

## Secondary structures of proteins adsorbed onto aluminum hydroxide: Infrared spectroscopic analysis of proteins from low solution concentrations

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Received 16 November 2005

Available online 24 January 2006

### Abstract

Comparative studies of the secondary structures of six model proteins, adsorbed onto aluminum hydroxide gel (Alhydrogel) or in aqueous solution, were carried out by Fourier transform infrared (FTIR) spectroscopy. The analysis of high-quality spectra of all six model proteins, with a broad range of secondary structure compositions, obtained at 15 mg/ml by the conventional method and at 0.5 and 1.0 mg/ml adsorbed to Alhydrogel revealed that adsorption onto hydrophilic surfaces of aluminum hydroxide particles did not alter the secondary structures of the proteins. The results of this study suggest that adsorbing proteins to Alhydrogel provides a means of obtaining FTIR spectra to study secondary structure and conformational changes of proteins in aqueous solution at very low concentrations. The new procedure effectively lowers the concentration requirement for FTIR studies of proteins in aqueous solutions by at least 40-fold, as compared with the conventional FTIR method. It permits FTIR study of proteins to be carried out in the same concentration range as is used for circular dichroism and fluorescence, thereby making it possible to compare structural information obtained by three commonly used techniques in protein biophysical characterization.

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**Keywords:** Aluminum hydroxide gel; Alhydrogel; Protein conformation; Protein secondary structure; FTIR; Hydrogen–deuterium exchange

Aluminum hydroxide gel (Alhydrogel) is one of the aluminum-containing adjuvants approved by the Food and Drug Administration for use in humans and has been formulated into various vaccine products for enhancing immunological effects, especially of protein antigens [1]. Structural studies of the adjuvant have shown that commercial Alhydrogel is a poorly soluble crystalline aluminum oxyhydroxide, mineralogically known as boehmite, which consists of corrugated sheets of octahedral aluminum [2–4]. The primary particles of aluminum hydroxide adjuvant are known to have needle-like or fibril morpholo-

gy, with a diameter of approximately 2 nm [3] and a high surface area [5,6]. The latter provides excellent adsorptive capability for protein antigens [7,8]. The adsorption of several proteins has been reported, including 1.6–3.1 mg/mg aluminum for bovine serum albumin (BSA)<sup>1</sup> [8–10], 1.9 mg/mg aluminum for  $\alpha$ -lactalbumin [11], 1.1 mg/mg aluminum for myoglobin [11], and 1.6–2.6 mg/mg aluminum for ovalbumin [9,12]. The mass of protein adsorbed

<sup>1</sup> Abbreviations used: BSA, bovine serum albumin; ATR–FTIR, attenuated total reflectance Fourier transform infrared; DSC, differential scanning calorimetric; rhIL-1ra, recombinant human interleukin-1 receptor antagonist; rhIgG1, a recombinant human immunoglobulin G1; sTNF-R1, soluble tumor necrosis factor receptor type-1; PBS, phosphate-buffered saline; FFT, fast Fourier transform; H–D, hydrogen–deuterium.

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to aluminum hydroxide adjuvant is influenced predominantly by electrostatic attractive forces; thus, it is affected by the isoelectric points and surface charges of proteins [10,13,14]. Hydrophobic forces are also thought to contribute to some degree to the adsorption of proteins to aluminum hydroxide gel [10].

Despite extensive studies of the mechanism [15–17], applications, and safety/toxicity [18,19] of aluminum hydroxide adjuvants, few studies have focused on the effects of adsorption on protein structure and stability due to the lack of suitable techniques. Using attenuated total reflectance Fourier transform infrared (ATR–FTIR) and fluorescence spectroscopic and differential scanning calorimetric (DSC) techniques, Jones and coworkers [9] reported that three model proteins—ovalbumin, lysozyme, and BSA—adsorbed to aluminum-containing adjuvants exhibited lower thermal stability than did their counterparts in aqueous solutions, indicating a destabilization of proteins' structures. However, no detailed structural information could be provided due to insufficient qualities of the ATR–FTIR spectra of the three model proteins. In earlier studies, it was reported that the secondary structures of proteins bound to silica were not grossly altered but had reduced thermal stabilities [20,21]. If the silica surface was modified to render it more hydrophobic, structural alterations were detectable. The extent of destabilization also increased as the hydrophobicity of the silica surfaces increased [20,21].

Because of the extensive use of aluminum salt adjuvants in human vaccine products, the importance of understanding the structure and stability of protein antigens when adsorbed onto them cannot be overemphasized. Unlike solid silica surfaces [20,21], the average dimensions of the primary crystallites of aluminum hydroxide, calculated at  $4.5 \times 2.2 \times 10.0$  nm along the crystallographic axes [6], are much smaller than most protein antigens. Furthermore, the surfaces of aluminum oxyhydroxide octahedral are hydrophilic in nature, and the particles are fully suspended in an aqueous environment [10,11,14]. Thus, the interaction model between the hydrophilic surfaces of the adjuvant and hydrophilic surfaces of proteins is not expected to cause structural perturbations of proteins. To test our hypothesis, we carried out comparative infrared spectroscopic studies of six model proteins: ovalbumin, cytochrome *c*,  $\alpha$ -chymotrypsinogen A, recombinant human interleukin-1 receptor antagonist (rhIL-1ra), a recombinant human immunoglobulin G1 (rhIgG1), and the 2.6 domain of the soluble tumor necrosis factor receptor type-1 (sTNF-R1, MW  $\sim$  10.5 kDa) in solution and adsorbed to Alhydrogel. In a newly developed procedure, the protein was bound to the Alhydrogel and concentrated by low-speed centrifugation to obtain a loosely packed protein–Alhydrogel complex. The new procedure reduced the initial concentration requirement for proteins in H<sub>2</sub>O-based solution to as low as 0.3 mg/ml and allowed collection of high-quality spectra using the conventional transmission method rather than

ATR–FTIR. The high-quality infrared spectral data presented here indicated clearly that the adsorption of six model proteins to aluminum hydroxide gel did not alter the secondary structures of the proteins.

