Surface diffusion of interacting proteins

Effect of concentration on the lateral mobility of adsorbed bovine serum albumin

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ABSTRACT Surface diffusion of bovine serum albumin adsorbed from aqueous solution to poly(methylmethacrylate) surfaces is significantly hindered by protein-protein lateral interactions. The long-time self diffusion coefficient measured by fluorescence recovery after pattern photobleaching decreases by approximately one order of magnitude as the surface area fraction occupied by protein increases from 0.10 to 0.69. Qualitative features of the surface concentration dependence of the self diffusion coefficient can be described by several recent models for lateral diffusion of interacting species. The mobile fraction is independent of the surface concentration, and both the self diffusion coefficient and the mobile fraction are constant between 15 min and 7 h of adsorption.

INTRODUCTION

Proteins confined to solid-liquid interfaces can influence the behavior of diverse biological and biomedical systems. For example, the structure and activity of adsorbed protein layers can determine the interaction between cultured cells and solid substrates, the biocompatibility of natural and synthetic materials, and the reliability of solid phase immunoassays. Despite their importance, the molecular determinants of the formation of these layers are not well understood. Early indications of the possibility of surface diffusion of adsorbed proteins (Burghardt and Axelrod, 1981) have recently been confirmed (Tilton et al., 1990), suggesting mechanisms for the possible development of molecular organization in adsorbed protein layers. Surface diffusion may enable randomly adsorbed proteins to assemble into organized structures at the solid-liquid interface, perhaps allowing nonrandom orientations, closest packing, patchwise aggregation, and phase changes in adsorbed monolayers.

In its numerous configurations, fluorescence recovery after photobleaching (FRAP) is commonly employed to measure two-dimensional diffusion. Whether applied to proteins adsorbed at solid-liquid interfaces, dispersed in membranes, or bound to membrane components, FRAP measures long-time self diffusion coefficients. Whereas the short-time diffusivity is determined by the protein-surface interaction, the diffusivity measured by FRAP depends on both protein-surface and protein-protein interactions. Unlike mutual diffusion, where repulsive interactions are rate enhancing, self diffusion is impeded by any interaction among diffusing species, repulsive or attractive. This produces a concentration-dependent attenuation of the self diffusion coefficient (Scalettar et al., 1988; Abney et al., 1989). Hindered self diffusion of interacting membrane proteins in fluid lipid layers (Tank et al., 1982; Peters and Cherry, 1982) and of monoclonal antibodies specifically bound to lipid haptens in supported phospholipid layers (Tamm, 1988; Wright et al., 1988; Subramaniam et al., 1986) has been demonstrated, but efforts to theoretically describe concentration-dependent self diffusion continue to outpace its experimental investigation. Attempts to reconcile theoretical predictions and experimental observations are rare. Often the models describe two-dimensional self diffusion of interacting membrane proteins, yet they are not bound by assumptions that either limit them to protein-lipid systems or exclude systems of proteins adsorbed at solid-liquid interfaces (for example, Abney et al., 1989; Minton, 1989; Saxton, 1982; Saxton, 1987). Current models typically do not consider specific interactions or hydrodynamic effects; hard disc interactions are sufficient to produce a strongly concentration-dependent self diffusion coefficient. Besides lateral interactions, other phenomena, such as the coexistence of mobile and immobile proteins, are common to self diffusion of both adsorbed and membrane-bound proteins. Thus, our findings relating to adsorbed proteins are also relevant to diffusive membrane processes.

As described in a previous publication, we determined that bovine serum albumin (BSA) molecules exhibited surface diffusion after adsorption to both poly(methylmethacrylate) (PMMA) and cross-linked poly(dimethylsiloxane) surfaces, in spite of being irreversibly adsorbed throughout the duration of the diffusion measurement (Tilton et al., 1990). On each material,

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mobile species shared the surface with proteins that remained immobile on the time scale of the experiment, suggesting a heterogeneity of adsorption states. In this communication, we present an experimental investigation of the effect of surface concentration on the self diffusion of BSA irreversibly adsorbed at the aqueous-PMMA interface. We find a significant hindrance of surface diffusion that increases with increasing surface concentration, owing to interactions among adsorbed proteins. We examine several recent models of two-dimensional self diffusion (Saxton, 1982; Saxton, 1987; van Beijeren and Kutner, 1985) and find that qualitative features of the surface concentration dependence can be described, but additional refinements will be required to improve the agreement between experiment and theory.

EXPERIMENTAL SYSTEM

We measured surface diffusion of fluorescent labeled BSA adsorbed to spin cast films of PMMA by a combination of total internal reflection fluorescence and fluorescence recovery after pattern photobleaching (FRAPP). A detailed description of the experimental apparatus, methods, and data analysis appears in the previous publication (Tilton et al., 1990). As discussed in that publication, our results were not affected by photo-induced artifacts. In the current experiments, as in our previously published work, an optically transparent polymer-coated microscope slide served as one wall of a flow cell and as the adsorption surface. The total internal reflection configuration employed in the experiments permitted us to continuously monitor the amount of adsorbed protein throughout the entire adsorption procedure, thereby ensuring reproducible conditions for photobleaching. By intersecting two coherent beams of 514.5 nm frequency-stabilized argon ion laser light at the point of total internal reflection at the polymer-protein solution interface, we monitored protein adsorption and performed FRAPP measurements with an interference fringe pattern in the evanescent wave. The characteristic diffusion length for FRAPP measurements, 9.1 or 12 μm for the experiments described here, was one-half the period of the fringe pattern. Analysis of FRAPP data provided both the fraction of mobile adsorbed proteins (f) and their self diffusion coefficient (D) (Tilton et al., 1990). The measured self diffusion coefficient applied only to the mobile proteins, rather than an average of all mobile and immobile proteins.

MATERIALS

BSA (A7511, <0.005% fatty acid from Sigma Chemical Co., St. Louis, MO) was fluorescently labeled by covalent attachment of eosin-5-isothiocyanate (EITC) (Molecular Probes, Eugene, OR) with an average of one EITC molecule per protein and dissolved in pH 7.4 phosphate buffered saline (10 mM phosphate, 150 mM NaCl). PMMA (Polysciences, Warrington, PA) was spin cast at 2,000 rpm onto cleaned microscope slides from 2 wt% solutions in toluene. Films prepared in this way are continuous and present an uncharged, hydrophobic, weakly hydrogen bonding surface to adsorbing proteins (Cheng et al., 1987).

METHODS

We adsorbed BSA to PMMA surfaces at 37°C from solutions in fully developed laminar flow at a wall shear rate of 102 s^{-1} in a flow cell. After adsorbing EITC-BSA for 40 min, we displaced it from the flow cell with unlabeled BSA. After adsorption for a total of 60 min, the intensity of fluorescence emitted from the adsorbed layer was constant, indicating that the fluorescent proteins were adsorbed irreversibly on the time scale of these experiments. This procedure ensured that the only source of fluorescent molecules was the population of irreversibly adsorbed BSA, preventing artifactual measurements of any dissolved or loosely bound EITC-BSA. Except where noted, we made FRAPP measurements after 60 min of adsorption in all experiments.

We varied surface concentration by adsorbing from solutions of different bulk protein concentration for equal durations. We used the relationship between surface concentration and bulk concentration previously established for BSA adsorption to PMMA for the same duration and under the same conditions (Tilton et al., 1990). To calculate the BSA area fraction corresponding to a particular surface mass concentration we assumed adsorption of elliposoidal molecules with the long dimension parallel to the surface. Monolayer "side-on" adsorption of BSA on polystyrene latices has been demonstrated by dynamic light scattering (Uzgiris and Fromageot, 1976; Fair and Jamieson, 1980). Based on the x-ray crystallographic structure of human serum albumin (Carter et al., 1989) and the hydrodynamic properties of BSA (Square et al., 1968), we considered BSA to be a 40 × 40 × 140 Å ellipsoid of molecular weight 66,700.

RESULTS

We measured the self diffusion coefficient of BSA adsorbed to PMMA at area fractions ranging from 0.10 to 0.69 of the available surface occupied by BSA. By extrapolating to zero surface concentration, according to a cubic polynomial least squares fit of the data, we determined that the self diffusion coefficient at infinite surface dilution was $D_0 = (5.6 \pm 0.5) \times 10^{-8}$ cm^2/s. Self diffusion coefficients, normalized by D_0, are presented as a function of surface concentration in Fig. 1, where each point corresponds to a single diffusion coefficient measurement on a different surface. The corresponding mobile fractions are plotted in Fig. 2. Whereas the self diffusion coefficient varied by approximately one order of magnitude over this range of area fractions, the fraction of mobile proteins remained constant at $f = 0.4 \pm 0.14$. There was considerable spread in the data for surfaces at 0.1 area fraction coverage. In these experiments, the largest diffusion coefficients were measured on those surfaces displaying the largest mobile fraction.

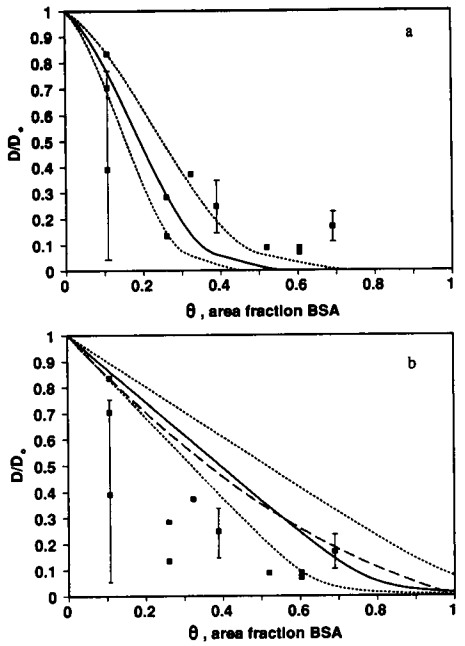

FIGURE 1 Diffusion coefficients are normalized by the extrapolated value at infinite surface dilution to allow comparison of the experimental values for BSA adsorbed to PMMA (points) and theoretical predictions. The area fraction of immobile obstacles is $(1 - f)\theta$, as explained in the text. (*a*) Predictions of Saxton; (*b*) Predictions of van Beijeren and Kutner, $\gamma = 1$ (*dashed line*); van Beijeren and Kutner, $\gamma = 100$ (*solid line*). Monte Carlo results of Saxton (not shown) are barely distinguishable from van Beijeren and Kutner for γ on the order of 10 or less. The Monte Carlo results for $\gamma = 100$ were not reported but are expected to closely resemble the van Beijeren and Kutner predictions. Representative error bars are 95% confidence intervals. Dotted lines represent the error limits for the model predictions based on the experimental error in the mobile fraction, *f*.

In addition to the role of surface concentration, we investigated the effect of adsorption time on lateral mobility at 0.6 area fraction. Both the self diffusion coefficient and the fraction of mobile proteins remained invariant between 15 min and 7 h of adsorption.

DISCUSSION

The strong dependence of the self diffusion coefficient of adsorbed BSA on its surface concentration can be described by a simple adaptation of the Saxton (1982) model of diffusion in an archipelago of impermeable patches. In the vicinity of the percolation threshold, continuous percolation theory provides convenient scaling relationships for two-dimensional diffusion coefficients as a function of the fraction of the available area blocked by "impermeable patches." These relationships fail far from the percolation threshold, where effective medium theo-

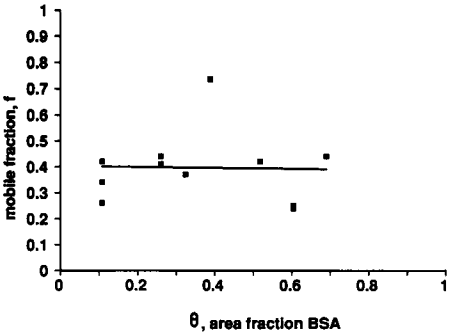

FIGURE 2 The fraction of mobile BSA molecules on PMMA, determined by FRAPP, is constant at 0.4 ± 0.14 over a wide range of surface concentrations.

ries are more successful (Saxton, 1982). To describe the behavior of two-dimensional self diffusion coefficients over a broad range of area fractions of completely impermeable lipid patches in membranes, Saxton developed a model incorporating the continuous percolation scaling relationship within a critical distance of the percolation threshold and the predictions of effective medium theory near zero area fraction. By interpolating between these two regimes with a cubic polynomial, he predicted the self diffusion coefficient of a tracer particle as a function of the area fraction occupied by impermeable patches.

We express this model not in terms of the area fraction of impermeable lipid patches but in terms of the area fraction occupied by immobile proteins, assuming they are immobile over time scales long compared with diffusion times. The appropriate area fraction of immobile obstacles is equal to $(1 - f)\theta$, where $1\text{-}f$ is the fraction of immobile proteins and θ is the total area fraction occupied by both mobile and immobile proteins. As shown in Fig. 2, f is constant at an average value of 0.4 ± 0.14. With this consideration, we plot the Saxton prediction of D/D_0 as a function of total occupied area fraction in Fig. 1. The sharp decrease in the self diffusion coefficient with increasing concentration is well described by this model, but we find no experimental evidence for a percolation threshold concentration for surface diffusion. In contrast with our results, the percolation threshold concept requires that long-range diffusion coefficients should vanish at concentrations exceeding the percolation threshold. Continuous percolation theory indicates a threshold coverage of 0.332 area fraction of immobile obstacles (corresponding to a percolation threshold at a total BSA area fraction $\theta = 0.55$). The disagreement between the model and the experimental results may reflect the assumption of complete immobility of the obstacles. If the obstacles were mobile, the percolation threshold would shift to higher concentrations or would not occur at all, depending on the

magnitude of their mobility. As discussed in Tilton et al. (1990), the apparently immobile proteins may be capable of slow diffusion with an upper bound on the self diffusion coefficient approximately two orders of magnitude smaller than that of the mobile species. There may also be a distribution of self diffusion coefficients that is more complicated than simple coexistence of "mobile" and "immobile" species but experimentally inaccessible.

By allowing for mobile background species, the lattice gas model of van Beijeren and Kutner (1985) predicts the absence of a threshold concentration for surface diffusion, given the appropriate ratio of the jump rates of tracer particles to background particles, expressed by the parameter $\gamma = D_0(\text{tracer})/D_0(\text{background})$. Predictions from this model are virtually indistinguishable from Monte Carlo simulations of tracer diffusion with mobile background particles (Saxton, 1987). As with the Saxton model of diffusion in an archipelago of immobile obstacles, we consider the area fraction of background particles to be the area fraction of apparently immobile proteins, $(1 - f)\theta$. In Fig. 1, the predictions from this model with $\gamma = 100$ (corresponding to the estimated upper limit of the diffusion coefficient for the "immobile" proteins) are compared with the FRAPP results. While the threshold concentration is absent, the extent of the surface concentration dependent hindrance of diffusion is underestimated. This most likely is due to the fact that these models consider only interactions between tracer and background species and neglect interactions among tracer species. These interactions would further hinder surface diffusion of tracers. For BSA adsorbed to PMMA, the mobile proteins represent 40% of the interacting species, and to consider these particles as tracers and ignore their interactions is to ignore a major fraction of the total interactions experienced by a diffusing protein. These interactions could be considered by modeling tracer diffusion with two different populations of background particles, one with a diffusivity equal to the tracer diffusivity, the other with a much lower diffusivity.

It must be noted that the surface concentrations in Fig. 1 represent only the labeled protein. At the lowest concentration, continued adsorption of unlabeled BSA during the 20 min displacement procedure may result in a true concentration (including unlabeled BSA) not more than 30% higher. At all the higher concentrations, adsorption was at or near the plateau before the displacement, so the reported concentrations accurately represent the entire adsorbed layer. The displacement procedure only altered the total coverage for the smallest surface coverage, thus our conclusions concerning the extent of the surface concentration dependence and the lack of a percolation threshold remain unchanged.

To support the interpretation of our FRAPP data in terms of coexisting mobile and apparently immobile adsorbed BSA, we also provide the lattice gas model predictions for $\gamma = 1$. In this case the appropriate area fraction is θ, the total occupied area fraction, because the concept of "background" and "tracer" species is somewhat arbitrary when all species are assumed to have the same diffusion coefficient. As opposed to a layer of coexisting mobile and immobile proteins, this describes a homogeneous adsorbed layer, and it clearly is not an adequate description of our results.

The origin of the coexistence of mobile and apparently immobile adsorbed BSA remains unclear. In addition to a distribution of conformational states or orientations of different intrinsic mobility, a distribution of states of aggregation on the surface is a possible explanation of this coexistence, if highly mobile monomeric proteins share the surface with immobilized aggregated proteins. An interesting comparison can be made to FRAPP measurements of monoclonal antibodies bound to lipid haptens in supported phospholipid bilayers (Tamm, 1988). At temperatures where the proteins were homogeneously dispersed on a supported bilayer, the mobile fraction remained constant over a wide range of concentrations, as in the current adsorbed protein system. At lower temperatures, where phase separation occurred and protein aggregates were visible by fluorescence microscopy, the proteins were immobilized. While this work identified protein aggregation as a means to restricted mobility, the behavior of antibodies bound to supported lipid layers is rather complex and appears to be quite sensitive to the components and handling of the particular experimental system. Other investigations indicate a variety of effects influencing the mobility of antibodies bound to supported lipid layers (Wright et al., 1988; Subramaniam et al., 1986).

The insensitivity of the mobile fraction of adsorbed BSA to changes in surface concentration and adsorption time suggests that an equilibrium aggregation process is not responsible for the presence of immobile adsorbed BSA molecules on the PMMA surface, although it is possible that a small variation in mobile fraction may not be evident given the scatter of the FRAPP results. The coexistence of mobile and immobile adsorbed proteins is likely a consequence of a distribution of BSA conformations or orientations having different surface binding strengths that are established during the initial contact of the proteins and the surface. The mobile fraction might also be related to a property of the polymer surface that would be insensitive to adsorption time or concentration.

In Tilton et al. (1990) we reported BSA surface area fractions exceeding 0.51, the jamming limit for random sequential adsorption of rectangular particles of aspect ratio 3.5 (Vigil and Ziff, 1989). Random sequential adsorption of rectangles can result in some degree of ordering, but only over small distances. While we must consider the possibilities that multilayer adsorption or

protein aggregation may be responsible for the attainment of surface concentrations exceeding the jamming limit, diffusion-mediated enhancement of order in the adsorbed layer is a more likely determinant of these large surface concentrations. The near certainty that BSA is adsorbed in a monolayer (Lyklema, 1984) and our current findings suggesting the improbability of BSA aggregation on the PMMA surface lead us to discard the first two possibilities in favor of the last. Surface diffusion apparently permits ordering of the adsorbed ellipsoidal BSA molecules, allowing more efficient molecular packing. Of course, more efficient protein packing may also result if some BSA molecules re-orient with their long axis perpendicular to the PMMA surface, but this is not expected to occur on a hydrophobic surface (Uzgiris and Fromageot, 1976; Fair and Jamieson, 1980).

CONCLUSIONS

Protein-protein lateral interactions significantly hinder the rate of surface diffusion of BSA adsorbed to PMMA, but they do not result in a percolation threshold for diffusion in the concentration range examined in this investigation. Comparison of the measured surface concentration-dependent self diffusion coefficient to the predictions from several models suggests that the main consequences of lateral interactions can be qualitatively explained with only excluded area effects. The models discussed in this communication have extremely simple foundations, neglecting interactions of finite range, as well as attractive or specific interactions. Inclusion of these interactions would contribute further to the hindrance of two-dimensional self diffusion and contribute to a more quantitative description of this system. Models treating the mobile species as tracer particles may be inadequate descriptions of the full extent of the concentration dependent hindrance of surface diffusion of adsorbed proteins. Due to the similarity of the fractions of mobile and immobile proteins in the BSA/PMMA system, interactions between the mobile species themselves should be included in addition to interactions between mobile and immobile species. More quantitative description of our results might be possible by the addition of interactions between mobile particles to the currently available lattice models or Monte Carlo simulations or to molecular dynamics simulations.

While the self diffusion coefficient of BSA adsorbed to PMMA is strongly dependent on the protein surface concentration, it is independent of adsorption time between 15 min and 7 h. The mobile fraction is independent of both surface concentration and adsorption time. The coexistence of mobile and apparently immobile proteins thus likely results from a distribution of conformations or orientations established in the early stages of adsorption and not from aggregation of adsorbed BSA.

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