Potential-dependent surface denaturation of BSA in acid media

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In contrast to previous reports claiming bovine serum albumin (BSA) denaturation at mercury surfaces, recently it has been shown that BSA and other proteins do not denature as a result of adsorption to the mercury electrodes at alkaline and neutral pH values. In this pH range, constant current chronopotentiometry (CPS) with mercury or solid amalgam electrodes can be used to distinguish between native, denatured and damaged BSA. Here we show that at acid pH values (around pH 4.5) native and denatured BSA yield almost the same CPS responses suggesting denaturation of native BSA at the electrode surface. Under these conditions BSA is, however, not denatured at the electrode at accumulation potentials (E_A values) close to the potential of zero charge, but at E_A values more negative than −0.8 V, after destabilization of the surface-attached BSA by electroreduction of some disulfide groups at about −0.48 V and by electric field effects at more negative potentials.

Introduction

Progress in genomics and particularly the completion of the DNA nucleotide sequences of hundreds of organisms including the human genome provides an abundance of information about the structure of individual genes.[1] These data inform us, however, little about the gene protein products. In contrast to genomics, proteomics deals with the large-scale studies of protein properties (expression level, post-translational modification, interactions, protein structures and their changes, *etc.*). Possible application of such information in the early detection of diseases and in new approaches in therapy evokes enormous interest of scientists and pharmaceutical companies in proteomics. Present proteomics requires new sensitive methods for the analysis of proteins. Electrochemistry has great potentialities in protein research, which has been so far little utilized. It is well known that some small conjugated proteins containing redox centers (usually metalloproteins) produce fast, reversible electrochemistry.[2,3] This electrochemistry of a relatively small group of proteins is therefore of limited use in proteomics.

Recently we have shown that the electroactivity of amino acid residues in proteins at carbon and mercury electrodes can be utilized in the analysis of practically all proteins,[4,5] including those important in biomedicine.[4–7] At carbon electrodes, tyrosine and tryptophan residues in peptides[4,5,8] and proteins[4,5,9,10] are oxidizable and well-developed signals can be obtained by means of various methods, such as square wave voltammetry[4,5,11,12] and constant current chronopotentiometry (CPS).[4,5,8] These signals do not, however, appear sufficiently sensitive to changes in protein structures (*e.g.* due to denaturation or changes in redox state).[13,14] On the other hand, we have shown that local and global changes in protein structures can be sensitively detected by CPS at mercury-containing electrodes using the so-called peak H.[4,5,14,15] This peak is due to the catalytic hydrogen evolution allowing determination of peptides and proteins at nanomolar and sub-nanomolar concentrations. In CPS the electrode is polarized by current, and the dependence of the potential E on time t is measured. The derivative of the E–t curve $(dE/dt)^{-1}$ against E is usually used for analytical purposes. Due to its catalytic nature, involving transfer of a large number of electrons per protein molecule, high current densities can be used. At such current densities very high rates of potential shifts are induced in the absence of electrode processes, which are by several orders of magnitude faster than those in usual linear sweep voltammetry. Very fast potential changes are of critical importance in the electrochemical determination of changes in protein structures.

At alkaline pH values, denatured forms of bovine serum albumin (BSA) and of some other proteins produced up to a 50-fold higher peak H than that of the corresponding native proteins.[16] Using high stripping currents (I_{str}) (inducing very high rates of potential changes) was critical for observing large differences between the signals of native and denatured BSA.[13,16–18] These large differences suggested that no significant surface denaturation of the studied (native) proteins took place at the hanging mercury drop electrode (HMDE) under the given conditions. Our results appeared in contradiction with the long time-held belief that commonly used metal electrodes, such as mercury, gold, platinum and silver, led to denaturation and irreversible adsorption of the resulting inactive protein.[2] Honeychurch[19] concluded in his review that proteins denature upon adsorption on mercury electrodes to give adsorbed layers of uniform thickness. This conclusion was based on the results of Scheller *et al.*[20] who measured BSA and eight other proteins by a.c. polarography/voltammetry using a dropping mercury electrode and HMDE as well as capacity–time curves in (unbuffered) 0.1 M KCl at pH 7 or pH 3. Under these conditions the surface area per amino acid residue of about 19 Å^2 corresponded roughly to the value of unfolded protein. Some reports, however, contradicted the above conclusions about the surface denaturation of proteins at mercury electrodes (*e.g.* refs 21 and 22). On the other hand the

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conditions used in these reports and in our recent experiments[13,16–18] were not exactly the same as those used by Scheller *et al.*[20]

In this paper we used peak H to investigate native and denatured BSA in the media used by Scheller *et al.*[20] as well as in acid buffers. We found that in difference to buffered alkaline and neutral media, where native and denatured BSA produced strikingly different responses,[18] in acid media (both unbuffered and buffered) native and denatured BSA produced almost identical peaks. On the other hand, in the acid media, native BSA was not denatured at the accumulation potentials, E_A values between 0 and −0.5 V; its denaturation occurred, however, at more negative E_A values or during the potential changes in the CPS experiments.

