EFFECTS OF ADSORPTION TO ALUMINUM SALT ADJUVANTS ON THE STRUCTURE AND STABILITY OF MODEL PROTEIN ANTIGENS

LaToya S. Jones†, Laura J. Peek*, Jonathan Power, Aaron Markham, Brian Yazzie and C. Russell Middaugh
*These authors contributed equally to this work.

From the Department of Pharmaceutical Sciences, University of Colorado Health Sciences Center; E-Mail: LaToya.Jones@UCHSC.edu

† Current address: Department of Pharmaceutical Sciences, University of Colorado Health Sciences Center; E-Mail: LaToya.Jones@UCHSC.edu

The effect of adsorption onto aluminum salt adjuvants on the structure and stability of three model protein antigens was studied using fluorescence and FTIR spectroscopies, as well as isothermal titration and differential scanning calorimetric techniques. Lysozyme was preferentially adsorbed to aluminum phosphate (Adju-Phos®) while ovalbumin and bovine serum albumin (BSA) were better adsorbed to aluminum hydroxide (Alhydrogel®). A linearized Langmuir adsorption isotherm was used to obtain information regarding the binding interactions between proteins and adjuvants. Binding energetics and stoichiometry data obtained from ITC measurements were complex. Based on the spectroscopic and DSC studies, the structure of all three proteins when adsorbed to the surface of an aluminum salt was altered in such a way to render the proteins less thermally stable. Besides the pharmaceutical significance of this destabilization, we consider the possibility that this phenomenon may facilitate the presentation of antigens and thus contribute to the adjuvant activity of the aluminum salts.

The interaction of proteins with surfaces is a widely recognized phenomenon of both physiological and technological significance. A particularly important example is the binding of protein antigens to aluminum salt adjuvants. Without the use of such adjuvants, proteins are usually only weakly immunogenic. The aluminum salts are currently the only such agents generally approved for use in vaccines for humans (1). Despite this widespread use, their mechanism of action is still poorly understood. Among a variety of non-mutually exclusive proposed mechanisms, roles as depots for antigen, induction of inflammatory responses and delivery of antigen into antigen presenting cells, are commonly proposed, with some evidence for each (2, 3).

The two most common aluminum salts employed as adjuvants are the phosphate and hydroxide forms. The salts themselves have been well characterized with aluminum hydroxide (Alhydrogel®) usually found in a crystalline state (4, 5) while aluminum phosphate exists in an amorphous form (5, 6). The points of zero charge (PZC, analogous to a macromolecule’s isoelectric point) are 4.0-5.5 and approximately 11 for the phosphate and hydroxide salts, respectively. In general, proteins seem to better adsorb to the oppositely charged salts through simple electrostatic effects (7) although apolar and ion displacement interactions may play a role as well (8).

What happens to the structure and stability of proteins when they are adsorbed onto the surface of the two commonly employed aluminum salts has not been well characterized. When proteins are adsorbed to solid surfaces, highly polar (including charged) interfaces tend to minimally perturb protein structure and stability, although exceptions are known. In contrast, more apolar surfaces often significantly alter the structure and stability of many proteins (9-12). The effect of the aluminum salts on protein
structure and stability is important from several perspectives. For example, to the extent that epitopes are conformational in nature, their retention (or alteration) may be critical for vaccine immunogenicity. The stability of protein antigens is equally important when they are stored for long periods prior to their use. The latter strongly impacts the utility of vaccines for use in the developing world where shipping under cold conditions can be problematic (13) and the use of vaccines against potential bioterrorism agents where storage for long periods in centralized locations may be critical for their effectiveness (14).

Therefore, in this study we have examined the effect of aluminum salts on the structure and stability of three proteins chosen to serve as models of typical protein antigens. Their interaction with both salts was tested and binding isotherms constructed for the better binding surface. The thermodynamics of this interaction were then characterized by isothermal titration calorimetry. Potential changes in structure of the three proteins were probed by front face fluorescence (to examine tertiary structure) and attenuated total reflectance Fourier transform infrared spectroscopy (to probe potential changes in secondary structure). Their thermal stability was then compared on and off the surface using the same two detection methods. In addition, differential scanning calorimetry was employed for the same purpose. The surprising finding is that the interactions of the proteins with the highly charged aluminum salts generally destabilized the proteins. The practical implications of these results are discussed and a potential significance for the mechanism of adjuvant action briefly considered.

EXPERIMENTAL PROCEDURES

Materials – Proteins (lysozyme, ovalbumin, and bovine serum albumin), MOPS, and MOPS sodium salt were purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification unless otherwise noted. Aluminum hydroxide adjuvant (Alhydrogel®, 2%) and aluminum phosphate adjuvant (Adju-Phos®) were purchased from Accurate Chemical and Scientific Corporation (Westbury, NY). Dialysis cassettes (10,000 MWCO) were obtained from Pierce Chemical Company (Rockford, IL). Distilled deionized water was used to prepare the buffer.

Preparation of protein stock solutions – Protein stock solutions were prepared in 10 mM MOPS (pH 7.4) buffer. Their concentrations were determined using published extinction coefficients and A280 measurements.

Binding isotherms – Protein working solutions in multiple concentrations were prepared at a concentration twice that of the desired final protein concentration. A stock adjuvant solution was prepared by combining the standard aluminum salt suspension with an appropriate volume of 10 mM MOPS (pH 7.4) buffer such that the final calculated aluminum concentration was 3.4 mg/ml. The calculation of aluminum concentration used data provided by the supplier in the lot specification sheet.

