Adsorption Behavior of Bovine Serum Albumin on Lowly Activated Anionic Exchangers Suggests a New Strategy for Solid-Phase Proteomics

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Received January 2, 2006; Revised Manuscript Received February 8, 2006

Diluted solutions of bovine serum albumin (BSA) (e.g., 0.1 mg /mL) do not form detectable protein large aggregates. Using gel-filtration experiments, we determined that a diluted solution of BSA is 97% monomeric BSA and 3% dimeric. The adsorption of this diluted BSA on highly activated anionic exchangers (e,g., having 40 μmol/wet g) keeps this mainly monomeric form. When supports activated with 2 μmol/wet g are used, only dimers become adsorbed to the support, accounting for 100% of the offered BSA. When the diluted BSA solution is offered to very mildly activated anionic exchangers (even only 0.125 μmol/wet g), an unexpected adsorption of most of the BSA on the support was also observed. These very slightly activated supports are only able to adsorb very large proteins or very large protein−protein complexes, larger than BSA dimers. In fact, a rapid cross-linking of the adsorbed BSA with dextran−aldehyde reveals the formation of very large BSA−BSA complexes with molecular mass higher than 500 000 Da, complexes that may be observed for soluble BSA with very high concentrations but are not detectable at 0.1 mg/mL. Moreover, the size of the aggregates strongly depends on the concentration of the ionized groups on the support: the less activated the supports are, the higher the sizes of the complexes. It seems that the interaction of the BSA molecules on the margins of the BSA aggregate with the groups on the support may stabilize the whole protein aggregate, although some components are not interacting with the support. Aggregates could account for more than 40% of the BSA in the solution after 50 h of incubation. However, only these large BSA aggregates were adsorbed in the support.

Introduction

Protein−protein interactions are important at almost every functional level of the cell including the structure of subcellular compartments, the transport machinery across biological membranes, the packaging of DNA into chromatin, the regulation of gene expression, and the transduction of intracellular signals. On the other hand, aberrant protein−protein interactions are implicated in many diseases.[1−16] Therefore, the study of protein complexes has become the object of intense research in many biological disciplines.[17−21]

The detection and purification of protein−protein complexes associated via very strong protein−protein interactions may be efficiently achieved by a variety of methods (such as affinity chromatography, gel filtration, and native and two-dimensional electrophoresis).[22−33] The problem is more intricate when the detection of a very weak or a transient protein−protein interaction is desired. These transient protein−protein interactions may play a critical role in cell physiology. However, the detection of a transient or weak protein−protein interaction remains technologically challenging. The transient protein complex may be the a minor component in the total isolated components because of its inherent instability.[34−35] Therefore, the ability to concentrate, purify, and identify very small traces of unstable complexes will be a useful tool in proteomic studies.

Very recently, it has been shown that the use of slightly activated ionic exchanger may help fulfill this goal:[36] This strategy has been developed to purify large proteins from small ones[37−40] and it is based in the mechanism of adsorption of these supports: a multipoint adsorption. This way, only large proteins are able to interact with several groups in the support placed at very large distances. If properly designed, the supports will selectively adsorb the protein complex while leaving individual components in the supernatant,[40] thus shifting the associated equilibrium toward complex formation.[36] The adsorbed complex may become more stable than the soluble one because of the synergy of the protein−protein interaction plus the interactions of each of the components of the large protein−protein complex with the active groups on the support.

Serum albumins are the most abundant proteins in the circulatory system in many organisms and function as carrier proteins. Serum albumins have a strong tendency to aggregate in solution.[41−50] In this paper, we have studied the effect of the presence of tailor-made ionic exchangers in this aggregation.

Materials and Methods

Materials. Cross-linked 4% agarose beads were kindly donated by Hispanagar SA (Burgos, Spain). Sodium periodate was from Merck (Darmstadt, Germany). Tris-HCl gradient (4−15%) ready-to-use gels were purchased from Bio-Rad. Ethylenediamine, sodium borohydride, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), bovine serum albumin (BSA) fraction IX, and dextran (20 kDa) from *Leuconostoc mesenteroides* were obtained from Sigma (St. Louis, MO). Trimethylaminoborane was purchased from Fluka (Buchs, Swizerland). All other reagents were of analytical grade.

