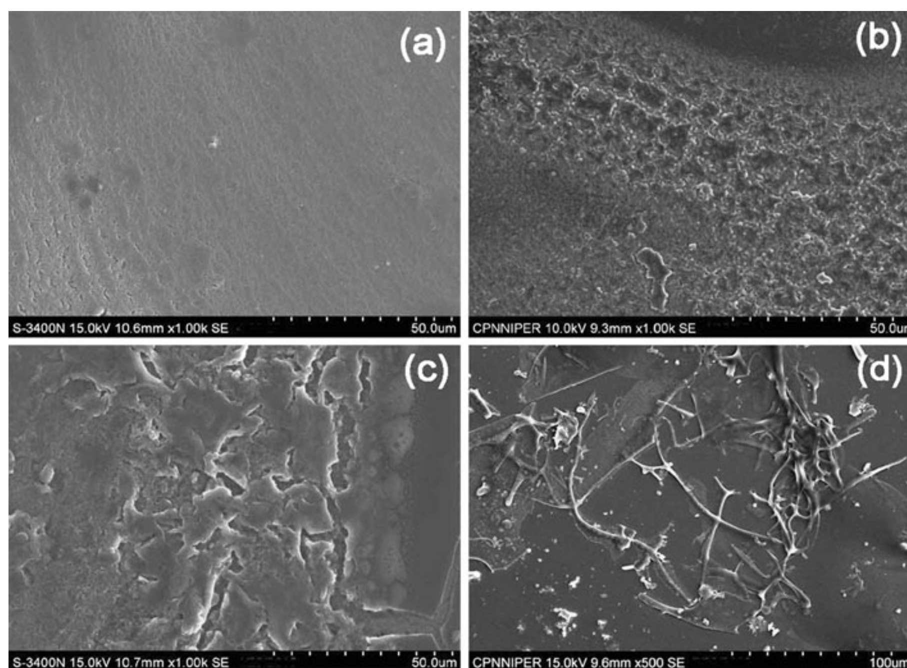

Fig. 5 Scanning electron microscopy of tetanus toxoid incubated under different incubation conditions. Tetanus toxoid was incubated for 15 h (**a**) and 2 days (**b**) in TTB condition and for 12 h (**c**) and 3 day (**d**) in TTS condition. Lyophilized tetanus toxoid was incubated under TTB and TTS conditions.

structure, whereas aggregate formation under TTB is a two-stage phenomenon where the 'sulphate-type' structure matures to form the 'type A' structure. Scanning electron microscopy of the aggregated samples was carried out to understand the morphology of the aggregates formed under the two conditions. The micrographs are recorded at two different time points for TTS and TTB: one is at the point where the fluorescence of ThT is maximal (Fig. 5a and c), and the second where both samples show a similar level (~ 80%) of aggregation (Fig. 5b and d). At the time point where the fluorescence of ThT had just reached the highest value, the morphology of the protein aggregates looked similar. No fibril formation was seen in either case. However, there was a dramatic difference when aggregation was allowed to proceed for a longer period. Although the extent of aggregation was similar in the two cases (Fig. 1b and c), an ordered fibrillar structure is seen only in the case of tetanus toxoid that has been exposed to a saturated solution of K_2CrO_4 in an environment of 86% RH (TTS). According to earlier reports, a uniform monolayer of water is not formed on the protein surface even under ideal conditions. Instead, it is concentrated on the charged amino acid residues (28,29). Fibrillar structures with lengths exceeding 100 μm are seen in this case. TTB exhibits a honeycomb-like structure probably due to non-uniform absorption of moisture by the protein. Thus, the differential effect of water on the aggregation pattern of tetanus toxoid is clear from these micrographs. Ordered structures result only in the case where water is absorbed uniformly by the solid protein sample, resulting in homogeneous distribution of water/moisture.

Changes in the Tertiary Structure of Tetanus Toxoid Upon Moisture Induced Aggregation

Changes in the three-dimensional structure of tetanus toxoid following moisture-induced aggregation were monitored by intrinsic fluorescence measurements. The intrinsic fluorescence of Trp depends upon the polarity of its local microenvironment or accessibility of Trp to the solvent. The emission intensity of Tyr, on the other hand, is largely independent of the microenvironment of the fluorophore. Thus, any change in the emission intensity or λ_{max} of a protein following aggregation largely reflects changes in the environment around the Trp residues. Tetanus toxoid contains 13 Trp and 79 Tyr residues. When a protein containing all three aromatic amino acid residues is excited at 280 nm, the changes in intrinsic fluorescence properties can be monitored with the main contribution from Trp residues (9). When excited at 280 nm, the fluorescence intensity maximum of TTS was found to decrease in a time-dependent manner (Fig. 6). This decrease was not simply because of the protein molecule precipitating out of solution, since a red shift in λ_{max} of emission intensity was also observed, indicating increased polarity around the fluorophores due to unfolding of the protein molecule (data not shown). When excited at 295 nm, the effect of the presence of Tyr residues on the emission spectrum could be eliminated and the emission spectra due to Trp only could be recorded (39). When the tetanus toxoid samples were excited at 295 nm, a decrease in fluorescence intensities was observed with increase in time of incubation for the

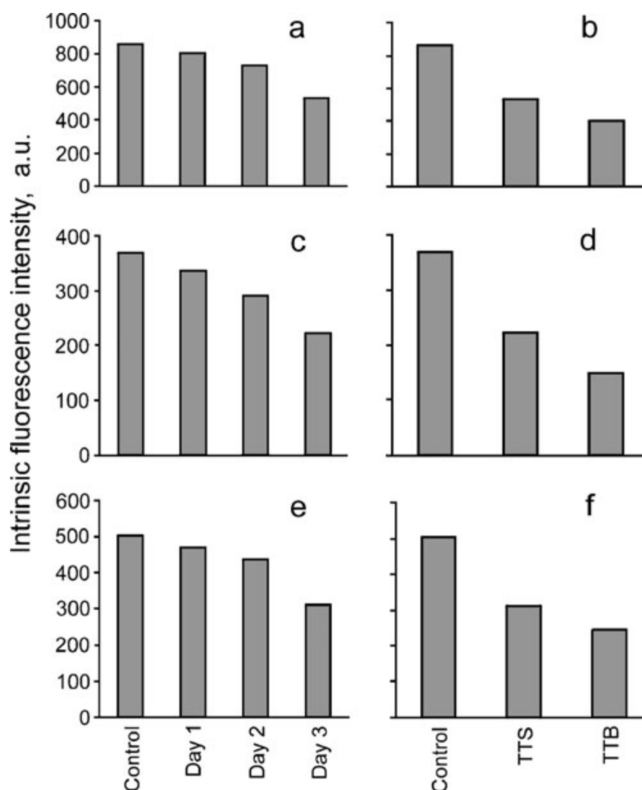

Fig. 6 Comparison of intrinsic fluorescence measurements for tetanus toxoid incubated under TTS and TTB. Emission intensities were recorded after excitation of the samples at 280 nm (**a**, **b**) and 295 nm (**c**, **d**). The difference between the emission spectra obtained after excitation at 280 nm and 295 nm was calculated. Plots e and f denote the changes in maximum intrinsic emission fluorescence intensities from the subtracted spectra. Plots a, c and e show the change in the fluorescence intensities of the TTS samples with time of incubation. In case of plots b, d and f, TTS denotes the sample incubated at 86% RH for 3 days. After excitation at 280 nm, emission was recorded in the range of 290–450 nm.

sample in the presence of a saturated solution of K_2CrO_4, similar to the results obtained with excitation at 280 nm (Fig. 6). A red shift in the λ_{max} of emission intensity was again observed (data not shown), confirming that the polypeptide chain unfolds around the Trp residues. Fluorescence measurement of tetanus toxoid samples incubated with added buffer as a source of moisture (TTB) also revealed similar properties. When the aggregated sample was excited at 280 or 295 nm, a decrease in fluorescence intensity as compared to the control sample was observed (Fig. 6). This was accompanied by a red shift in both the cases, indicating the unfolding of the polypeptide chain during aggregation and increased polarity around the Trp residues during unfolding. Since both Tyr and Trp residues contribute to the emission intensity of tetanus toxoid when the samples are excited at 280 nm, and since the number of Tyr residues is much larger than that of Trp residues in tetanus toxoid, a change in the spectrum due to Tyr residues will provide a better idea of the structural changes that the protein undergoes following aggregation. We determined the changes in the microenvironment around the Tyr residues by subtracting the spectrum obtained after excitation at 295 nm (Trp only) from the emission spectrum obtained after excitation at 280 nm (contribution from Trp and Tyr). As can be seen (Fig. 6), the subtracted emission intensities of TTS samples decreased with increase in the time of incubation as compared to the control sample, and after three days were only marginally higher than the emission intensities of TTB samples. Thus, Tyr residues were less exposed and less available under both conditions of aggregation. Amino acid analysis had shown the involvement of Tyr crosslinking in the formation of aggregates under both conditions. Thus, in both cases (TTS and TTB), Tyr residues participate in the formation of covalent bonds which result in aggregates of tetanus toxoid under both conditions. This reduces the concentration of Tyr residues to be excited, decreasing their emission intensities.

Comparison Between the Two Methods

Water plays a crucial role in preserving the structure and function of a protein. On one hand, water is required for maintaining the three-dimensionally folded architecture of a protein. Thus, the removal of water (e.g. during freeze-drying) leads to reduced stability of the protein. On the other hand, the storage stability of pharmaceutical proteins can be enhanced by removing water, i.e. drying them, since water acts as a catalyst or participant in almost all protein degradation reactions. Thus, understanding the nature of changes in the protein as a function of its hydration level is important. The nature of aggregates formed in the two cases and the mechanism of aggregation involved therein are, albeit slightly, different. Although the structure of the initial protein subjected to aggregation is the same, the structure of the protein at the 'moisture-equilibrated' stage

Table 1 Comparison of Properties of Aggregates Formed Under Different Conditions of Moisture Exposure

Parameter	Native protein	Aggregated protein (80% aggregation)		Comments
		TTS	TTB	
Water content	3.12% w w^{-1}	89.6%, w w^{-1}	38.3%, w w^{-1}	Amounts of water adsorbed are drastically different, indicating different modes of interaction
Residual antigenicity	100%	20.4%	47%	Different values at the same level of aggregation, indicating difference in conformation of protein under different incubation conditions
Free amine groups	27.5 $nmolmg^{-1}$	8.4 $nmolmg^{-1}$	16.9 nmolmg–^{1}	Involvement of covalent bonds in the formation of aggregates in both cases but up to different extents.
Free thiol groups	6.8 μMmg^{-1}	4.5 μMmg^{-1}	5.6 μMmg^{-1}	Modification of Cys residues occurs in both cases; the extent of modification is different
Amino acid residues with marked changes				Involvement of His and Tyr residues in the formation of aggregates; the extent of involvement is different
• His	154 $nmolmg^{-1}$	120 $nmolmg^{-1}$	123 $nmolmg^{-1}$	
• Tyr	151 $nmolmg^{-1}$	179 $nmolmg^{-1}$	168 $nmolmg^{-1}$	
Spectrofluorimetric changes	λ_{em} = 327 nm	330 nm	331 nm	Red shift indicating increase in hydrophobicity around the fluorophore following aggregation in both cases
Secondary structure changes and ThT reactivity	–	Maxima in β- sheet content and ThT fluorescence reached at the same time	Increase in β−sheet content trails the increase in ThT intensity	Changes in secondary structure and β-amyloid character do not follow the same trend in both cases
Morphology	–	Fibrillar structure	Honeycomb-like structure	More amount of adsorbed water leads to a more ordered structure in case of TTS; non-uniform distribution of water leads to a disordered aggregate in case of TTB
Mechanism of aggregation	–	Non-disulphide-mediated covalent crosslinking with comparatively more covalent contribution	Non-disulphide-mediated covalent crosslinking with comparatively less covalent contribution	In the case of TTS, a higher amount of covalent character involving the epitope-determining region of the toxoid leads to an ordered aggregation of the protein; in the case of TTB, at a similar level of aggregation, the amount of covalent contribution and hence ordered structure of the aggregate is lower.

is different when the protein is exposed to moisture in two different ways. In a majority of the cases, 'accelerated stability' studies to mimic the long-term effect of humidity on the storage stability of a protein are carried out using either of these two routes. The solid protein is incubated for a defined length of time by adding minute quantities of water/buffer to it. Alternately, the solid protein is incubated in an environment of defined RH by incubating it in the presence of a saturated solution of a known salt. A comparison of the nature of the aggregates formed in the two cases is almost never carried out. In this work, we have performed accelerated storage studies by exposing solid tetanus toxoid to moisture under both of these conditions (Table I). The stability parameters of the toxoid were monitored after dissolution of the (aggregated) protein in a suitable buffer. It has recently been shown that the tetanus toxoid encapsulated in PLGA microparticles aggregated via non-disulphide-mediated covalent bonds (27). This matches with our results obtained with TTS and TTB. The amount of Tyr content in the aggregated sample was also found to increase. Tyr is one of the major players in the formaldehyde-mediated aggregation pathway, and its level has been found to increase in PLGA-encapsulated tetanus toxoid (16,27). The loss in antigenicity observed in the case of TTS (residual antigenicity of 20.4%) is of a similar level that has been observed in the case of the encapsulated protein present in the polymeric PLGA microspheres (residual antigenicity of 17%) (27) and is much lower than 47% residual antigenicity that has been observed with TTB in the present case. In the case of TTS (at 86% RH), the absorbed moisture is distributed uniformly throughout the protein sample. In the case of TTB, on the other hand, water is preferentially absorbed by a part of the protein sample, resulting in non-uniform distribution of water. These results are also supported by scanning electron microscopy in which fibrillar structure is seen only in the case of TTS and not with TTB at similar levels of aggregation.

Measurement of changes in secondary structure by far UV-CD spectroscopy showed a reduction in the α-helical content and increase in β-sheet content with the time of incubation. The lag periods were different in the two cases. Changes in the tertiary structure of polymer-encapsulated tetanus toxoid, as monitored by fluorescence spectroscopy, showed a second peak at 445 nm (20), whose intensity increased with the time of incubation. This peak, which is attributed to the formation of an oxidation product of Trp, was missing in our case. The red shift in the λ_{max} of the original peaks (at 331 nm for TTB and at 330 nm for TTS) was due to the partial unfolding of the protein molecule. Thus, the aggregate of tetanus toxoid formed under TTS resembles a population where water is distributed more evenly in the aggregated protein than in case of TTB condition.

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