Equal volumes of the protein working solution and the adjuvant working solution were combined in 1.5 ml microcentrifuge tubes. Based on preliminary binding studies, Alhydrogel® was used with BSA and ovalbumin solutions, while Adju-Phos® was added to lysozyme solutions. The suspensions were mixed gently by end-over-end rotation at 4 °C for 15-30 minutes. (A previous study of the kinetics of adsorption indicated that protein adsorption was complete within this time period.) The samples were then centrifuged at a force of 14,000 g for 2-5 minutes. The supernatant was assayed for protein concentration by measuring the absorbance at 280 nm. The amount of protein bound was determined by subtracting the amount of protein remaining in solution from the amount introduced initially.

Isothermal titration calorimetry – Isothermal titration calorimetry studies were conducted by titrating protein solutions or buffer into a sample cell containing the adjuvant. Titrations were performed in triplicate using a CSC model 4200 isothermal titration calorimeter (Calorimetry Sciences Corporation, American Fork, Utah). All protein solutions were dialyzed into 10 mM MOPS (pH 7.4) buffer, and the dialysis buffer was used in preparation of the adjuvant solutions. The adjuvant stock suspensions were diluted with dialysate buffer to yield the desired aluminum concentration. For lysozyme titrated into Adju-Phos®, two lysozyme solutions were necessary, the concentrations of
which were 13 and 10 mg/ml. The corresponding Adju-Phos® concentrations were 1.0 and 0.4 mg Al/ml, respectively. The titration of BSA into Alhydrogel® employed 19 mg/ml BSA and 1 mg Al/ml of Alhydrogel®. Ovalbumin at 13 mg/ml was titrated into 1.8 mg Al/ml of Alhydrogel®. All titrations were conducted at 25 ºC. A 10 µl injection volume was employed and the sample equilibrated for 300 sec between injections. The adjuvant suspension in the sample cell was stirred at a constant speed of 300 rpm while the sample reached thermal equilibrium prior to titration and during titration of the protein or buffer.

The ITC data was analyzed using BindWorks™ (Calorimetry Sciences Corporation, American Fork, Utah). Prior to fitting the data to appropriate binding isotherms, heats of dilution were subtracted from all protein titration data. For BSA and ovalbumin, the average heat of the buffer titration was subtracted from all heats observed during titration of the protein. The heat of the final injection of lysozyme into Adju-Phos® was used to correct the lysozyme data for dilution heat contributions. The data for each protein/adjuvant system was fit to a model of multiple independent binding sites.

**Differential scanning calorimetry** – Differential scanning calorimetry (DSC) was used to determine the thermal transition temperatures (T_m) of each protein in solution and adsorbed onto an aluminum salt adjuvant. A high-throughput capillary differential scanning calorimeter (MicroCal LLC, Northampton, MA) was used for the measurements. Samples containing protein completely adsorbed onto the surface of the adjuvant were prepared as described for the protein adsorption study. DSC experiments were performed using lysozyme, ovalbumin and BSA in 10 mM MOPS (pH 7.4) buffer at 0.5, 1.0 and 1.0 mg/ml protein, respectively. For DSC studies in the presence of adjuvant, 1 mg Al/ml Adju-Phos® and 4 mg Al/ml Alhydrogel® were employed. It was necessary to increase the BSA concentration to 2 mg/ml for DSC investigations of the adsorbed protein, while 0.5 mg/ml lysozyme and 1.0 mg/ml ovalbumin were sufficient. Samples and reference buffer solutions were loaded in triplicate into a 96-well plate, which was thermostated at 10 ºC. An autosampler equipped with a 2.5 ml Hamilton GASTIGHT® syringe was utilized to load the DSC cells and clean the cells between experiments.

Samples were mixed by loading and expelling the syringe three times to ensure that the suspensions were well dispersed. A scan rate of 90 ºC/hour was used to scan from 10 ºC to 90 ºC for lysozyme and 10 ºC to 100 ºC for BSA and ovalbumin. Buffer and adjuvant samples were examined separately to evaluate the independent behavior of each.

DSC data was analyzed using Origin v. 7.0 (OriginLab Corporation, Northampton, MA). The thermogram for buffer alone was subtracted from the thermograms of the proteins in solution prior to further processing. Similarly, the thermogram of the appropriate adjuvant alone was subtracted from the thermograms of the adsorbed proteins. Lysozyme thermograms were analyzed using a single peak, 2-state model. For BSA and ovalbumin, both in solution and in the case of ovalbumin adsorbed to Alhydrogel®, transition temperatures were determined by fitting the data to a 2-state model using two peaks. A six peak model provided the best fit for BSA adsorbed to Alhydrogel®.

**Fluorescence spectroscopy** – Fluorescence spectroscopy employing front-face sampling geometry was used to probe changes in tertiary structure of the protein as a function of temperature for each protein in solution as well as adsorbed to an adjuvant. This geometry was necessary because of the opacity of the adjuvant suspensions. For this purpose, right angle triangular cuvettes were used. Samples of protein adsorbed onto the adjuvant were prepared as described previously. For all studies, a protein concentration of 0.2 mg/ml was used. For samples containing adjuvant, the aluminum concentration was 1.7 mg/ml. Samples containing adjuvant were allowed to settle in the cuvettes for approximately 16 hours at 4 ºC to minimize variability due to settling of the adjuvant during the course of the experiment. An excitation wavelength of 280 nm was employed, and emission spectra were recorded from 290 nm to 400 nm using a QuantaMaster spectrofluorometer equipped with a Peltier thermostatted cuvette holder (Photon Technologies International, Lawrenceville, NJ). Data were collected every 1 nm at a scanning rate of 1 nm/s. Full spectra were obtained every 2.5 ºC from 10 to 90 ºC with 300 s of thermal equilibration at each temperature. The data was processed using Origin v. 7.0. Initially, an 11-
point Savitsky-Golay smooth was applied to all spectra. The peak positions were then determined using first derivative analysis.