Methods. All experiments were performed in triplicate and error did not exceed 5%.

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10.1021/bm060002x CCC: $33.50
Published on Web 03/16/2006

1. Preparation of Monoaminoethyl-N-aminoethyl- (MANAE-)Agarose. The protocol to prepare these supports was previously described by Fernández-Lafuente et al.,[52] but using glyoxyl-agarose with different activation degrees was used[36] and the supports were blocked with formaldehyde as described in ref 36. Briefly, agarose was activated with glycidol, oxidized with sodium periodate to get the desired concentration of glyoxyl groups, and further incubated in the presence of 1 M ethylenediamine at pH 10 to modify all the glyoxyl groups. Finally, formaldehyde modification of the amino groups prevented any undesired further aldehyde−support reaction.

2. Adsorption of BSA on Aminated Supports. Aminated support (1 mL) was suspended in 10 mL of BSA (from 0.1 to 1 mg/mL) in 5 mM sodium phosphate at pH 7.0 and 25 °C. During adsorption, samples were withdrawn from the supernatant and the protein concentration was determined by Bradford's method.[53] Periodically, samples of the matrix with the adsorbed protein were withdrawn and cross-linked following the procedures described below.

3. Cross-Linking of the Adsorbed or Soluble BSA.

3.1. Preparation of Dextran−Aldehyde. Standard cross-linking solution was prepared according to ref 51. Sodium periodate (4.36 g) was added to 50 mL of 50 kDa dextran (33.3 mg/mL) in distilled water. After 2 h, the oxidized dextran was extensively dialyzed against distilled water at 4 °C. The optimized cross-linking solution was similar, but a 3-fold increment of dextran and sodium periodate concentration was used, yielding a final aldehyde−dextran concentration of 100 mg/mL.

3.2. Cross-Linking of Soluble BSA. A volume of 5 mL of BSA samples (0.1 or 1 mg/mL) was mixed with 5 mL of standard cross-linking solution in the presence of 150 mM trimethylaminoborane during 8 h at 25 °C. Then, the mixture was reduced by addition of solid sodium borohydride to a final concentration of 3 mg/mL at pH 10 and 25 °C. After 30 min, the pH was decreased to pH 7 by addition of diluted HCl. The large excess of aldehyde−dextran avoided unspecific protein−protein cross-linking.[51]

3.3. Cross-Linking of Adsorbed BSA. The standard assay was similar to that described in ref 36, with 5 mL of suspension containing the matrix and 5 mL of standard cross-linking reagent mixed in the presence of 150 mM trimethylaminoborane for 24 h.

In the optimized protocol, developed to accelerate the cross-linking reaction by increasing the dextran concentration, 1 g of the matrix with the adsorbed BSA (vacuum-dried but not washed) was added to 5 mL of cross-linking solution in the presence of 150 mM trimethylaminoborane during 8 h at 25 °C[51] (concentration of aldehyde−dextran was 9-fold higher than in the standard case).

After this, the matrices were washed with distilled water to eliminate soluble BSA molecules and dextran. Reduction was performed as described above. The low protein load of the support prevents any undesired protein−protein cross-linking.

4. Gel-Filtration Assays. Gel-filtration analysis was performed on a glass column packed with agarose 4BCL (column bed volume = 100 mL). The column was previously equilibrated with 500 mL of the elution buffer (50 mM sodium phosphate, pH 7.0). All experiments were carried out at 25 °C with a flow rate of 0.5 mL/min employing an isocratic pump (Pharmacia) and detection of the absorbance at 280 nm (UV detector, Pharmacia). The eluted samples were collected in 1 mL aliquots, and protein concentration was determined by Bradford's method.[53]

Results and Discussion

Aggregation of Soluble BSA. When a BSA solution of 10 mg/mL was passed through a gel-filtration matrix, it eluted in two major peaks (data not shown). To avoid loss of BSA aggregation due to dilution during elution, BSA was cross-linked by use of aldehyde−dextran.[51] Figure 1 shows that 30% of 10 mg/mL BSA is eluted in dimer form, and to a lesser extent a third peak (accounting for less than 1%) is observed, which corresponds to higher degrees of BSA aggregation. With 0.1 mg/mL BSA, the percentage of cross-linked protein that was eluted as a dimer is much lower, accounting for less than 3%, and larger aggregates could be not detected. Dextran−aldehyde had been proposed as a optimized method to stabilize the protein complexes,[36] avoiding artificial cross-linking of nonrelated proteins.

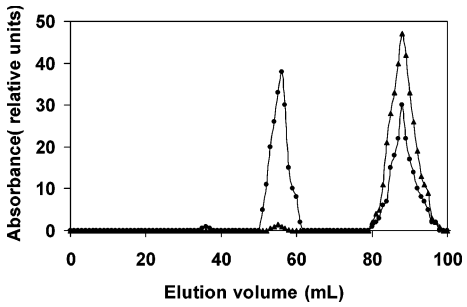


Figure 1. Gel-filtration analysis of aldehyde−dextran-treated BSA. Samples (1 mL) of BSA, either 10 mg/mL (●) or 0.1 mg/mL (▲) in 5 mM sodium phosphate buffer, pH 7, were treated with aldehyde−dextran as described in ref 36. The 10 mg of BSA/mL solution was diluted to have the same protein concentration as the 0.1 mg/mL solution. Both solutions were loaded in a glass column packed with 100 mL of agarose 4 BCL. Other details are described under Methods.

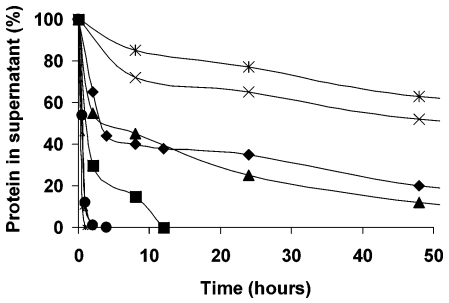


Figure 2. Adsorption of BSA (0.1 mg/mL) on MANAE-agarose 4BCL with different activation degrees. Adsorption was performed in 5 mM sodium phosphate at pH 7.0 and 25 °C. Other specifications are described under Methods. (+) 40, (●) 20, (■) 5, (▲) 2, (◆) 1, (×) 0.250, or (∗) 0.125 μmol/g of MANAE-agarose.,

This experiment reveals that BSA molecules have a trend to form aggregates and that the percentage and maximum size of these aggregates could be controlled by the BSA concentration, as previously described.[41−50]

To study the effect of tailor-made ionic exchangers in the aggregation of BSA, we used 0.1 mg/mL BSA solution, which exists primarily as monomers.

Adsorption of BSA on MANAE Supports with Different Activation Degrees. Figure 2 shows that 100% of the BSA was fully and rapidly adsorbed on supports having 40, 20, or 5 μmol of MANAE groups/g of matrix. At lower density, for example, with 1 or 2 μmol of MANAE groups/g of matrix, full immobilization was still achieved but with slower kinetics. Matrix with 0.25 or 0.125 μmol of MANAE groups/g of matrix adsorbed only a fraction of BSA in a biphasic pattern. At this low density of 0.25 and 0.125 μmol of MANAE groups, the first fraction accounts for 30% and 15% of the available BSA, respectively, which binds relatively rapidly in 10 h. After a second slower phase of binding, the total absorbed protein reaches a maximum of 45% and 30%, respectively, after 50 h. The adsorption rate was progressively slower between 50 and 80 h, during which another 10% of the BSA bound to the matrix. No further adsorption was observed between 80 and 120 h. The adsorption of BSA on these supports was not expected. These very slightly activated supports have been reported to be unable to adsorb even very large proteins such as lactase from *Thermus*

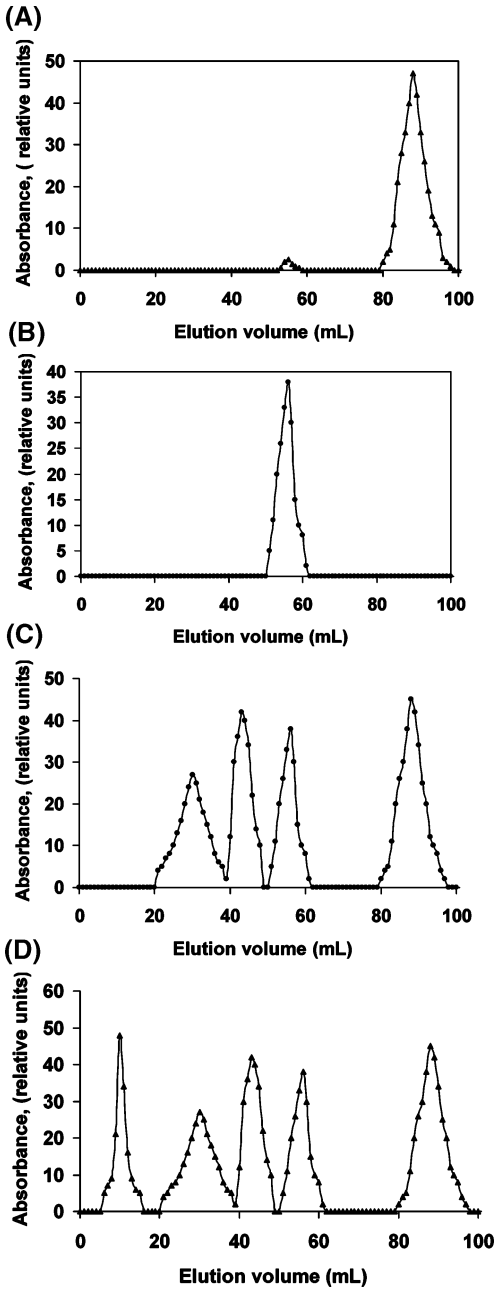


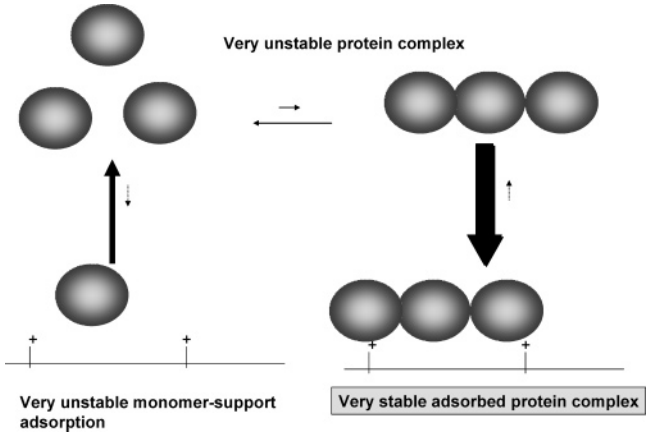
Figure 3. Gel-filtration analysis of BSA adsorbed on MANAE-agarose and further treated with aldehyde−dextran. BSA (1 mL, 0.1 mg/mL) at pH 7 was offered to MANAE-agarose and the adsorbed protein was cross-linked by aldehyde−dextran (20 kDa) as described in ref 36. Different activation degrees of the support were assayed: (A) 40, (B) 2, (C) 1, or (D) 0.125 μmol/g.

sp.,[38] a protein much larger than BSA dimers. In fact, the lowest activated supports used in this experiments presented only 1 MANAE group per ~350 000 A^2, reducing the likelihood of two BSA molecules interacting with two residues in the support.

Gel-Filtration Analysis of the Adsorbed Proteins. After cross-linking of the proteins adsorbed on the different supports as described,[36] the protein was desorbed from the supports and the eluted proteins were utilized in gel-filtration experiments (see Figure 3). The proteins eluted from the support activated with 40 μmol of MANAE/g of support were very similar in size to aggregates formed with cross-linked soluble enzyme.

For supports with 2 μmol of MANAE/g of matrix, the only detectable peak corresponded to a dimer of BSA molecules. Thus, at this density of MANAE groups, we converted 100% of the monomeric BSA to dimeric aggregates via selective adsorption to the matrix. This result agrees with other reports on other proteins.[36]

Scheme 1. Use of Tailor-Made Support to Shift, Concentrate, and Purify Very Weak BSA Oligomeric Complex



For supports activated at a lower density (e.g., 1 μmol of MANAE/g of support), it was possible to detect different sizes of aggregates, some of them with a molecular weight higher than that of the dimer, in addition to dimers and monomers. This was surprising, considering that the monomer form of BSA could not be detected with MANAE groups at 2 μmol/g of matrix.

Similarly, at lower densities, for example, 0.125 μmol of MANAE/g of support, many different sizes of aggregates of BSA were observed, with visible peaks corresponding to proteins with a molecular weight greater than the multimers observed with 1 μmol of MANAE/g of support and also including monomers and dimers.

The presence of monomers and dimers when very low-density MANAE groups are used is difficult to explain. With high-density MANAE groups on the matrix, any BSA molecule in the solution (aggregate or not) becomes adsorbed to the surface by multipoint intermolecular interactions with the activated matrix surface.

When the density of the MANAE groups is decreased (e.g., 2 μmol/g), individual molecules can no longer be adsorbed on the support while dimers can; the association equilibrium shifts to dimer formation via the selective adsorption of the dimers. The dimer is stabilized by both interprotein interactions (BSA−BSA) and intermolecular interaction between the BSA molecule and the MANAE groups on the matrix. In this way, BSA after cross-linking may be recovered in a dimeric form. In both cases, 100% protein adsorption is observed.

By this logic, the aggregate size is determined by the spacing of the MANAE groups (Scheme 1). At lower degrees of activation, for example, less than 2 μmol/g, only complexes those are larger than dimers should be adsorbed on the support. These multimer complexes should be stabilized by the BSA−BSA interactions plus the interactions of the molecules at the margins of the aggregate with the MANAE groups. At these low densities of MANAE groups, some association−dissociation equilibrium may exist, except if 100% of BSA molecules are adsorbed. Thus, when we cross-linked the aggregates in the experiments above, our cross-linking conditions may not have been optimal to rapidly enough capture the aggregates. This could explain the presence of monomer and dimer peaks in the gel-filtration studies when a very low activated support is used, while they are missing for medium-activated supports.

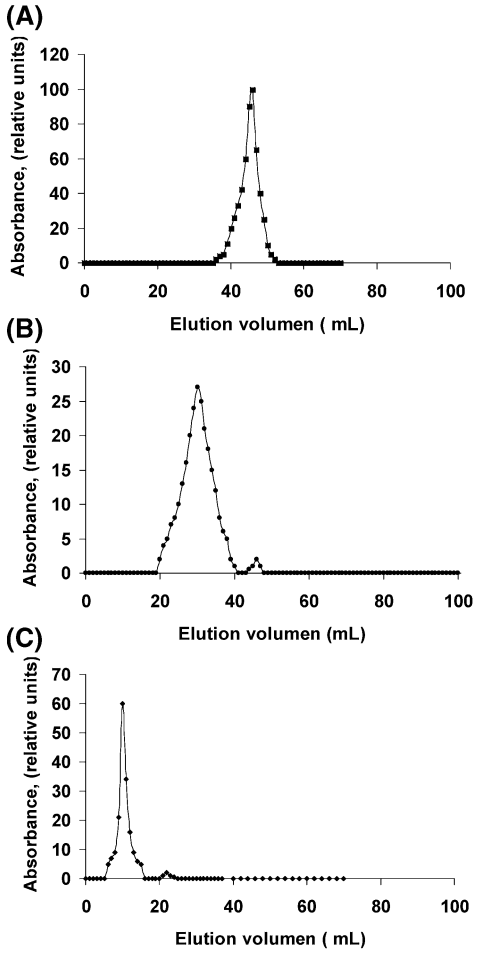
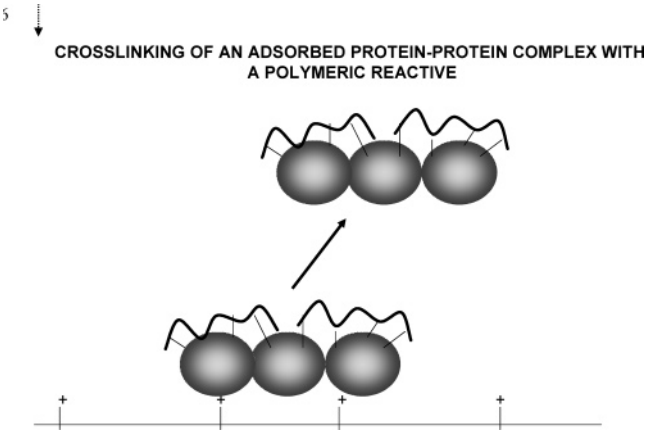


Figure 4. Gel-filtration analysis of BSA adsorbed on MANAE-agarose and further treated with aldehyde−dextran.BSA (1 mL, 0.1 mg/mL) at pH 7 was offered to MANAE-agarose and the adsorbed protein was cross-linked by aldehyde−dextran (20 kDa) following the new protocol described under Methods. Different activation degrees of the support were assayed: (A) 2, (B) 1, or (C) 0.125 μmol/g.

Scheme 2. Use of Aldehyde Dextran to Cross-Link Adsorbed BSA Oligomeric Complex



To verify this hypothesis, the cross-linking conditions were modified (see Methods) to increase the dextran concentration, which accelerates the cross-linking step (Scheme 2).

Figure 4 shows the results from this protocol. For supports activated with 2 μmol/g, results were identical to those observed for the standard cross-linking protocol,[36] that is, only dimers can be observed. However, for support activated at 1 μmol/g, where previously many different sizes of protein aggregates were isolated (Figure 4), we observed protein multimers of four BSA molecules, with only some traces of slightly smaller aggregates.

Similarly, proteins eluted from 0.125 μmol/g activated matrix reveals a main peak (about 50% of the total BSA) with a molecular mass greater than 500 kDa and traces of some smaller aggregates. The >500 kDa aggregates correspond to multimers of more than eight BSA molecules. The traces of the smaller aggregate peaks may be the consequence of suboptimal instantaneous cross-linking of the adsorbed proteins or slight heterogeneity in the distances among the MANAE groups on the support that allows smaller aggregates to adsorb. Thus, at 0.125 μmol of MANAE groups/g of matrix, we shifted the equilibrium of monomeric BSA to large stable multimers.

Discussion

In this example, the use of several anionic exchanger supports with varying degrees of activation has allowed us (1) to shift the association equilibrium of monomeric BSA to multimerization and (2) to control the size of the protein aggregate formed.

This protein exhibits a tendency to form large aggregates,[31−40] although under diluted conditions, only a small percentage of dimers can be visualized.

As previously described,[36] we have been able to shift the equilibrium from 97% monomer/3% dimer to 100% dimer by using a support with 2 μmol of MANAE/g. At this density, the synergy of the weak interactions of the MANAE groups and the BSA−BSA interactions is able to stabilize a BSA dimer on the support. For supports with significantly lower activation, the aggregate needs to grow to a size that can be stabilized by multipoint interactions with the MANAE groups on the matrix.

This situation is different than the one previously described,[36] where each individual molecule forming the complex was interacting with the support. Now some of the units of the aggregate are fixed in their position only by the interaction with other BSA molecules. However, the breakage of this association should promote full breakage of the aggregate and desorption of the BSA molecules that are interacting with the support (Scheme 1). Thus, the synergy of many weak interactions, that individually are not enough to give relevant concentration of the complex, permits us to shift the equilibrium and yields a high percentage of aggregate. However, the adsorption of the subunits at the end of the aggregate must compensate and stabilize the whole complex, and in this case the adsorption rate is very slow and the yield of the adsorbed protein did not reach 100% of the BSA molecules, although very high percentages of the initial BSA molecules are transformed in the aggregate (a yield of 50% of the octamer was achieved for the lowest activated support). Nevertheless, on the support there are only aggregates because they are the only proteins that can be adsorbed. Moreover, the size of the aggregate is controlled by the distance among the groups in the support: addition of new BSA molecules after those that are interacting with the support should be stabilized only by the weak BSA−BSA interaction. The breakage or formation of this interaction will have no effect on the adsorption of the BSA aggregate to the support; therefore it has no influence on the final stability of the complex and may have an equilibrium similar to that of the soluble protein. Therefore, if this did not occur in solution, it will not occur in the solid phase.

The results presented in this paper suggest that "solid-phase proteomics" may be a promising tool in the study of protein complexes.

Acknowledgment. This work was supported by "Ramon y Cajal" Spanish contract and an EC Project (MATINOES G5RD-CT-2002-00752). The help of Dr. A. Berenguer, Dr. Niroshan Ramachandran, and Dr. Bhupinder Bullar during the writing of the manuscript is gratefully recognized.

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BM060002X