Results and discussion

Native and denatured BSA in 0.1 M KCl and at acid pH values

Scheller *et al.*[20] used for their measurements (unbuffered) 0.1 M KCl, pH 7 or pH 3 as background electrolytes. These media are not the best for signals due to the catalytic hydrogen evolution (known to increase with the buffer capacity);[23,24] we used them, however, to compare our data with those of Scheller *et al.*[20] In 0.1 M KCl, pH 7 we did not obtain the CPS peak H but only poorly developed inflections with 100 nM BSA in both native and denatured states (Fig. 1a). Such absence of a well-developed peak H might be due to uncontrolled changes in pH in the vicinity of the electrode surfaces and/or to an insufficient amount of protons in the solution for fast proton recombination in the adsorbed protein. On the other hand, in buffered 50 mM sodium phosphate, pH 7 a well-developed peak of native BSA and a much higher peak of denatured BSA appeared.[18]

In 0.1 M KCl, pH 3, peaks of native and denatured BSA did not substantially differ from each other (Fig. 1b). To be sure that the similarity of responses of native and denatured BSA obtained at pH 3 was not due to the unbuffered medium or to its too acidic pH we repeated the measurements in 50 mM sodium acetate pH 4.5. We obtained practically identical peaks of native and denatured BSA (Fig. 1c), which were much higher and better developed than those produced in 0.1 M KCl, pH 3 (Fig. 1b), as expected for well-buffered acetate medium. These results suggest that at acid pH the protein is probably denatured at the electrode surface. Such surface denaturation may occur already at the accumulation potential (E_A −0.1 V) or during the following electrode polarization to negative potentials.

BSA is not significantly denatured at E_A −0.1 V

To determine whether BSA was denatured during its adsorptive accumulation (for 60 s at E_A −0.1 V) we performed the adsorptive transfer (*ex situ*) analysis,[4,25] in which the accumulation of BSA was separated from the electrode process. Considering our earlier finding that at alkaline and neutral pHs the heights of peak H of native and denatured BSA greatly differ[13,16–18] we used 50 mM sodium phosphate, pH 7 as a background electrolyte to test the conformational state of the surface-attached BSA.

100 nM BSA was adsorbed from the electrolytic cell and the BSA-modified electrode was transferred to another cell containing only the blank background electrolyte. After adsorbing BSA from 50 mM sodium acetate, pH 4.5, followed by transfer of the BSA-modified electrode to blank 50 mM phosphate, pH 7.0 (background electrolyte in which the CPS responses of native and denatured BSA greatly differ[18]), a large difference between peaks of native and denatured BSA was obtained (Fig. 2a). If the BSA-modified electrode was transferred into 50 mM sodium acetate, pH 4.5, native and denatured BSA yielded almost the same peaks (Fig. 2b). These results suggest that (i) no significant irreversible surface denaturation of BSA took place during the protein accumulation at pH 4.5, but (ii) such denaturation occurred at more negative potentials during the CPS measurement at pH 4.5 in a time interval much shorter than 60 s.

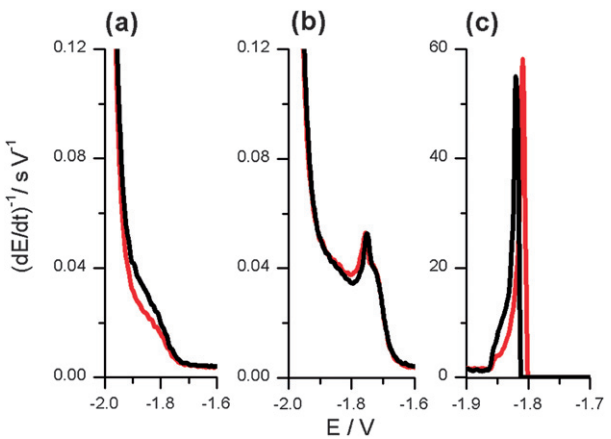

Fig. 1 Peak H of native BSA (red) and urea-denatured BSA (black) in 0.1 M KCl, pH 7 (a); in 0.1 M KCl, pH 3 (b); and in 50 mM sodium acetate, pH 4.5 (c) in the presence of 56 mM urea. 100 nM BSA was adsorbed on the HMDE for the accumulation time t_A, 60 s, at the accumulation potential E_A −0.1 V, from the supporting electrolyte with stirring at 1500 rpm then chronopotentiograms were recorded (*in situ*) with the stripping current I_{str} −25 μA (a,b); I_{str} −40 μA (c) at 18.5 °C.

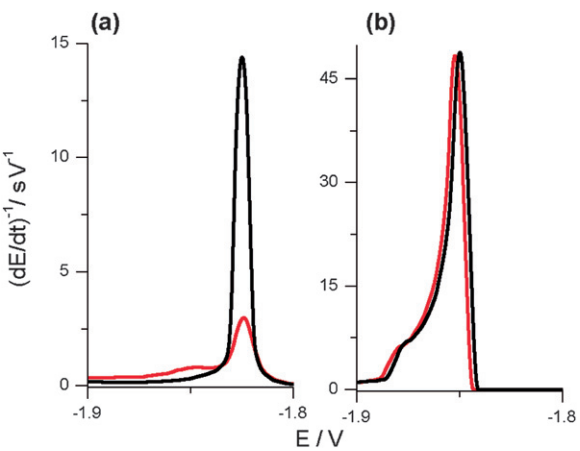

Fig. 2 *Ex situ* peak H of native (red) and denatured BSA (black). BSA was adsorbed at the HMDE from 50 mM sodium acetate pH 4.5 in presence of 56 mM urea; the BSA-modified electrode was transferred to 50 mM sodium phosphate buffer, pH 7 (a) or 50 mM sodium acetate, pH 4.5 (b), where the chronopotentiograms were recorded. Other details are as in Fig. 1c.

BSA is denatured at negatively charged electrode surface

Dependence on accumulation potential. To understand better the influence of the electrode potential on the surface-attached BSA we measured the dependence of peak H of native and denatured BSA on the accumulation potential, E_A.

Denatured BSA. As in the previous experiments (Fig. 2a) BSA was adsorbed from 50 mM sodium acetate, pH 4.5, for t_A 60 s, but the E_A varied between +0.1 and −1.6 V. From E_A +0.1 to about −0.8 V the peak height of the denatured BSA gradually increased; at more negative E_A values a decrease followed, reaching the original value at $E_A \approx -1.1$ V (Fig. 3a–c,f). At more negative E_A values such as −1.4 V this peak greatly decreased (Fig. 3d) suggesting that at these potentials the amount of adsorbed BSA molecules decreased because of strong repulsion of BSA from the electrode surface. At E_A −1.6 V the peak split into two peaks (H-1d with E_p −1.75 V and H-2d, E_p −1.90 V) (Fig. 3e). This peak splitting might be due to various reasons, such as the co-existence of differently oriented BSA molecules at the electrode charged to highly negative potentials, or reduction of some protein groups by nascent hydrogen, *etc*. More work will be necessary to understand better this phenomenon. At E_A −1.6 V both peaks were small (Fig. 3e), their heights corresponding to about 25% of peak H height at E_A values between 0.1 and −1.2 V suggesting that at E_A values around −1.6 V repulsion forces prevented stronger BSA adsorption.

Native BSA. Between E_A +0.1 V and −0.6 V the peak H of native BSA was much smaller than that of the denatured BSA, changing only slightly with E_A (Fig. 3a,b,f); at more negative E_A values it grew steeply (Fig. 3c,f), and between E_A −1.0 and −1.4 V the height of this peak was close to that of denatured BSA. A sharp decrease of this peak at E_A values more negative than −1.4 V differed only slightly from that of the denatured BSA (Fig. 3f). At E_A values −1.5 and −1.6 V, peaks of native and denatured BSA were almost identical (Fig. 3e). Striking increase of peak H of native BSA between E_A values −0.7 and −1.0 V (Fig. 3f) may indicate conformational change in the surface-attached BSA, which occurred at pH 4.5 during the BSA accumulation at the electrode charged to these and more negative E_A values. No such change was observed with native BSA in alkaline media at any E_A between 0 and −1.4 V.[13]

Nature of BSA surface denaturation

What is the nature of this BSA conformation change at the electrode surface and why did it occur at acid pH? At acid pH, orientation of the BSA molecule at the electrode surface appears favorable for the reduction of at least some of the BSA disulfide bonds.[19,26] Among the BSA 17 disulfide bonds, several of the exposed bonds are reduced at the mercury electrodes. Moreover, chemical reduction of native BSA induced changes in the protein structure resulting in a large increase in peak H, similar to BSA denaturation.[17] Square wave voltammetry of native and denatured BSA in 50 mM sodium acetate, pH 4.5 showed reduction peaks at *ca.* −0.5 V[19] (not shown) but no sign of electroreduction of the BSA disulfide bonds between −0.7 and −1.4 V. Thus electroreduction of some disulfide bonds at −0.5 V may contribute to the observed effects (Fig. 3) by destabilizing the BSA molecule, but the surface denaturation between E_A −1.0 and −1.4 V has to be due to additional factors. Recently we observed at neutral pH an ionic strength-dependent structural transition in surface-attached BSA induced by the electric field.[18] The electric field can cause alteration of the charge distribution in the adsorbed protein brought about by shifts in the acid–base equilibrium towards the ionized forms,[27] polarization of hydrogen bonds, alignment of the molecular dipoles and displacement of the charged residues.[28] Acid BSA surface denaturation may thus take place (in the protein attached to the negatively charged electrode surface) at the background electrolyte pH values much higher than those necessary for BSA

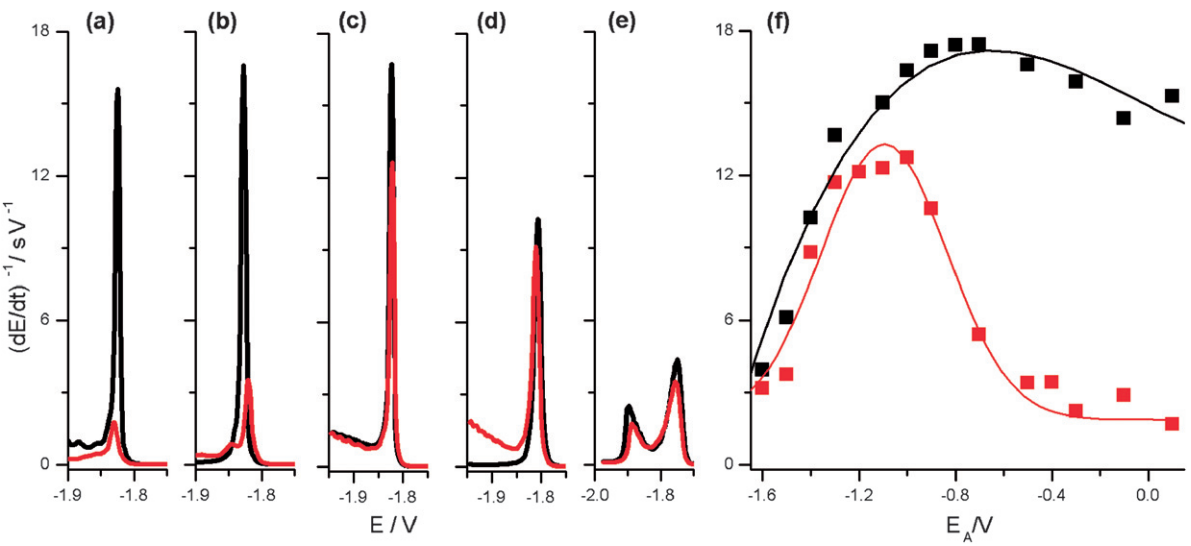

Fig. 3 100 nM native (red) and urea-denatured (black) BSA was adsorbed at different accumulation potentials, E_A −0.1 V (a), −0.5 V (b), −1.1 V (c), −1.4 V (d) and at −1.6 V (e) from 50 mM sodium acetate pH 4.5 in presence of 56 mM urea followed by transfer of the BSA-modified HMDE to 50 mM sodium phosphate, pH 7. Dependence of peak H height on accumulation potential (f). Other details are as in Fig. 2b.

denaturation in solution (about pH 2.7).[29] Earlier it was shown that relatively slow (around 60 s) surface denaturation of chromosomal DNA took place at mercury electrodes at neutral pH.[30–32] In contrast, surface denaturation of DNA at weakly acid pHs was much faster (>1 s).[33]

Experimental

Materials

Bovine serum albumin (BSA) and urea were purchased from Sigma-Aldrich Chemical Co. All other chemicals used were of analytical grade, solutions were prepared from triply distilled water. pH values of unbuffered KCl solutions were adjusted by the addition of diluted HCl or KOH to the appropriate pH's.

Apparatus and electrochemical procedures

Electrochemical measurements were performed with an AUTOLAB Analyzer (EcoChemie, Utrecht, Netherlands) in connection with VA-Stand 663 (Metrohm, Herisau, Switzerland); an HMDE (area of drop 0.4 mm^2) was used as a working electrode in the standard cell with a three-electrode system. An Ag/AgCl/3 M KCl electrode served as the reference electrode and a platinum wire as the auxiliary electrode. Experiments were carried out in a thermostated cell at 18.5 °C open to air.

Adsorptive Stripping (AdS, *in situ*). The working electrode was immersed into the electrolytic cell containing the protein solution in the background electrolyte for the accumulation time, t_A (usually 60 s) at the accumulation potential, E_A −0.1 V with stirring (1500 rpm), and the chronopotentiograms were recorded.

Adsorptive Transfer Stripping (AdTS, *ex situ*)[4,25]. BSA was adsorbed at E_A −0.1 V for t_A 60 s, under stirring, then the BSA-modified HMDE was washed and transferred into a blank background electrolyte.

Parameters of derivative CPS analysis. The initial potential, E_i was −0.1 V, the potential negative limit, E_f was −2 V and the stripping current, I_{str} was −40 μA, if not stated otherwise.

Denaturation of proteins

Denaturation was performed by incubating 14.4 μM BSA in 0.1 M Tris–HCl, pH 7.4 with 8 M urea at 4 °C overnight. Then the protein solution was diluted to the final protein concentration (usually about 100 nM; final concentration of urea usually was 56 mM) and immediately measured.

Conclusions

CPS analysis of BSA and other proteins at alkaline and neutral pH showed relatively small electrocatalytic responses (peak H) of their native forms as compared to large responses of their denatured forms.[13,16–18] Increasing the buffer concentration from 50 mM to 200 mM at neutral pH resulted in an increase of peak H of native BSA to the height of denatured BSA, suggesting that an ionic strength-driven BSA structural transition took place at the electrode surface.[18] No changes in peak H of native BSA were observed as a result of the protein adsorption at E_A −0.1 V. The BSA surface denaturation took place only at more negative potentials. Thus the behavior of native BSA in 200 mM sodium phosphate, pH 7, can be compared to that in 50 mM sodium acetate, pH 4.5, shown in this paper (Fig. 2). In both media (i) BSA denatured at the electrode charged to negative potentials and (ii) the surface denaturation was so fast that even the CPS working with very high rates of potential changes was not able to avoid this denaturation. Our results[13,16–18] suggest that the long time-held view that proteins are denatured when adsorbed at mercury electrodes was too general. At mercury electrode surfaces, proteins can be denatured but such denaturation depends on a number of factors, including electrode charge, pH, ionic strength, temperature, time and properties of the given protein. So far we have not observed any protein denaturation, resulting from 60 s adsorption of BSA at the HMDE at open-circuit potential. Application of electrochemical methods working with very fast potential changes during the electrode polarization makes it thus possible to study changes in protein conformation, if proper solution conditions are chosen. It is, however, important that the electrochemical method of choice should be able to measure protein-catalyzed hydrogen evolution even under conditions of very fast potential changes. Our preliminary results show that the increase of voltammetric electrocatalytic peaks of some proteins and other biomolecules with scan rate (at low scan rates) is followed by a decrease at higher scan rates.[34] It is interesting that irreversible adsorption of native BSA at the HMDE does not significantly change the native state of the protein. Thus a BSA-modified HMDE can be easily prepared and transferred to different medium to be subjected to various kinds of interaction and (*ex situ*) electrochemical measurements. It should be stressed that peak H of proteins can be obtained not only with an HMDE, but also with solid (dental) amalgam electrodes.[5,35] This finding opens the door to application of peak H in highly parallel analysis of proteins for proteomics and biomedicine. Such an application appears particularly interesting in relation to the recent results showing the sensitivity of peak H to pre-aggregation and aggregation of α-synuclein in Parkinson's disease[6] and amino acid exchange in mutant tumor suppressor protein p53.[5]

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