**FTIR spectroscopy** – The secondary structure of the proteins in solution and adsorbed onto the surface of the adjuvants was assessed using Fourier transform infrared (FTIR) spectroscopy. Samples containing an adjuvant were prepared as described previously for protein adsorption studies. The concentration of lysozyme, ovalbumin and BSA in 10 mM MOPS (pH 7.4) buffer employed in solution studies was 17.7, 20.5 and 24.0 mg/ml, respectively. For lysozyme adsorbed to Adju-Phos®, the protein and aluminum concentrations were 2 and 4 mg/ml, respectively. The concentrations of BSA and ovalbumin adsorbed to Alhydrogel® (4 mg/ml aluminum) were 5.8 and 4.4 mg/ml, respectively. The amount of protein adsorbed to the adjuvant was determined by measuring A280 of the supernatant following centrifugation. The supernatant was then removed and discarded. The remaining slurry was smeared uniformly onto a ZnSe ATR crystal. An attenuated total reflectance (ATR) cell using a 45° ZnSe trough plate with volatile liquid cover was employed. Data were collected using 256 scans with a resolution of 4 cm⁻¹ with a Nicolet Magna 560 FTIR spectrometer equipped with an MCT/A detector. A thermal A.R.K. temperature controller (Spectra-Tech, Inc., Shelton, CT) was used to vary the temperature every 5 °C from 25 to 75 °C. Samples were equilibrated for seven minutes at each temperature. In addition to samples containing protein, thermal melt spectra were acquired for 10 mM MOPS (pH 7.4) buffer and adjuvant alone.

For spectra of proteins in solution, buffer spectra at each temperature were subtracted using Omnic® v. 4.1A software (Nicolet, Madison, WI). In the case of samples containing an adjuvant, spectra of the adjuvant alone at each temperature were subtracted from the spectra of protein adsorbed to the adjuvant. The subtracted data were then baseline-corrected and zeroed between 1800 and 2400 cm⁻¹. A Savitsky-Golay smoothing function was applied to each spectrum using 7-13 points, depending on the quality of the data. Smoothed data was copied into Excel, and the ratio of absorbances at two frequencies (see below) was calculated.

**RESULTS**

**Thermodynamics of protein adsorption** – The amount of each protein that can be adsorbed to selected aluminum salt adjuvants was determined by constructing adsorption isotherms (Figure 1A-C). In 10 mM MOPS (pH 7.4) buffer, all proteins adsorbed to the predicted (based on the protein’s pI and adjuvant’s PZC) aluminum salt adjuvant. Lysozyme (pI ~11.0) adsorbed to Adju-Phos® while BSA (pI ~4.9) and ovalbumin (pI ~4.6) adsorbed onto Alhydrogel®. There was no significant adsorption of lysozyme onto Alhydrogel® nor ovalbumin and BSA onto Adju-Phos® under the same conditions (data not shown).

The adsorption data was analyzed by linearizing the adsorption isotherms as previously described (Table 1) (7, 15). The adsorptive capacity is a measure of the maximum mass of protein adsorbed per mass of aluminum. The adsorptive coefficient describes the strength of the interaction. The adsorptive capacity and adsorptive coefficient of the lysozyme/Adju-Phos® complex was the smallest at 1.4 mg lysozyme/mg aluminum and 0.47 ml/mg, respectively. At the other extreme was BSA/Alhydrogel®, having an adsorptive capacity of 2.2 mg BSA/mg aluminum and an adsorptive coefficient of 3.1 ml/mg. The adsorptive capacity and coefficient of the ovalbumin/Alhydrogel® system are 1.6mg/mg and 1.6 ml/mg, respectively. The experimental values for the adsorptive capacities agree well with previously published adsorptive capacities for the same protein/adjuvant combinations in water (7, 16). The experimental adsorptive coefficients were lower than those previously published (7, 16). Iyer et al, however, have reported variations over several orders of magnitude for adsorptive coefficients for the same combinations of protein and adjuvant (16).

The interactions between the proteins and aluminum salts were also characterized by ITC (Table 2). The binding stoichiometry determinations are roughly similar to the adsorptive capacities calculated from the linearized adsorption isotherms. The binding constants, K, are analogous to the adsorptive coefficients and show similar relationships among the three proteins with the highest value being for...
the BSA/Alhydrogel® system and the lowest for lysozyme/Adju-Phos®.

The binding enthalpy and free energy for each protein/adjuvant pair were directly determined from the data and were used to calculate corresponding entropy changes (Table 2). The interaction between BSA and Alhydrogel® is entropically driven while being enthalpically unfavorable. The opposite is observed for the binding between ovalbumin and Alhydrogel®. For the lysozyme/Adju-Phos® system, both enthalpy and entropy contribute favorably to the interaction.