## Materials and methods

### Materials

Cytochrome *c* (horse heart, C7752), ovalbumin (chicken egg white, A-5503),  $\alpha$ -chymotrypsinogen A (bovine pancreas, C4879), and deuterium oxide (99.9 atom% D) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and used without further purification. Pharmaceutical quality rhIL-1ra, IgG1, and sTNF-R1 were generous gifts from Amgen [22]. Alhydrogel (2%) is the product of Brenntag (Denmark).

### Sample preparation

The stock solutions of cytochrome *c*, ovalbumin, and  $\alpha$ -chymotrypsinogen A were prepared by dissolving lyophilized protein powders in phosphate-buffered saline (PBS, 10 mM phosphate buffer with 140 mM NaCl, pH 7.4) and filtering with 0.22- $\mu$ m syringe filters. rhIL-1ra was obtained as a concentrated protein solution (220 mg/ml) in 10 mM citrate/140 mM NaCl/0.5 mM EDTA (pH 6.5) and was dialyzed against PBS buffer (pH 7.4) overnight prior to use. IgG1 and sTNF-R1 were obtained as concentrated protein solutions in 20 mM sodium acetate (pH 5.0). The concentrations of protein stock solutions were determined by UV–vis spectroscopy using the extinction coefficients as follows:  $E_{410\text{ nm}} = 1.06 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  for ferricytochrome *c* [23],  $A_{280}^{1\%} = 6.9$  for ovalbumin,  $A_{282}^{1\%} = 20.0$  for  $\alpha$ -chymotrypsinogen A [24],  $A_{280}^{1\%} = 7.7$  for rhIL-1ra [25],  $A_{280}^{1\%} = 14.0$  for rhIgG1, and  $A_{280}^{1\%} = 11.4$  for sTNF-R1 as determined by the method described previously [26].

Aluminum hydroxide gel was washed with a fivefold volume of the respective buffer to be used for each protein and precipitated by centrifugation with a benchtop centrifuge at 5000 rpm for 2 min. After discarding the supernatant, the Alhydrogel was resuspended in the buffer to the original concentration (2%). Aliquots of protein stock solutions were diluted to 0.5 and 1.0 mg/ml and then mixed with aluminum hydroxide gel at a ratio of 0.1 ml aluminum hydroxide gel/ml protein solution and incubated at room temperature for 30 min. The protein–Alhydrogel mixtures were then precipitated with a benchtop centrifuge at 5000 rpm for 2 min. The adsorption of proteins onto aluminum hydroxide gel was confirmed spectroscopically by measuring the protein concentrations before and after Alhydrogel precipitation (data not shown).

### FTIR spectroscopy

Infrared spectra were measured using an ABB Bomem (Que., Canada) MB-series FTIR spectrometer.

For studying the structure of proteins adsorbed to the adjuvant, the protein–Alhydrogel was loaded into a Bio-Cell (Bio Tools) with  $\text{CaF}_2$  windows having a 6.5- $\mu\text{m}$  fixed path length well. For investigating protein structure in solution using the conventional method, the protein solutions were loaded into a liquid cell (P/H 2500) with  $\text{CaF}_2$  windows and a 6- $\mu\text{m}$  spacer. For each spectrum, a 128-scan interferogram was collected in a single-beam mode with a  $4\text{ cm}^{-1}$  resolution. The reference spectrum was recorded under identical scan conditions with aluminum hydroxide gel suspended and precipitated in corresponding medium in the cell. The protein spectra were obtained according to previously described criteria and water subtraction procedures [27,28]. If residual water vapor signals were present in the spectrum of protein, they were removed by subtracting the spectrum of gaseous water. Second-derivative spectra were obtained with a seven-point Savitsky–Golay derivative function. Second-derivative spectra were baseline corrected as described previously [29]. Final spectra were treated with a  $2\times$  fast Fourier transform (FFT) interpolate function and plotted with SigmaPlot 8.0 software (Systat Software).

#### Hydrogen–deuterium exchange

To eliminate any concentration-dependent differences, the protein sample for adsorption to aluminum hydroxide gel was prepared by adding 100  $\mu\text{l}$  of Alhydrogel (2%) to 1 ml of ovalbumin solution at the same concentration (15 mg/ml) as used for the solution sample and incubated for 30 min at room temperature. After discarding the supernatant, the precipitated ovalbumin–Alhydrogel complex was first rinsed with PBS and then resuspended into another 1-ml aliquot of the same buffer, followed by centrifugation and resuspension as described above to remove any unbound and loosely bound protein molecules. Hydrogen–deuterium (H–D) exchange experiments were carried out by mixing one part protein solution (15 mg/ml) with three parts  $\text{D}_2\text{O}$ ; in the case of protein adsorption to Alhydrogel, one part precipitated protein–Alhydrogel complex was mixed with three parts  $\text{D}_2\text{O}$ . The resulting samples were loaded into a liquid infrared cell with a 25- $\mu\text{m}$  spacer. The spectra were measured in a kinetic mode with a 32-scan average per subfile at 2-min intervals. A 40-s lag time between the start of sample mixing and spectral recording was observed. The spectrum of fully deuterated ovalbumin was obtained by dissolving the lyophilized protein powder in PBS– $\text{D}_2\text{O}$  buffer and incubating the sample at room temperature for 1 h, followed by incubation in a 50  $^\circ\text{C}$  water bath for 1 h. The fraction of unexchanged amide hydrogens ( $X$ ) was calculated using the equation [30]

$$X = (A_{\text{II}} - A_{\text{II}\omega}) / (A_{\text{I}\omega})$$

where  $A_{\text{I}}$  and  $A_{\text{II}}$  are the absorbance maxima of the amide I and amide II bands of ovalbumin exposed to  $\text{D}_2\text{O}$  solution, respectively;  $A_{\text{II}\omega}$  is the amide II absorbance maximum of fully deuterated ovalbumin; and  $\omega$  is the  $A_{\text{II}0}/A_{\text{I}0}$  ratio,

with  $A_{\text{II}0}$  and  $A_{\text{I}0}$  being the respective absorbance maxima for the amide II and amide I bands of the undeuterated ovalbumin in  $\text{H}_2\text{O}$ -based buffer.

## Results

To obtain high-quality infrared spectra of proteins in aqueous solution by the conventional FTIR spectroscopic method, high concentrations ( $>12\text{ mg/ml}$ ) of protein generally are required to compensate for the extremely thin cell path length used for spectral measurement. Fig. 1 presents a series of primary and second-derivative spectra of cytochrome *c*, free from interference of gaseous water, measured at 3, 6, 9, 12, and 15 mg/ml using the conventional method with a 6- $\mu\text{m}$  spacer. It clearly demonstrates that as the protein concentration decreases, the quality of the second-derivative spectra deteriorates as a result of the decreasing signal/noise ratio, especially in the protein conformation-sensitive amide I region. The second-derivative-enhanced band components in the amide I region distort as the interference of white noise intensifies; this would affect the accuracy of data interpretation involving the secondary structures and conformational changes of proteins.

The concentration requirement of the conventional method for high-quality spectral data has made FTIR spectroscopy useless in conformational studies of proteins at low concentrations such as those relevant to vaccine studies and poorly soluble protein therapeutics. It also makes the direct comparison between two commonly used

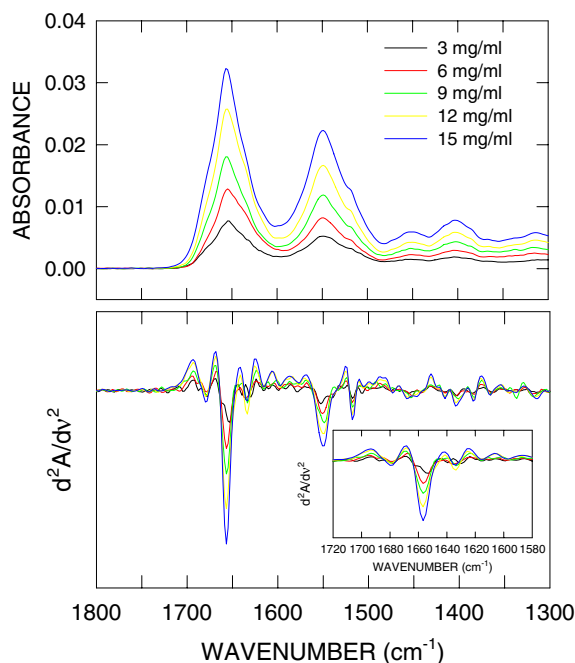


Fig. 1. High-quality primary and second-derivative spectra of cytochrome *c* acquired at 3, 6, 9, 12, and 15 mg/ml. All spectra are free from the interference of gaseous water. The spectrum of aqueous water was subtracted from the spectra of the protein using the procedure described in Materials and methods. The inset shows the second-derivative spectra of cytochrome *c* in the amide I region.

spectroscopic techniques, FTIR and circular dichroism spectroscopy, somewhat uncertain. To lower the protein concentration requirement without compromising the quality of the FTIR spectra, we developed a new procedure for concentrating protein from dilute samples that involves adsorbing proteins onto aluminum hydroxide adjuvant, concentrating the complex by centrifugation at low speed, and measuring the FTIR spectra of the protein–Alhydrogel complex in an aqueous environment.

Fig. 2 shows the second-derivative spectra of avian ovalbumin in the amide I region acquired with the conventional method at 15 mg/ml concentration and with the new Alhydrogel method at initial protein concentrations of 1.5, 1.0, 0.5, and 0.3 mg/ml. Avian ovalbumin contains both  $\alpha$ -helix (the band near  $1657\text{ cm}^{-1}$ ) and  $\beta$ -sheet structures (the bands near  $1638$  and  $1626\text{ cm}^{-1}$ ) in similar amounts [31]; thus, it serves as an excellent model protein for demonstrating the usefulness of the new procedure. This result clearly shows that ovalbumin molecules adsorbed onto the surface of aluminum hydroxide adjuvant retain their native conformation. In addition, it demonstrates the excellent quality of the second-derivative spectra for samples prepared at initial protein concentrations as low as 0.3 mg/ml, which are comparable to the spectrum obtained by the conventional method at 15 mg/ml concentration.

To determine whether or not the conformations of proteins with a broad range of secondary structural compositions are altered when adsorbed to aluminum hydroxide

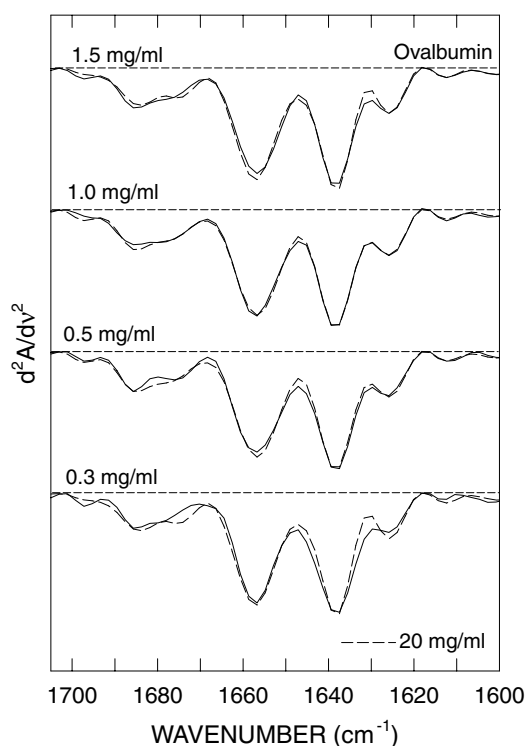


Fig. 2. Comparisons of the second-derivative spectra of ovalbumin in amide I region obtained at 15 mg/ml concentration by the conventional method and at 1.5, 1.0, 0.5, and 0.3 mg/ml concentrations by the Alhydrogel method.

adjuvant, we selected several proteins, including cytochrome *c*,  $\alpha$ -chymotrypsinogen A, rhIL-1ra, rhIgG1, and sTNF-R1, as model proteins and conducted detailed comparisons of the second-derivative spectra acquired at 15 mg/ml by the conventional method and at 0.5 and 1.0 mg/ml by the Alhydrogel method. This group of model proteins includes proteins with predominantly  $\alpha$ -helical (cytochrome *c*),  $\beta$ -sheet ( $\alpha$ -chymotrypsinogen A, rhIL-1ra, and rhIgG1), and random coil (sTNF-R1) structures.

Fig. 3 presents the second-derivative amide I spectra of cytochrome *c*,  $\alpha$ -chymotrypsinogen A, rhIL-1ra, rhIgG1, and sTNF-R1. For cytochrome *c*, IL-1RA, and rhIgG1, the spectra obtained for samples adsorbed onto the adjuvant from 0.5 and 1.0 mg/ml initial protein concentrations are nearly superimposable with the spectra acquired at 15 mg/ml in solutions, indicating similar secondary structures, types, and contents, for the proteins adsorbed to aluminum hydroxide adjuvant as their counterparts in solutions. Thus, for these proteins, there is no measurable alteration in the secondary structure when adsorbed to aluminum hydroxide adjuvant.

However, there are noticeable differences between the spectra acquired at different concentrations with or without

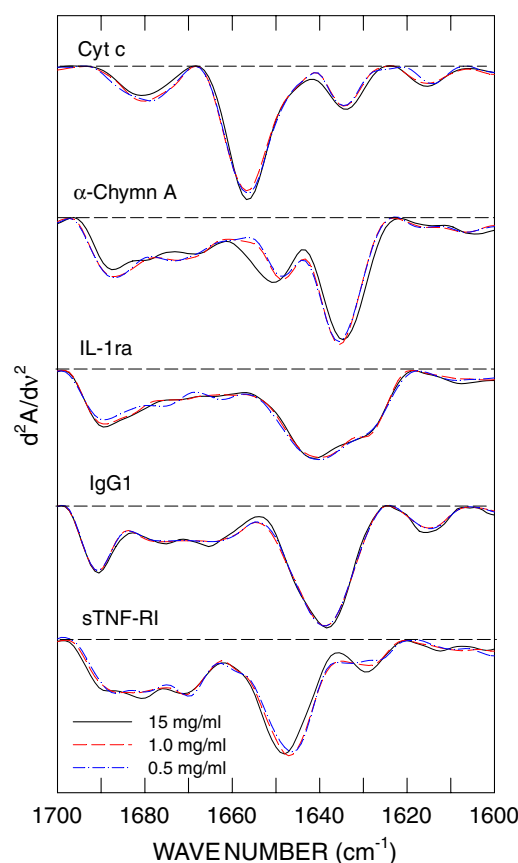


Fig. 3. Comparisons of the second-derivative spectra of cytochrome *c* (Cyt *c*),  $\alpha$ -chymotrypsinogen A ( $\alpha$ -Chymn A), rhIL-1ra (IL-1ra), rhIgG1 (IgG1), and sTNF-R1 in the amide I region obtained at 15 mg/ml concentration by the conventional method and at 0.5 and 1.0 mg/ml concentrations by the Alhydrogel method. rhIgG1 was in 20 mM sodium phosphate (pH 7.0), and sTNF-R1 was in 10 mM sodium acetate (pH 5.92).

adsorption to aluminum hydroxide adjuvant for  $\alpha$ -chymotrypsinogen A and, to a lesser degree, sTNF-R1. The spectral differences are observed at the region assigned to random coil ( $1648 \pm 2 \text{ cm}^{-1}$ ) for both  $\alpha$ -chymotrypsinogen A and sTNF-R1 and at the regions assigned to  $\beta$ -sheet structures (near  $1638$  and  $1690 \text{ cm}^{-1}$ ) for  $\alpha$ -chymotrypsinogen A (Fig. 3). Although those changes do not alter the assignments of the secondary structures, they do suggest somewhat different microenvironments being experienced by those structures. In the case of  $\alpha$ -chymotrypsinogen A, the band assigned to the random coil (unordered structure) is much narrower in the spectra measured at lower initial concentrations (0.5 and 1.0 mg/ml) adsorption onto Alhydrogel and has a lower frequency than that measured at a higher concentration (15 mg/ml) in solution. Furthermore, the spectral differences are highly reproducible; thus, it is unlikely that they were produced by experimental error.

To determine whether the differences in  $\alpha$ -chymotrypsinogen A structure were due to the initial low concentrations of protein used for the adsorbed samples, the structure of protein adsorbed to Alhydrogel from solution at 15 mg/ml was studied. After precipitating the aluminum hydroxide gel and removing supernatant, the protein–Alhydrogel complex was washed with PBS buffer to remove any unbound protein molecules in the solution. The upper panel of Fig. 4 presents the overlays of the second-derivative amide I spectra of  $\alpha$ -chymotrypsinogen A adsorption to Alhydrogel at 15 mg/ml and in solution at 15 mg/ml as well as the spectra of  $\alpha$ -chymotrypsinogen A adsorption to Alhydrogel from solutions initially containing 0.5 and 1.0 mg/ml protein. It shows that the spectra

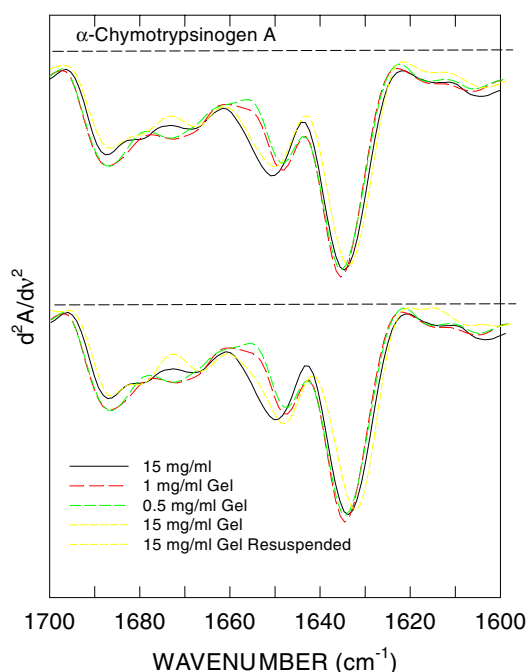


Fig. 4. Comparison of the second-derivative spectra of  $\alpha$ -chymotrypsinogen A acquired at 15 mg/ml concentration by both the conventional and Alhydrogel methods (upper panel) and effect of wash and resuspension (lower panel).

of the protein at 15 mg/ml with adsorption to Alhydrogel are nearly identical to those without adsorption to Alhydrogel. This result demonstrates unequivocally that the spectral differences between spectra measured at 15 mg/ml and at 0.5 and 1.0 mg/ml are due to a concentration-dependent conformational change in  $\alpha$ -chymotrypsinogen A, not to a protein–Alhydrogel interaction.

To determine whether the protein molecules adsorbed to aluminum hydroxide adjuvant at a high concentration (15 mg/ml) could change their conformation to that observed at lower concentrations, we resuspended the protein–Alhydrogel complex in PBS buffer for 1 h and then reconcentrated the protein–Alhydrogel complex for spectral analysis. The lower panel of Fig. 4 shows the comparison between the spectrum of the protein after resuspension and the spectra recorded at 15, 1, and 0.5 mg/ml. The second-derivative amide I spectrum of the resuspended protein revealed a transitional spectral pattern between the high and low concentrations. This observation suggests that the conformation of the protein molecules adsorption onto aluminum hydroxide adjuvant is flexible and that it changes slowly in response to changes in the environment. Alternatively, the protein may be in equilibrium with that in solution, and some of the protein may release from the Alhydrogel and then rebind with the conformation of the lower concentration protein.

So far, we have demonstrated that the adsorption onto aluminum hydroxide adjuvant does not significantly alter the native conformations of six model proteins with a broad range of secondary structural compositions. However, we wanted to determine whether the structural alteration by absorbing to aluminum hydroxide gel manifests in another process, namely H–D exchange. It has been reported previously that although ribonucleases A and S exhibited nearly identical second-derivative amide I spectral patterns, they revealed differences in their structural dynamics during H–D exchange [32]. We performed an H–D exchange experiment using ovalbumin as our model protein and compared the rates of amide proton exchange of the protein in solution and adsorption onto Alhydrogel.

Fig. 5 shows the overlays of selected primary spectra of ovalbumin recorded at 0, 2, 10, 20, 40, 60, and 120 min (starting time for data collection) immediately after mixing with  $\text{D}_2\text{O}$  at 1:3 ratios. Fig. 6 presents the second-derivative spectra in the amide I region calculated from the spectra shown in Fig. 5. The spectra in the upper panel are for ovalbumin in solution mixed with  $\text{D}_2\text{O}$ , and the spectra in the lower panel are for ovalbumin adsorbed onto Alhydrogel and mixed with  $\text{D}_2\text{O}$ . The spectra of the protein in  $\text{H}_2\text{O}$ -based buffer and in the fully deuterated form in  $\text{D}_2\text{O}$ -based buffer are included for comparison. As shown in Fig. 5, the protein amides I and II band maxima shifted to lower frequencies on mixing with  $\text{D}_2\text{O}$ . A time-dependent intensity change was observed between the amide II band in  $\text{H}_2\text{O}$  ( $\sim 1547 \text{ cm}^{-1}$ ) and the amide II' band in  $\text{D}_2\text{O}$  ( $\sim 1453 \text{ cm}^{-1}$ ), and this is typical for the exchange of amide protons to deuterons. The rates of amide proton

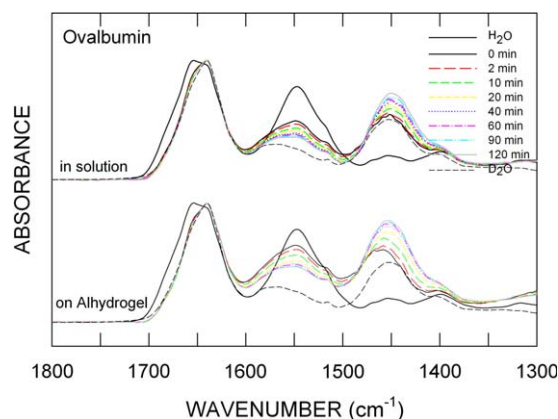


Fig. 5. H–D exchange of ovalbumin in solution or adsorption to aluminum hydroxide gel. The H–D exchange experiments were carried out by mixing the protein, in solution or adsorption to Alhydrogel, to D<sub>2</sub>O at a ratio of 1:3. The spectra of ovalbumin in PBS buffer and fully deuterated form in D<sub>2</sub>O-based buffer are included for comparison. The time points were referred to as scan starting times. There was a 40-s lag time between the start of H–D exchange and the first spectral measurement. The amide I absorbance maxima of all spectra were normalized to the same intensity for easier comparison. The preparation of ovalbumin–Alhydrogel is described in Materials and methods.

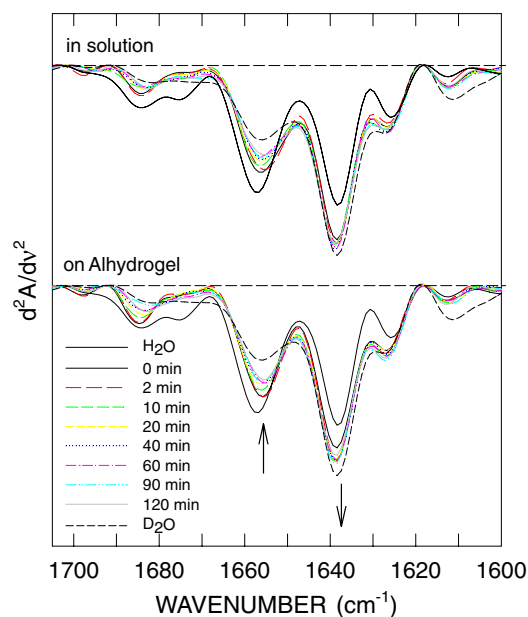


Fig. 6. Second-derivative amide I spectra of ovalbumin during H–D exchange as functions of time. The spectra were calculated from the primary spectra shown in Fig. 5. The spectra of ovalbumin in H<sub>2</sub>O-based buffer and fully deuterated in D<sub>2</sub>O-based buffer are included for comparison. The arrows present the direction of intensity change as a function of time.

exchange calculated from both the primary spectra and the second-derivative spectra show a slower exchange for the ovalbumin adsorbed to aluminum hydroxide gel than for the ovalbumin in solution (Fig. 7). The slower amide proton exchange rate for ovalbumin adsorption to Alhydrogel is expected due to the immobilization of the protein molecules on the surfaces of aluminum hydroxide particles. No sign of secondary structure abnormality, such as an

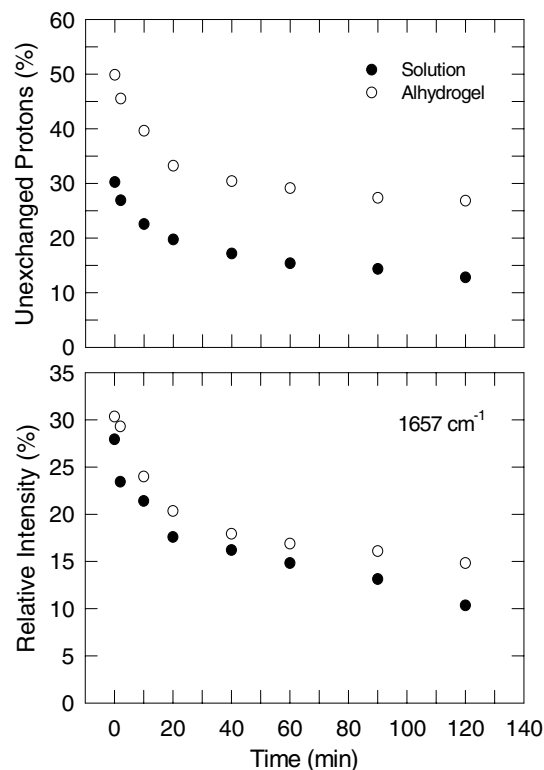


Fig. 7. Fraction of unchanged amide protons and relative intensity at 1657 cm<sup>-1</sup> for ovalbumin as a function of time at 22 °C. The fraction of unexchanged amide protons was calculated as described in Materials and methods. The relative intensity change at 1657 cm<sup>-1</sup> was calculated from the second-derivative spectra using the intensity in H<sub>2</sub>O as 100% and the intensity in the fully deuterated state in D<sub>2</sub>O as 0%.

elevated level of random coil structure, was observed in ovalbumin adsorption onto aluminum hydroxide gel other than the slower rate of amide proton exchange resulting from the protein immobilization. The amide proton exchange data further confirm our observation that adsorption onto aluminum hydroxide gel does not alter the native conformations of the model proteins.

It is noteworthy that because of the aluminum hydroxide gel precipitation step during the sample preparation, the Alhydrogel method does not reduce the total amount of protein required for each measurement to produce a high-quality infrared spectrum. The concentrations of proteins for the Alhydrogel experiments are the protein solution concentrations before adsorption onto aluminum hydroxide particles, not the actual concentrations for the spectral measurements. However, this method does effectively reduce the concentration requirement of the starting materials. From the experimental data currently available, the gel precipitation procedure does not alter the conformation of protein adopted at a given protein concentration when aluminum hydroxide adjuvant was applied.

## Discussion

The results presented in this study strongly suggest that the interactions between the aluminum hydroxide gel and

the six model proteins do not alter the secondary structures of these proteins. These results indicate that the interactions between the hydrophilic surfaces of aluminum hydroxide particles and globular protein may be similar to those between the hydrophilic surfaces of silica and the hydrophilic surfaces of proteins reported previously [20,21,33]. Although previous studies suggest that protein adsorption onto either material results in decreased thermal stability of the proteins [9,20,21,33], neither results in substantial alteration of protein secondary structure. Previous studies have indicated that the interactions between aluminum hydroxide particles and proteins are dominated by electrostatic attractive forces [10,13,14]. The small particle sizes, in nano scales, and full suspension in aqueous solution of the aluminum hydroxide adjuvant may also allow for binding to proteins without resulting in protein conformational changes.

The H–D exchange data for ovalbumin suggest that immobilization of protein molecules onto aluminum hydroxide gel particles retards the amide proton exchange of the protein. However, it does not prevent the amide proton exchange from taking place. The spectral data shown in Fig. 4 also suggest that there are some degrees of freedom for the adsorbed protein to change its conformation responding to the changes in the environment. These observations further strengthen our conclusion that the conformations of proteins adsorbed to aluminum hydroxide gel are in their native states.

Previously, ATR–FTIR spectroscopy was used to study three model proteins—ovalbumin, BSA, and lysozyme—adsorbed onto aluminum salt adjuvants [9]. The protein structural analysis by ATR–FTIR, however, has been known to suffer severe drawbacks, including nonproportionality and spectral distortion [34]. Even when caution is observed, the quality of the spectra obtained by ATR–FTIR might not be sufficient for further analysis by deconvolution, especially second-derivative analysis. Although lysozyme and BSA were not included in our model system, ovalbumin was used in both studies. We noticed that comparative structural analysis was not conducted in the earlier study due to insufficient quality of the ATR–FTIR spectra of samples adsorbed to the aluminum salt adjuvants. The conclusion that protein conformation was perturbed on adsorption onto aluminum hydroxide adjuvant was based mainly on a thermal denaturation studies. Although changes in thermal stability can be associated with changes in the conformation of a given protein [35], we did not see this in the current study. In addition, the decrease in thermal stability of ovalbumin adsorbed onto aluminum hydroxide in a heated aqueous environment might not result from structural destabilization of the protein; instead, it may result from potentially higher thermal stress experienced by protein molecules at the surfaces of crystals than in solution. Changes in thermal stability of ovalbumin adsorption onto aluminum hydroxide gel may be manifested in other ways. Further study on thermal stability of model proteins using the new procedure is needed.

FTIR spectroscopy is one of the widely used vibrational spectroscopic methods in protein structural analysis [28,36,37]. It is extremely sensitive to the conformational changes of proteins induced by various factors such as temperature, pH, and added chemicals and solvents. It has been used to study the secondary structure of proteins not only in the soluble native state [28,37,38] but also in precipitated states, both native (e.g., salted out) and denatured by various factors such as heat and chemical stresses [29,39–41]. However, to obtain high-quality FTIR spectra of proteins for detailed structural analysis, relatively high concentrations (>12 mg/ml in H<sub>2</sub>O-based solution) of protein samples are required for all commercial FTIR instruments. In comparison, other spectroscopic techniques, such as circular dichroism, typically use a sample concentration range of 0.1–2.0 mg/ml.

The high concentration requirement for the FTIR spectroscopic analysis of proteins is due primarily to the high absorbance of water in the amide I region and the requirement for extremely thin cell path lengths (6–10  $\mu\text{m}$ ). The thin cell path length is used to avoid the strong absorbance of the O–H bending mode of water molecules in the region between 1600 and 1700  $\text{cm}^{-1}$  [27,28], which overlaps with the conformation-sensitive amide I region of proteins [36,37]. The concentration requirement renders the technique unsuitable for the secondary structural and conformational studies of proteins in aqueous solution at low concentrations. The use of an ATR apparatus would not improve the quality of the spectra of proteins under these circumstances [34].

To overcome the shortcomings of FTIR spectroscopy in studies of low concentrations of proteins, we developed a new procedure by precipitating protein adsorbed to aluminum hydroxide gel using low-speed centrifugation and obtaining a loosely packed and fully hydrated protein–Alhydrogel complex. The new procedure effectively lowered the concentration requirement for FTIR studies of secondary structure and conformational changes of proteins in aqueous solutions by at least 40-fold as compared with the conventional FTIR method. It greatly expands the concentration range of FTIR spectroscopic applications of protein studies, thereby making it possible to study concentration-dependent conformational changes of proteins. Most important, the new procedure permits FTIR measurement of protein samples to be carried out in the same concentration range as those used by circular dichroism and fluorescence, thereby making it possible to directly cross-reference among three commonly used techniques in protein studies.

## References

- [1] R. Edelman, Vaccine adjuvants, *Rev. Infect. Dis.* 2 (1980) 370–383.
- [2] F.R. Vogel, M.F. Powell, A compendium of vaccine adjuvants and excipients, *Pharm. Biotechnol.* 6 (1995) 141–228.
- [3] S. Shirodkar, R.L. Hutchinson, D.L. Perry, J.L. White, S.L. Hem, Aluminum compounds used as adjuvants in vaccines, *Pharm. Res.* 7 (1990) 1282–1288.

- [4] K. Damodaran, P.R. Rajamohanam, D. Chakrabarty, U.S. Racherla, V. Manohar, C. Fernandez, J.P. Amoureux, S. Ganapathy, Triple-quantum magic angle spinning (27)Al NMR of aluminum hydroxides, *J. Am. Chem. Soc.* 124 (2002) 3200–3201.
- [5] J.D. Kubicki, S.E. Apitz, Molecular cluster models of aluminum oxide and aluminum hydroxide surfaces, *Am. Mineral.* 83 (1998) 1054–1066.
- [6] C.T. Johnston, S.L. Wang, S.L. Hem, Measuring the surface area of aluminum hydroxide adjuvant, *J. Pharm. Sci.* 91 (2002) 1702–1706.
- [7] H. Masood, J.L. White, S.L. Hem, Relationship between protein adsorptive capacity and the X-ray diffraction pattern of aluminum hydroxide adjuvants, *Vaccine* 12 (1994) 187–189.
- [8] S.J. Seeber, J.L. White, S.L. Hem, Predicting the adsorption of proteins by aluminium-containing adjuvants, *Vaccine* 9 (1991) 201–203.
- [9] L.S. Jones, L.J. Peek, J. Power, A. Markham, B. Yazzie, C.R. Middaugh, Effects of adsorption to aluminum salt adjuvants on the structure and stability of model protein antigens, *J. Biol. Chem.* 280 (2005) 13406–13414.
- [10] R.H. al-Shakhshir, F.E. Regnier, J.L. White, S.L. Hem, Contribution of electrostatic and hydrophobic interactions to the adsorption of proteins by aluminium-containing adjuvants, *Vaccine* 13 (1995) 41–44.
- [11] J.M. Heimlich, F.E. Regnier, J.L. White, S.L. Hem, The in-vitro displacement of adsorbed model antigens from aluminum-containing adjuvants by interstitial proteins, *Vaccine* 17 (1999) 2873–2881.
- [12] J.V.J. Rinella, J.L. White, S.L. Hem, Effect of anions on model aluminum-adjuvant-containing vaccines, *J. Colloid Interface Sci.* 172 (1995) 121–130.
- [13] R.H. al-Shakhshir, F.E. Regnier, J.L. White, S.L. Hem, Effect of protein adsorption on the surface charge characteristics of aluminium-containing adjuvants, *Vaccine* 12 (1994) 472–474.
- [14] M.F. Chang, J.L. White, S.L. Nail, S.L. Hem, Role of the electrostatic attractive force in the adsorption of proteins by aluminum hydroxide adjuvant, *PDA J. Pharm. Sci. Technol.* 51 (1997) 25–29.
- [15] J.L. Grun, P.H. Maurer, Different T helper cell subsets elicited in mice utilizing two different adjuvant vehicles: the role of endogenous interleukin 1 in proliferative responses, *Cell. Immunol.* 121 (1989) 134–145.
- [16] M. Ulanova, A. Tarkowski, M. Hahn-Zoric, L.A. Hanson, The common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism, *Infect. Immunol.* 69 (2001) 1151–1159.
- [17] J.M. Brewer, M. Conacher, C.A. Hunter, M. Mohrs, F. Brombacher, J. Alexander, Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling, *J. Immunol.* 163 (1999) 6448–6454.
- [18] P.O. Ganrot, Metabolism and possible health effects of aluminum, *Environ. Health Perspect.* 65 (1986) 363–441.
- [19] R.K. Gupta, E.H. Relyveld, E.B. Lindblad, B. Bizzini, S. Ben-Efraim, C.K. Gupta, Adjuvants: a balance between toxicity and adjuvanticity, *Vaccine* 11 (1993) 293–306.
- [20] D.T. Brandau, E.Q. Lawson, C.F. Schubert, N.K. Day, K. Matsuno, C.R. Middaugh, The interaction of cryoimmunoglobulins with a model surface, *Mol. Immunol.* 28 (1991) 1019–1026.
- [21] K. Matsuno, R.V. Lewis, C.R. Middaugh, The interaction of gamma-crystallins with model surfaces, *Arch. Biochem. Biophys.* 291 (1991) 349–355.
- [22] B.A. Kerwin, B.S. Chang, C.V. Gegg, M. Gonnelli, T. Li, G.B. Strambini, Interactions between PEG and type I soluble tumor necrosis factor receptor: modulation by pH and by PEGylation at the N-terminus, *Protein Sci.* 11 (2002) 1825–1833.
- [23] J. Babul, E. Stellwagen, Participation of the protein ligands in the folding of cytochrome *c*, *Biochemistry* 11 (1972) 1195–1200.
- [24] D.M. Abradi, P.E. Wilcox, Chemical derivatives of  $\alpha$ -chymotrypsinogen: III. Reaction with *N*-acetyl-DL-homocysteine thiolactone, *J. Biol. Chem.* 235 (1960) 396–404.
- [25] B.S. Kendrick, B.S. Chang, T. Arakawa, B. Peterson, T.W. Randolph, M.C. Manning, J.F. Carpenter, Preferential exclusion of sucrose from recombinant interleukin-1 receptor antagonist: role in restricted conformational mobility and compaction of native state, *Proc. Natl. Acad. Sci. USA* 94 (1997) 11917–11922.
- [26] C.N. Pace, F. Vajdos, L. Fee, G. Grimsley, T. Gray, How to measure and predict the molar absorption coefficient of a protein, *Protein Sci.* 4 (1995) 2411–2423.
- [27] A. Dong, W.S. Caughey, Infrared methods for study of hemoglobin reactions and structures, *Methods Enzymol.* 232 (1994) 139–175.
- [28] A. Dong, P. Huang, W.S. Caughey, Protein secondary structures in water from second-derivative amide I infrared spectra, *Biochemistry* 29 (1990) 3303–3308.
- [29] A. Dong, S.J. Prestrelski, S.D. Allison, J.F. Carpenter, Infrared spectroscopic studies of lyophilization- and temperature-induced protein aggregation, *J. Pharm. Sci.* 84 (1995) 415–424.
- [30] A.D. Barksdale, A. Rosenberg, Acquisition and interpretation of hydrogen exchange data from peptides, polymers, and proteins, *Methods Biochem. Anal.* 28 (1982) 1–113.
- [31] A. Dong, J.D. Meyer, M.C. Manning, J.F. Carpenter, Comparative FTIR and CD spectroscopic analysis of alpha 1-proteinase inhibitor and ovalbumin in aqueous solution, *Arch. Biochem. Biophys.* 383 (2000) 148–155.
- [32] A. Dong, R.M. Hyslop, D.L. Pringle, Differences in conformational dynamics of RNase A and S as observed by infrared spectroscopy and hydrogen–deuterium exchange, *Arch. Biochem. Biophys.* 333 (1996) 275–281.
- [33] G.E. Katzenstein, S.A. Vrona, R.J. Wechsler, B.L. Steadman, R.V. Lewis, C.R. Middaugh, Role of conformational changes in the elution of proteins from reversed-phase HPLC columns, *Proc. Natl. Acad. Sci. USA* 83 (1986) 4268–4272.
- [34] M.E. Goldberg, A.F. Chaffotte, Undistorted structural analysis of soluble proteins by attenuated total reflectance infrared spectroscopy, *Protein Sci.* 14 (2005) 2781–2792.
- [35] A. Dong, T. Randolph, J.F. Carpenter, Entrapping intermediates of thermal aggregation in  $\alpha$ -helical proteins with low concentration of guanidine–hydrochloride, *J. Biol. Chem.* 275 (2000) 27689–27693.
- [36] S. Krimm, J. Bandekar, Vibrational spectroscopy and conformation of peptides, polypeptides, and proteins, *Adv. Protein Chem.* 38 (1986) 181–364.
- [37] H. Susi, D.M. Byler, Resolution-enhanced Fourier transform infrared spectroscopy of enzymes, *Methods Enzymol.* 130 (1986) 291–311.
- [38] W.K. Surewicz, H.H. Mantsch, New insight into protein secondary structure from resolution-enhanced infrared spectra, *Biochim. Biophys. Acta* 952 (1988) 115–130.
- [39] S.D. Allison, A. Dong, J.F. Carpenter, Counteracting effects of thiocyanate and sucrose on chymotrypsinogen secondary structure and aggregation during freezing, drying, and rehydration, *Biophys. J.* 71 (1996) 2022–2032.
- [40] Y. Kim, J.S. Wall, J. Meyer, C. Murphy, T.W. Randolph, M.C. Manning, A. Solomon, J.F. Carpenter, Thermodynamic modulation of light chain amyloid fibril formation, *J. Biol. Chem.* 275 (2000) 1570–1574.
- [41] A.A. Ismail, H.H. Mantsch, P.T.T. Wong, Aggregation of chymotrypsinogen: portrait by infrared spectroscopy, *Biochim. Biophys. Acta* 1121 (1992) 183–188.