Differential scanning calorimetry – Differential scanning calorimetry was used to investigate the effect of adsorption on the thermal stability of the proteins. Differences in T_m’s and thermograms are observed when the proteins are adsorbed onto the adjuvant in comparison to the protein alone in solution under the same conditions (Table 3 and Figures 2A-2C). The T_m of lysozyme is decreased by more than 10 ºC (i.e., from ~73 ºC to ~59 ºC) when the protein is adsorbed onto the surface of Adju-Phos®. In both situations, however, there is only a single transition observed (Figures 2.A.i. and 2.A.ii.). Although the T_m’s for ovalbumin in solution and adsorbed to Alhydrogel® are essentially identical, the transition profile is significantly altered (Table 3 and Figures 2.B.i. and 2.B.ii.). The thermograms of both ovalbumin samples were best modeled by two component transitions centered at approximately 70 and 78 ºC. In the absence of the adjuvant, the 78 ºC peak is clearly dominant (Figure 2.B.i.). When the protein is adsorbed onto Alhydrogel®, however, there is a larger contribution from the lower peak (T_m ~ 70 ºC) (Figure 2.B.ii.). The thermodynamic stability of BSA is the most profoundly perturbed by adsorption to an adjuvant (Table 3 and Figures 2.C.i. and 2.C.ii.). Unbound BSA has a major transition centered at 78 ºC (Figure 2.C.i.). When the protein is adsorbed onto Alhydrogel®, there are multiple transitions now present underlying a large, broad peak spanning from approximately 22 to beyond 85 ºC (Figure 2.C.ii.).

Tertiary structure stability—Front surface fluorescence spectroscopy was used to assess changes in tertiary structure of the model protein antigens adsorbed to the aluminum salts. At 10 ºC, the position of the emission maximum for lysozyme in solution is at 337.5 nm, indicating that on average, the tryptophan residues are substantially buried (Figure 3.A.i.). When the protein is adsorbed onto the surface of Adju-Phos® at 10 ºC, there is a very small blue shift in the emission maximum. As the temperature is increased, the peak position of the protein in solution remains essentially unchanged up to 65 ºC, at which point the peak is significantly red-shifted. In contrast, the fluorescence emission peak position of the adsorbed lysozyme gradually increases from 10 to 50 ºC, at which point there is a significant increase in the slope of the change. Overall, the data suggest that the tertiary structure of lysozyme is perturbed upon adsorption to Adju-Phos®, which results in a major decrease in the thermal stability of the protein. These changes are much less evident in the intensity changes of the emission peak (Figure 3.A.ii.).

Like lysozyme, ovalbumin’s tryptophan residues also exist in a relatively apolar environment when the protein is in solution (emission max at 332 nm) (Figure 3.B.i). When ovalbumin is adsorbed onto the surface of Alhydrogel®, there is again a slight blue shift in the fluorescence emission peak position at 10 ºC (to 331 nm). In the presence of the adjuvant, the emission peak position remains essentially unchanged between 10 and 90 ºC. This would seem to imply that the tertiary structure of ovalbumin is stabilized on the surface of the adjuvant. When the fluorescence intensities at 332 and 331 nm of ovalbumin in the absence and presence of the adjuvant, are compared however, as a function of temperature, the opposite conclusion is suggested (Figure 3.B.ii.). The change in slope at approximately 75 ºC seen for the protein in solution coincides with the transition observed in the emission peak position curve. In contrast, there is a sharp decrease in fluorescence intensity observed at 25 ºC for the adsorbed ovalbumin. Thus, surface binding seems to permit a thermally induced event in the bound protein not seen in the protein in solution.

BSA in solution possesses an emission maximum at approximately 338 nm at 10 ºC (Figure 3.C.i.). When BSA is adsorbed onto Alhydrogel®, this peak is blue shifted to 327 nm and remains at this position from 10-90 ºC. With increasing temperature, the fluorescence emission peak of BSA in solution exhibits a blue-shifted
transition region above 35 °C. The emission peak of this protein in the post-transition region is near 327 nm, the same position seen for adsorbed BSA at all temperatures. This suggests that the post-transition state of BSA in solution may be similar to the conformational state of the protein adsorbed to Alhydrogel®. Abolition of a transition in the adsorbed protein is seen in plots of fluorescence intensity versus temperature as well (Figure 3.C.ii.).

**Secondary structure stability**—ATR-FTIR was employed to monitor changes in secondary structure of the model proteins in solution and adsorbed to aluminum salt adjuvants as a function of temperature. Potential alterations to the protein’s secondary structure were monitored in the Amide I region (1600-1700 cm⁻¹) of FTIR spectra. For lysozyme in solution, a shoulder begins to appear at ~1624 cm⁻¹ as the temperature is increased (Figure 4A). This suggests increased β-sheet structure (relative to α-helix) forming with increasing temperature. When adsorbed to Adju-Phos® (Figure 4B), the spectrum of lysozyme is significantly altered compared to the solution spectrum. The 1653 cm⁻¹ (α-helix) peak present in the spectra of lysozyme in solution appears to have shifted to ~1640 cm⁻¹ suggesting an increase in β-sheet structure at the expense of the α-helix upon adsorption. As the temperature increases, a sharper peak begins to emerge at ~1640 cm⁻¹ for the adsorbed protein. At 65 °C, a second peak appears at ~1620 cm⁻¹, suggesting the presence of intermolecular β-sheet due to aggregation. At higher temperatures, this peak dominates the 1640 cm⁻¹ signal. This is the same position at which the shoulder gradually emerges for lysozyme in solution. A plot of the ratio of absorbances at 1620 cm⁻¹ and 1640 cm⁻¹ as a function of temperature reveals a significant transition for lysozyme adsorbed to Adju-Phos® between 60 and 70 °C (Figure 4C). For lysozyme in solution, a much more subtle transition occurs over the same range. This approach was found to be superior to deconvolution or the use of derivatives in displaying differences as a function of temperature.

The shape of the spectrum of BSA adsorbed to Alhydrogel® (Figure 5B) is also distinguishable from that of the protein in solution (Figure 5A). Based on the ratio of the absorbances at 1624 and 1647 cm⁻¹, BSA appears to be less ordered when adsorbed to the adjuvant (Figure 5C). As the temperature is increased above ~35 °C, the secondary structure of BSA adsorbed to Alhydrogel® gradually changes, gaining β-sheet content. Major changes in the spectrum of the protein in solution are only seen at higher temperatures (Figure 5A). Above 70 °C, a shoulder at ~1624 cm⁻¹ emerges. Based on a plot of the ratio of absorbances vs. temperature, actual secondary structure changes of BSA in solution begin at ~65 °C. This result suggests a greater percentage of β-sheet at higher temperatures.

The results for ovalbumin are similar to those for BSA with the spectrum of the adsorbed protein different from that in solution (Figures 6A and 6B). As the temperature increases above 25 °C, the secondary structure of adsorbed ovalbumin gradually changes toward more α-helical character with a corresponding loss in β-sheet (Figure 6B and 6C). A change in secondary structure is not observed until ~75 °C for the protein in solution (Figures 6A and 6C). The change in secondary structure observed at this temperature suggests an increase in β-sheet. As seen for BSA, the data for ovalbumin suggest that the protein becomes conformationally altered when adsorbed to the adjuvant causing it to become less thermally stable.

**DISCUSSION**

Although aluminum salts are the most commonly used adjuvants in vaccines, what happens to the antigens when adsorbed to their surfaces has never been fully characterized. An understanding of how adsorption to these salts affects the structure and stability of proteins is essential to their use as vaccines as well as the mechanism of adjuvant mediated immune stimulation.

The adjuvant to which each protein preferentially adsorbs in this study is in agreement with the prediction that the protein with the highest pI (lysozyme) will best adsorb to the salt having the lowest PZC (Adju-Phos®), and vice versa. Although the two acidic proteins have a greater binding affinity for Alhydrogel® than does lysozyme for Adju-Phos®, it appears that approximately 1-2 mg protein can be adsorbed per mg aluminum, regardless of the protein or
adjuvant. This suggests that while both adjuvants have a similar number of binding sites, there may be more than one mechanism of binding.

A calorimetric analysis reveals distinct binding characteristics for each protein-adjuvant system. The interaction between BSA and Alhydrogel® is entropically driven and enthalpically unfavorable. The favorable entropy term suggests a release of water and counter ions from the surface of the protein and adjuvant upon adsorption consistent with either electrostatic or apolar interactions or both being involved in the binding process. Surprisingly, adsorption of ovalbumin onto Alhydrogel® is enthalpically driven and entropically unfavorable. The simplest interpretation for these results is that protein conformational effects are dominating the characteristic thermodynamic changes produced by the direct electrostatic effects. Lysozyme adsorption onto Adju-Phos® is both enthalpically and entropically favorable arguing for a variety of contributions to the thermodynamics of the binding process.

DSC demonstrates that proteins adsorbed to the surface of aluminum salts have significantly reduced thermal stabilities compared to the proteins in solution. These changes were determined to be irreversible in all cases. The extent to which the proteins are destabilized on the surface varies among the proteins, with the least affected being ovalbumin and the most BSA. To better understand the nature of this destabilization, we used intrinsic Trp fluorescence and ATR-FTIR spectroscopies to see whether the DSC changes reflected alterations in tertiary and secondary structures, respectively. Both methods suggest that extensive perturbation of the protein’s structure is occurring upon interaction with the surfaces of the insoluble salts.

The stability of BSA is most affected by adsorption onto an aluminum salt adjuvant. Although the thermogram for BSA in solution can be fit to two components, at least five are necessary to describe the thermogram of the adsorbed protein, with the transitions beginning just above 30 °C. This result combined with the changes in the FTIR spectra at lower temperatures suggests that the relatively invariant fluorescence emission peak position is not due to the adoption of a stable conformation. One possible explanation is that BSA adsors onto the adjuvant in such a way that the tryptophan residues remain inaccessible to the solvent throughout the temperature range investigated. A second possible explanation is that BSA microaggregates resulting in shielding of the protein’s Trp. Both explanations are plausible given that (1) BSA is a hydrophobic protein; (2) it is subject to self-association (17); (3) the observed blue shift of the protein in solution at intermediate temperatures is consistent with aggregation; and (4) the ITC data are consistent with a role for apolar interactions.

The stability of ovalbumin is the least affected by adsorption. Although the Tm’s (as determined by DSC) of the protein both in solution and adsorbed are essentially identical, FTIR data indicate very different thermal stabilities. Additionally, fluorescence intensity data suggest that adsorption causes the protein to undergo a conformational change at approximately 25 °C (Figure 3.B.ii.). Fluorescence peak position data indicate that the change does not result in a dramatic change in the environment of ovalbumin’s tryptophan residues (Figure 3.B.i.). These changes may be the result of a small conformational change of sufficient magnitude to account for an increase in population of the state with the lower Tm when ovalbumin is adsorbed relative to the unbound protein. Significant differences are observed in the stability of lysozyme in solution and adsorbed to Adju-Phos®. FTIR spectra clearly show major structural alterations occurring at temperatures above 65 °C for the protein on the surface (Figures 4B and 4C). The changes in secondary structure are much more subtle for the protein in solution.

The presence of the band at 1620 cm⁻¹ in the FTIR spectrum of lysozyme adsorbed to Adju-Phos suggests that lysozyme may be aggregating at higher temperatures. A brief study was conducted to determine whether the aggregation of lysozyme was occurring on the surface of the adjuvant, or if the protein was being desorbed at elevated temperatures followed by aggregation in solution. The study determined that no protein was desorbed from the adjuvant at higher temperatures; therefore, the aggregation must be occurring on the surface. This is also supported by the lack of a 1620 cm⁻¹ band in the FTIR spectrum of lysozyme in solution. Although it seems counterintuitive that the adsorbed protein would tend to aggregate while the protein in solution
does not, the structural change that occurs when the protein is adsorbed to the aluminum salt may, in fact, make the protein more susceptible to aggregation.

The extent of destabilization of these three proteins on these apparently highly polar surfaces is somewhat surprising (9-12). One possibility is that in optimizing interaction of the polar surface of the protein with that of the adjuvant, structural alterations and subsequent destabilization are induced. Another possibility is that the surface of the aluminum salts is actually somewhat apolar due to charge neutralization by counter-ion binding. Both explanations are consistent with the variable thermodynamics and the complex conformational equilibria observed. A third possibility is that the behavior seen reflects changes in the dynamic behavior of the adsorbed proteins. It seems reasonable to postulate that the distribution of native states is significantly perturbed upon surface localization with certain micro-states trapped by surface contact. The latter possibility is under active investigation in our laboratory.

Since stability is necessary for pharmaceuticals, the destabilization observed for protein antigens on the surface of aluminum salt adjuvants raises potential concerns regarding vaccine stability. For example, the structural alteration that occurs when the protein adsorbs to the adjuvant could make the protein more susceptible to chemical alterations such as deamidation and oxidation. In addition, the modified structure may enhance aggregation of the antigen on the surface of the adjuvant as described above. Therefore, it becomes critical that the long-term stability of the antigen be evaluated not only in solution, but on the surface of the adjuvant as well.

An interesting possibility for how this phenomenon may have a positive implication on the vaccine is that the reduced stability of the surface bound proteins in some way contributes to their enhanced antigenicity. The mechanism by which aluminum salt adjuvants promote a better immune response is not well established. At least three mechanisms are commonly considered (2, 3). One idea is that the formation of a depot at the site of injection allows the antigen to be gradually released, thereby extending the time possible for the antigen to interact with antigen presenting cells and lymphocytes. A second proposed mechanism is attributed to the particulate nature of the aluminum salt. Since particles smaller than 10 µm are more easily phagocytosed by macrophages, neutrophils and dendritic cells (18), uptake of the antigen adsorbed to an adjuvant should be increased (relative to antigen in solution) thereby improving the efficiency of antigen presentation. The third suggested mechanism of action is by a direct stimulation of the immune system through enhanced cytokine production. Aluminum salt adjuvants are known to primarily stimulate a humoral response. More specifically, it is believed that this class of adjuvants does so by stimulation of the production of Th2 cytokines. This current work suggests a fourth possibility.

Previous studies have demonstrated a correlation between protein antigen stability and proteolytic susceptibility. Thai et al employed cathepsin L and Pronase E to show that in a series of homologous antigens, the least stable was the most easily degraded and the most antigenic (19). Enzyme inhibitors eliminated this effect, confirming the relationship between stability and proteolytic susceptibility. Ametani et al demonstrated that the less stable variant of β-lactoglobulin was more antigenic (20). Similarly, the antigenicity of hen egg white lysozyme was found to increase as the stability of the derivatives decreased (21). The results in all cases suggest that antigen presentation efficiency is inversely related to antigen stability.

Thus, an alternative hypothesis for the mechanism of immune stimulation by aluminum salt adjuvants is suggested by this work. The data presented herein demonstrate that protein structure often becomes destabilized upon adsorption to the surface of certain aluminum salts. These physical changes in adsorbed proteins may make them more susceptible to proteolytic processing by the immune system, resulting in enhanced antigen presentation.

Acknowledgements—Financial support for this research was provided by the Madison & Lila Self Graduate Fellowship for L. Peek and the PhRMA Foundation for L. Jones.
REFERENCES

1. Vogel, F. R., and Powell, M. F. (1995) *Pharm. Biotechnol.* **6**, 141-228.
2. Lindblad, E. B. (2004) *Immunol. Cell Biol.* **82**, 497-505.
3. Gupta, R. K. (1998) *Adv. Drug Deliv. Rev.* **32**, 155-172.
4. Johnston, C. T., Wang, S. L., and Hem, S. L. (2002) *J. Pharm. Sci.* **91**, 1702-6.
5. Shirodkar, S., Hutchinson, R. L., Perry, D. L., White, J. L., and Hem, S. L. (1990) *Pharm. Res.* **7**, 1282-8.
6. Burrell, L. S., Johnston, C. T., Schulze, D., Klein, J., White, J. L., and Hem, S. L. (2000) *Vaccine* **19**, 275-81.
7. Seeber, S. J., White, J. L., and Hem, S. L. (1991) *Vaccine* **9**, 201-3.
8. Iyer, S., Robinett, R. S., HogenEsch, H., and Hem, S. L. (2004) *Vaccine* **22**, 1475-9.
9. Steadman, B. L., Thompson, K. C., Middaugh, C. R., Matsuno, K., Vrona, S., Lawson, E. Q., and Lewis, R. V. (1992) *Biotechnology and Bioengineering* **40**, 8-15.
10. Matsuno, K., Lewis, R. V., and Middaugh, C. R. (1991) *Arch. Biochem. Biophys.* **291**, 349-55.
11. Katzenstein, G. E., Vrona, S. A., Wechsler, R. J., Steadman, B. L., Lewis, R. V., and Middaugh, C. R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4268-72.
12. Brandau, D. T., Lawson, E. Q., Schubert, C. F., Day, N. K., Matsuno, K., and Middaugh, C. R. (1991) *Mol. Immunol.* **28**, 1019-26.
13. Brandau, D. T., Jones, L. S., Wiethoff, C. M., Rexroad, J., and Middaugh, C. R. (2003) *J. Pharm. Sci.* **92**, 218-31.
14. Fauci, A. S. (2001) *Clin. Infect. Dis.* **32**, 675-85.
15. Jendrek, S., Little, S. F., Hem, S., Mitra, G., and Giardina, S. (2003) *Vaccine* **21**, 3011-8.
16. Iyer, S., HogenEsch, H., and Hem, S. L. (2003) *Pharm. Dev. Technol.* **8**, 81-6.
17. Bulone, D., Martorana, V., and San Biagio, P. L. (2001) *Biophys. Chem.* **91**, 61-9.
18. Gupta, R. K., Rost, B. E., Relyveld, E., and Siber, G. R. (1995) in *Vaccine Design: the Subunit and Adjuvant Approach* (Powell, M. F., Newman, M. J., eds.) pp 229-248, Plenum Press, New York.
19. Thai, R., Moine, G., Desmadril, M., Servent, D., Tarride, J. L., Menez, A., and Leonetti, M. (2004) *J. Biol. Chem.* **279**, 50257-66.
20. Ametani, A., Sakurai, T., Katakura, Y., Kuhara, S., Hirakawa, H., Hosoi, T., Dosako, S., and Kaminogawa, S. (2003) *Biosci. Biotechnol. Biochem.* **67**, 1507-14.
21. So, T., Ito, H. O., Koga, T., Watanabe, S., Ueda, T., and Imoto, T. (1997) *J. Biol. Chem.* **272**, 32136-40.
FIGURE LEGENDS

Figure 1: Adsorption isotherms. The amount of protein bound to adjuvant was determined by subtracting the amount of protein remaining in solution from the amount of protein added. (A) Lysozyme adsorption onto Adju-Phos®. (B) Ovalbumin adsorption onto Alhydrogel®. (C) BSA adsorption onto Alhydrogel®.

Figure 2: DSC thermograms and peak fits comparing thermal stabilities of proteins in solution to adsorbed protein. Tₘ’s are summarized in Table 3. (A.i.) Lyszoyme in solution. (A.ii.) Lysozyme adsorbed onto Adju-Phos®. (B.i.) Ovalbumin in solution. (B.ii.) Ovalbumin adsorbed onto Alhydrogel®. (C.i.) BSA in solution. (C.ii.) BSA adsorbed onto Alhydrogel®.

Figure 3: Comparison of tertiary structure stabilities determined by front face fluorescence spectroscopy of proteins in solution (■) and adsorbed proteins (▲). (A.i.) Peak position of lysozyme fluorescence emission maximum as a function of temperature. (A.ii.) Lysozyme fluorescence intensity at a fixed wavelength (■=337 nm; ▲=336 nm) as a function of temperature. (B.i.) Peak position of ovalbumin fluorescence emission maximum as a function of temperature. (B.ii.) Ovalbumin fluorescence intensity at a fixed wavelength (■=332 nm; ▲=331 nm) as a function of temperature. (C.i.) Peak position of BSA fluorescence emission maximum as a function of temperature. (C.ii.) BSA fluorescence intensity at a fixed wavelength (■=338 nm; ▲=327 nm) as a function of temperature.

Figure 4: Comparison of secondary structure stabilities determined by ATR-FTIR of lysozyme in solution and adsorbed to Adju-Phos®. Amide I and II regions of FTIR spectra as a function of temperature for (A) protein in solution and (B) adsorbed protein. (C) Ratio of absorbances (1620 cm⁻¹/1641 cm⁻¹) as a function of temperature for lysozyme in solution (■) and adsorbed onto Adju-Phos® (▲).

Figure 5: Comparison of secondary structure stabilities determined by ATR-FTIR of BSA in solution and adsorbed to Alhydrogel®. Amide I and II regions of FTIR spectra as a function of temperature for (A) protein in solution and (B) adsorbed protein. (C) Ratio of absorbances (1624 cm⁻¹/1647 cm⁻¹) as a function of temperature for BSA in solution (■) and adsorbed onto Alhydrogel® (▲).

Figure 6: Comparison of secondary structure stabilities determined by ATR-FTIR of ovalbumin in solution and adsorbed to Alhydrogel®. Amide I and II regions of FTIR spectra as a function of temperature for (A) protein in solution and (B) adsorbed protein. (C) Ratio of absorbances (1653 cm⁻¹/1637 cm⁻¹) as a function of temperature for ovalbumin in solution (■) and adsorbed onto Alhydrogel® (▲).
Table 1: Linearized Langmuir adsorption isotherm analysis.

| Protein (pI)   | Adjuvant      | Ads$_{\text{max}}$ (mg protein/mg aluminum) | K$_{\text{ads}}$ (ml/mg) |
|----------------|---------------|-------------------------------------------|--------------------------|
| Lysozyme (11.0) | Adju-Phos®  | 1.4 ± 0.1                                  | 0.47 ± 0.03              |
| Ovalbumin (4.6)  | Alhydrogel®  | 1.6 ± 0.1                                  | 1.6 ± 0.3                |
| BSA (4.9)        | Alhydrogel®  | 2.2 ± 0.1                                  | 3.1 ± 0.5                |

Table 2: Binding Energetics obtained from Isothermal Titration Calorimetry.

| Protein       | n (mol protein/mol Aluminum) [mg/mg] | $K_a$ (M$^{-1}$)  | $\Delta H$ (kJ/mol) | $\Delta G$ (kJ/mol) | $\Delta S$ (J/mol K) |
|---------------|-------------------------------------|------------------|---------------------|---------------------|-----------------------|
| BSA           | 7.6e-4 ± 2e-5 [1.9]                  | 7.8e6 ± 3.4e6    | 95 ± 5              | -39 ± 1             | 450 ± 17              |
| Ovalbumin     | 6.3e-4 ± 3e-5 [1.0]                  | 5.1e5 ± 1.6e5    | -46 ± 6             | -33 ± 1             | -44 ± 20              |
| Lysozyme      | 2.1e-3 ± 7e-4 [1.12]                 | 7.0e4 ± 2.7e4    | -15 ± 6             | -28 ± 1             | 42 ± 20               |

Table 3: Average calorimetric T$_m$s of the model protein antigens in solution and adsorbed onto aluminum salt adjuvants (N=3).

| Protein     | Adjuvant                  | T$_m$ (average ± SD) (°C) |
|-------------|---------------------------|---------------------------|
| Lysozyme    | None                      | 72.5 ± 0.2                |
|             | Adju-Phos®                | 59.0 ± 0.9                |
| Ovalbumin   | None                      | 71.0 ± 0.4                |
|             | Alhydrogel®               | 69.7 ± 0.1                |
|             |                           | 77.7 ± 0.2                |
| BSA         | None                      | 71.4 ± 1.1                |
|             | Alhydrogel®               | 32.9 ± 2.9                |
|             |                           | 49.1 ± 1.4                |
|             |                           | 65.4 ± 0.2                |
|             |                           | 79.0 ± 0.6                |
|             |                           | 86.3 ± 0.2                |

A peak at (92.0 ± 0.1 °C) appears in thermograms of both adsorbed ovalbumin and adsorbed BSA. Although it was included as a 6th peak to fit the adsorbed BSA DSC data, it is not listed in this table.
Figure 1

(A) Lysozyme in solution (mg/ml)

(B) Ovalbumin in solution (mg/ml)

(C) BSA in solution (mg/ml)
Figure 2

(A.i.)

![Graph A.i.](image)

Temperature (°C)

Cp (kcal/mole/°C)

(A.ii.)

![Graph A.ii.](image)

Temperature (°C)

Cp (kcal/mole/°C)

(B.i.)

![Graph B.i.](image)

Temperature (°C)

Cp (kcal/mole/°C)

(B.ii.)

![Graph B.ii.](image)

Temperature (°C)

Cp (kcal/mole/°C)

(C.i.)

![Graph C.i.](image)

Temperature (°C)

Cp (kcal/mole/°C)

(C.ii.)

![Graph C.ii.](image)

Temperature (°C)

Cp (kcal/mole/°C)
Figure 3

(A.i.)

Peak position of emission maximum (nm)

Temperature (°C)

(A.ii.)

Fluorescence Intensity (normalized)

Temperature (°C)

(B.i.)

Peak position of emission maximum (nm)

Temperature (°C)

(B.ii.)

Fluorescence Intensity (normalized)

Temperature (°C)

(C.i.)

Peak position of emission maximum (nm)

Temperature (°C)

(C.ii.)

Fluorescence Intensity (normalized)

Temperature (°C)
Figure 4

(A) Wavenumber (cm$^{-1}$) vs. Absorbance (offset) for different temperatures.

(B) Wavenumber (cm$^{-1}$) vs. Absorbance (offset) for different temperatures.

(C) Temperature (°C) vs. Ratio of Absorbances (1620 cm$^{-1}$/1641 cm$^{-1}$) for different temperatures.
Figure 5

(A) and (B) show the absorption spectra at different temperatures, with peaks at specific wavenumbers. (C) displays the ratio of absorbances at 1624 cm\(^{-1}\) and 1647 cm\(^{-1}\) against temperature. The ratio increases with increasing temperature.
Figure 6

(A) and (B) show the absorbance spectra for different temperatures. The absorbance (offset) is plotted against the wavenumber (cm\(^{-1}\)) for temperatures ranging from 25°C to 75°C. The spectra change with temperature, indicating changes in protein structure and stability.

(C) illustrates the ratio of absorbances (1653 cm\(^{-1}\) to 1637 cm\(^{-1}\)) as a function of temperature. The ratio increases with temperature, suggesting a change in the ratio of protein structures.
Effects of adsorption to aluminum salt adjuvants on the structure and stability of model protein antigens
LaToya S. Jones, Laura J. Peek, Jonathan Power, Aaron Markham, Brian Yazzie and C. Russell Middaugh

J. Biol. Chem. published online January 31, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M500687200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts