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Protein Structure-Sensitive Electrocatalysis at Dithiothreitol-Modified Electrodes

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Abstract: Dithiothreitol (DTT)−mercury and DTT−solid amalgam electrodes are proposed for protein microanalysis by means of constant current chronopotentiometric stripping (CPS). At the DTT-modified hanging mercury drop electrode (DTT-HMDE), proteins at nanomolar concentrations produce the CPS peak H, which is due to the protein catalyzed hydrogen evolution. Self-assembled monolayers (SAMs) of DTT at the electrode surface protected surface-attached proteins from the electric field-driven denaturation, but did not interfere with the electrocatalysis. Using CPS peak H, native and denatured forms of bovine serum albumin (BSA) and of other proteins were easily distinguished. On the other hand, in usual slow scan voltammetry (scan rates between 50 mV/s and 1 V/s), the adsorbed BSA behaved as fully or partially denatured. BSA-modified DTT-HMDE was exposed to different potentials, E_B for 60 s, followed by CPS measurement. Three E_B regions were observed, in which either BSA remained native (A, −0.1 to −0.3 V), was denatured (B, −0.35 to −1.4 V), or underwent desorption (C, at potentials more negative than −1.4 V). At potentials more positive than the reduction potential of the DTT Hg−S bond (∼ −0.65 V against Ag|AgCl|3 M KCl), the densely packed DTT SAM was impermeable to $[Ru(NH_3)_6]^{3+}$. At more negative potentials, the DTT SAM was disturbed, but under conditions of CPS (with very fast potential changes), this SAM still protected the protein from surface-induced denaturation. Thiol-modified Hg electrodes in combination with CPS represent a new tool for protein analysis in biomedicine and proteomics.

Introduction

In early the 1990s, a remarkable increase started in electrochemical analyses of nucleic acids,[1] related to progress in genomics, and particularly in the Human Genome Project.[2] Following advances in proteomics[3−6] and biomedicine requires new sensitive and simple methods for protein analysis. Since the beginning of the 1970s, electrochemistry of proteins dealt predominantly with conjugated proteins containing nonprotein redox centers yielding reversible electrode processes.[7−11] This important field of protein electrochemistry was, however, limited by its nature to a very small fraction among the tens of thousands of proteins occurring in nature.

The first paper on electrochemical (polarographic) analysis of proteins was published in 1930 by Heyrovsky and Babicka.[12] It was shown that proteins are able to catalyze hydrogen evolution at the dropping mercury electrode producing the so-called presodium d.c. polarographic wave. The wave, which appeared too close to the background discharge, was poorly developed and thus considered of little analytical use. The history of the research into electrochemical signals of proteins due to the catalytic hydrogen evolution has been reviewed.[13−16]

Recently, we have shown that using constant current chronopotentiometric stripping (CPS) analysis well-resolved electrocatalytic signal (peak H) is produced at bare mercury electrodes by all tested proteins regardless of the presence or absence of redox centers in their molecules.[17−19] Peak H differs from the previously studied electrochemical signals of proteins[13,15] particularly (i) by its ability to detect proteins down to nanomolar and subnanomolar concentrations[17,19−26] and (ii) by its high

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sensitivity to local and global changes in protein structures[17−19,21−24,27] and to protein redox states.[19,20,27] In adsorptive transfer stripping (*ex situ*) experiments,[18,28] 3−10 μL drops of protein solution are sufficient for the analysis with HMDE, thus, allowing protein determination at femtomole levels.[29] It has been further shown that amino acid residues containing labile protons, such as cysteine, lysine, and arginine, can be involved in the electrocatalysis responsible for peak H.[18,20,27] Their participation in the electrode process is affected not only by their accessibility in the given protein conformation, but also by ionic conditions, including pH, nature, and concentration of ions in the background electrolyte, and so forth. In early experiments with dropping mercury electrode it was possible to observe differences between the polarographic waves of native and denatured proteins[14,15,30] but later, when dropping mercury electrode was replaced by stationary electrodes, it was concluded that proteins are denatured when adsorbed at metal surfaces (including bare mercury).[31−33] Only few results contradicted this conclusion.[18,34] For example, it was shown that urease and alcohol dehydrogenase adsorbed at bare liquid mercury or copper amalgam foil retained their enzymatic activity.[34]

We have shown that, depending on ionic conditions[22] and the speed of electrode charging to negative potentials,[17] proteins can either retain their native structures, or undergo denaturation at bare mercury electrodes[18,22,23] (further, surface denaturation). In particular, we have shown that BSA and other proteins behaved as native in 50 mM but not in 200 mM sodium phosphate, pH 7; in the latter medium, native and denatured BSA forms displayed almost identical responses.[22] We explained the surface denaturation of proteins at higher ionic strength by the effect of the electric field on the structure of the protein immobilized at the electrode surface.

Earlier it was shown that alkanethiols[35,36] and thiolated DNA[37] form impermeable, pinhole-free SAMs on Hg surfaces in short time intervals. Modification of gold electrodes with alkanethiol SAM's has been widely used in the analysis of conjugated proteins, that is, those containing nonprotein electroactive redox centers.[38] Protein catalyzed hydrogen evolution,[15] which is responsible for peak H, has not been, however, observed with gold electrodes but solely with mercury electrodes.[13,15,18,28]

In this paper, we propose thiol-modified Hg electrodes with surfaces that can be engineered with chemical groups suitable for immobilization of a given protein (as with SAM-modified Au electrodes). In contrast to Au electrodes (which have been applied predominantly to a small group of conjugated proteins),[7−10,31] thiol-modified Hg electrodes offer a new quality, that is, detection of practically all proteins by means of peak H, which sensitively reflects changes in protein structure. To prevent direct contact of proteins with the mercury surface, we created a dithiothreitol (DTT) SAM at the hanging mercury drop electrode (HMDE) surface (DTT-HMDE). We chose DTT because this reducing agent is frequently added to protein solutions at millimolar concentrations to keep proteins in their reduced state. We immobilized BSA and other proteins at DTT-HMDE and showed (i) that they produce peak H and (ii) that native BSA, which underwent surface denaturation at the bare HMDE, displayed no sign of denaturation when immobilized at DTT-HMDE under otherwise the same conditions.

Experimental Section

Materials. Bovine Serum Albumin (BSA), Human Serum Albumin (HSA), rabbit aldolase from rabbit muscle, cytochrome c from bovine heart, gamma globulin from bovine blood, and guanidinium chloride were purchased from Sigma-Aldrich Chemical Co. Recombinant glutathione-*S*-transferase was expressed from *Escherichia coli* and purified by courtesy of Dr. M. Brázdová. All other chemicals were of analytical grade, solutions were prepared from triply distilled water.

Apparatus. Electrochemical measurements were performed with an AUTOLAB Analyzer (EcoChemie, Utrecht, The Netherlands) in combination with VA-Stand 663 (Metrohm, Herisau, Switzerland); hanging mercury drop electrode (HMDE) or polished silver Solid Amalgam Electrode (p-AgSAE) was used as the working electrode in a standard cell in a three-electrode system. An Ag|AgCl|3 M KCl electrode served as the reference electrode and a platinum wire as the auxiliary electrode. CPS experiments were carried out at room temperature, open to air.

Procedures. DTT-Modified Electrodes. HMDE was immersed in 1 mM DTT for accumulation time, t_A 60 s at accumulation potential, E_A −0.1 V. Freshly prepared DTT-modified HMDE (DTT-HMDE) was immediately used for CPS or voltammetric measurements. Polished silver Solid Amalgam Electrode (p-AgSAE) was prepared as described[39] and modified by DTT in the same way as DTT-HMDE, but using t_A 120 s. In *Adsorptive Stripping* (*in situ*)[18] experiments, bare HMDE or DTT-HMDE were immersed into the electrolytic cell (containing the protein in the background electrolyte) at E_A −0.1 V, for t_A (usually 60 s), followed by CPS or voltammetric measurements. In *Adsorptive Transfer* (AdT, *ex situ*) *stripping*,[18] the protein was adsorbed from a 5 μL drop of protein solution followed by washing of the protein-modified electrode and its transfer to the electrolytic cell with blank background electrolyte (not containing any protein) to perform CPS.

Protein Denaturation. A total of 1 mg/mL of protein in 0.1 M Tris-HCl, pH 7.5 with 6 M GdmCl was incubated overnight at 4 °C, if not stated otherwise. Electrochemical measurements were

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performed immediately after dilution of the denatured protein. Proteins were diluted to 100 nM or higher concentrations as indicated in the text. No attempt was made to remove GdmCl from the protein solution, but its (nondenaturing) concentration was kept constant (70 mM). This procedure enabled us to measure the protein immediately after the dilution, thus, preventing the protein aggregation frequently occurring shortly after removal of the denaturing conditions.

Protein Reduction. A total of 1 mg/mL of BSA in 0.1 M Tris-HCl, pH 7.5 with 1 mM DTT was incubated overnight at 4 °C.

Results and Discussion

CPS of Native and Denatured BSA at DTT-Modified Mercury Electrode. To analyze BSA, we prepared DTT-HMDE by immersing the electrode in 1 mM DTT at accumulation potential, E_A −0.1 V for accumulation time, t_A 60 s (for more details see Supporting Information). Freshly prepared DTT-HMDE was washed and immediately immersed in 100 nM BSA, 0.2 M McIlvaine buffer, pH 7.0, in presence of 70 mM of guanidinium chloride for t_A 60 s at E_A −0.1 V, followed by recording of the chronopotentiogram. GdmCl-denatured BSA produced a well-developed peak H with peak potential, E_p of −1.75 V (Figure 1b), while native BSA yielded almost no signal under the same conditions, suggesting that a high peak H produced by native BSA at the bare HMDE (Figure 1a) resulted from the contact of BSA with the bare mercury electrode surface. Analogous results were obtained also with urea-denatured BSA in 0.2 M sodium phosphate, pH 7 (not shown). Using DTT-HMDE, peak H of 100 nM BSA greatly increased with increasing buffer capacity (Figure S2a) as well as with decreasing pH (Figure S2b). This behavior is similar to that obtained at bare HMDE, which is typical for the catalytic hydrogen evolution.[15,16,40] For example, at pH 5, this peak was >100-fold higher than at pH 7.6. Under the same conditions, peak S (due to the reduction of the Hg−S bond) did not increase (Figure S2b).

Native and Denatured BSA Forms Are Adsorbed at DTT-HMDE. Absence of peak H (Figure 1b) or presence of only very small peak in native BSA can be explained either by negligible adsorption of native BSA at the electrode or by inaccessibility of the catalytic sites in the surface-attached protein molecules. Using bare HMDE and linear sweep voltammetry (LSV) at alkaline pH, earlier we observed very small differences between LSV peak heights of denatured and native BSA, in contrast to large differences between CPS peak H of these two species under the same conditions.[17] These results suggested that both native and denatured BSA were adsorbed at the electrode but slow scan voltammetry was rather inefficient in differentiating native BSA from its denatured form. We further found that in the studied stripping current (I_{str}) range, peak H of native BSA was substantially smaller than that of denatured BSA. Shifting I_{str} in the cathodic direction resulted in an increase of the ratio of peak heights of denatured/native BSA forms,[17] suggesting that the rate of potential changes in the CPS experiment (about 1000 V/s in Figures 1−4) is important for recognition of native BSA from its denatured form. Here, we used cyclic voltammetric stripping to measure native and denatured BSA at DTT-HMDE. At the scan rate 50 mV/s, native and denatured forms of 1 μM BSA yielded well-developed peaks at −1.74 V differing only slightly from each other (Figure 1d). At 1 V/s scan rate, the peak of native BSA was about 2.5-fold smaller than that of denatured BSA (Figure 1e). The ratio of peak heights of denatured/native BSA at 1 V/s still greatly differed from CPS, which produced peak H of native BSA approximately 75-fold smaller as compared to that of denatured BSA under similar conditions (Figure 1b). These results show, in agreement with the previous measurements on bare HMDE,[17] that also at DTT-HMDE native BSA is adsorbed to about the same extent as denatured BSA, but the former may become denatured during slow potential scanning to negative potentials.

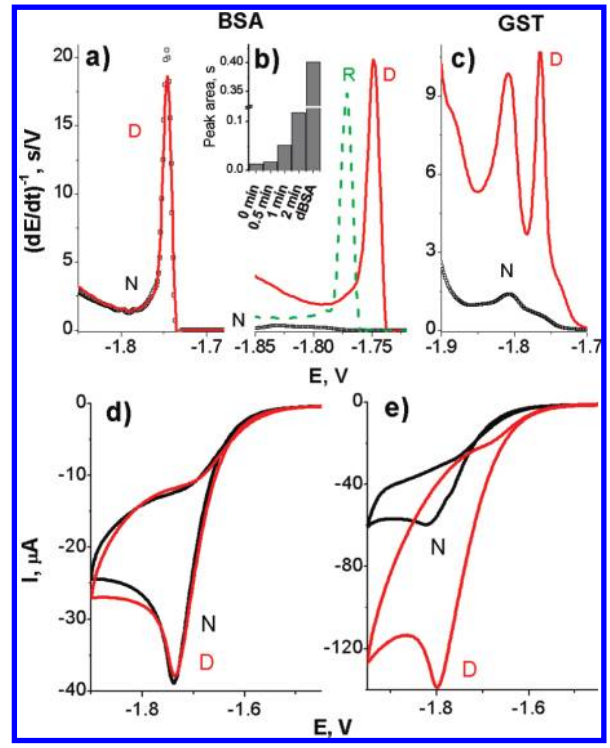

Figure 1. (a and b) Conventional (*in situ*) constant current chronopotentiometric stripping (CPS) peak H of 100 nM native BSA (N, black), DTT-reduced BSA (R, green), and guanidinium chloride (GdmCl)-denatured BSA (D, red) (a) at bare and (b) at DTT-modified HMDE (DTT-HMDE) in 0.2 M McIlvaine buffer, pH 7. GdmCl (70 mM) was present in all samples; stripping current, I_{str} −70 μA. (Inset) Dependence of peak H area on BSA treatment with GdmCl. 14.4 μM BSA in 0.1 M Tris-HCl, pH 7.8 was treated with 2 M GdmCl at 37 °C for 0, 30, 60, 120 s, and overnight; samples were withdrawn in the indicated time intervals. After this treatment, the protein solution was 36-fold diluted and immediately used for CPS measurements. (c) Adsorptive transfer (*ex situ*) CPS peak H of 5 μM native (N, black), and urea-denatured (D, red), glutathione-*S*-transferase (GST) with 5 mM DTT at DTT-HMDE. The DTT-modified HMDE was immersed in 5 μM GST for t_A 180 s at open current potential. Then, the GST-modified DTT-HMDE was washed and transferred into 50 mM sodium phosphate, pH 7.0, to perform the CPS analysis; I_{str} −10 μA. (d and e) AdT stripping cyclic voltamograms of native (N, black) and denatured BSA (D, red) at DTT-modified HMDE at scan rates of (d) 50 mV/s and (e) 1 V/s. In this experiment, 1 μM BSA was adsorbed at DTT-HMDE from 0.2 M McIlvaine buffer, pH 7, for t_A 60 s. The *ex situ* procedure was applied to prevent additional BSA adsorption during the potential scanning.

BSA Is Denatured at DTT-HMDE in a Wide Range of Negative Potentials. To learn in which potential region the BSA surface denaturation may take place, we first adsorbed native BSA on DTT-HMDE at accumulation potential, E_A −0.1 V for t_A 60 s and exposed it to different potentials E_B for t_B 60 s. Between E_B −0.1 and −0.3 V (region A), almost no change in peak H height occurred, while between −0.35 and −0.50 V, a steep increase in this peak took place (Figure 2). Between E_B −0.5 and −1.4 V (region B), peak H height remained almost constant, but at more negative E_B (region C), peak H steeply decreased. Similar decrease in the region C was observed also

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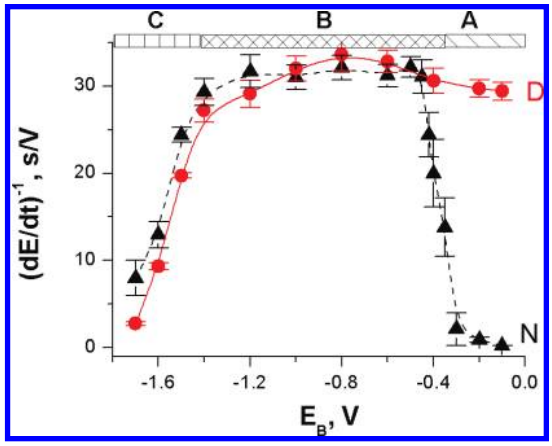

Figure 2. Dependence of peak H height of native (black) and denatured (red) BSA on potential E_B. BSA (200 nM) was adsorbed at DTT-HMDE at E_A −0.1 V for t_A 60 s and exposed to different potentials E_B for t_B 60 s in 0.2 M McIlvaine buffer, pH 7, with 70 mM GdmCl. Results represent mean (± SD) obtained at least from 3 independent measurements. More details are in the text and in Figure 1.

with denatured BSA, suggesting that this decrease is due to desorption of BSA from the electrode charged to highly negative potentials for 60 s. Figure 2 shows that peaks H of "native" BSA at E_B more negative than −1.0 V are higher than those of the denatured BSA; this is particularly well visible at E_B −1.7 V, where peak H of native BSA is about 3-fold higher than that of denatured BSA. It is probable that BSA, which was denatured secondarily at the electrode, does not form a surface layer identical to that of BSA, which was denatured in solution (with 6 M GdmCl).

In region B, peak heights of native and denatured BSA were about the same, suggesting that 60 s contact of native BSA with the electrode charged to potentials of the region B resulted in the BSA denaturation. In contrast, in region A, the heights of peak H of native BSA were much smaller than those of denatured BSA suggesting that surface-induced denaturation of BSA in this region was negligible. The steep increase in peak height of native BSA took place between E_B −0.35 and −0.50 V, that is, at potentials slightly more positive than peak S (Figure S1), which indicates reduction of the DTT Hg−S bond. These results suggest that prolonged contact of the DTT SAM with the electrode charged to potentials of region B resulted in destruction of the SAM (formed at more positive potentials) followed by denaturation of BSA in 0.2 M McIlvaine buffer at the mercury surface (with destroyed DTT layer), similar to surface denaturation at bare HMDE at comparable buffer concentrations.[22]

We may thus conclude that formation of the DTT SAM at the HMDE is sufficient for preventing significant BSA denaturation in the CPS experiments (Figure 1b), but prolonged exposure of the DTT-modified electrode to potentials more negative than −0.4 V destroys the DTT SAM at the electrode surface (Figure 2) and enables BSA denaturation (at potentials more negative than in region B). Similarly, in voltammetry disturbances in the DTT SAM may occur during slow potential scanning over the region B (Figure 1e). In contrast to these results, surface denaturation of BSA at negatively charged bare HMDE observed at neutral pH (in 0.2 M but not in 50 mM buffers, pH 7.4)[22] and at acid pH values (even at low ionic strengths)[23] was much faster.

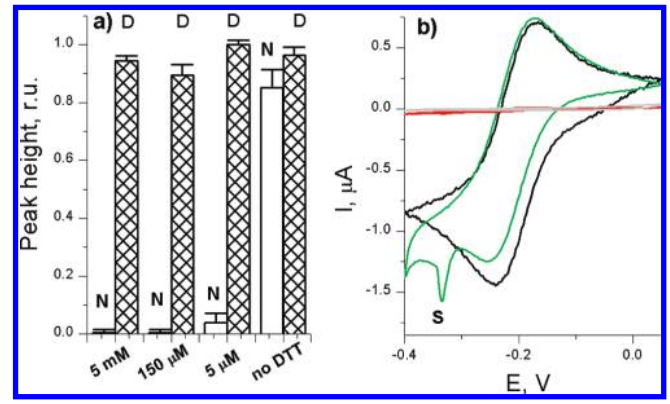

Figure 3. (a) Bar graph of relative peak H heights of 100 nM BSA (in presence of 70 mM GdmCl) at DTT-HMDEs prepared with different concentrations of DTT. Native BSA (N) and denatured BSA (D) at bare and DTT-HMDEs prepared by treating HMDE with 5 μM, 150 μM and 5 mM DTT in 0.2 M McIlvaine, pH 7. The peak H height of denatured BSA at the bare HMDE was taken as 1. Results represent mean (± SD) obtained at least from 3 independent measurements. (b) Cyclic voltammograms of 1 mM $[Ru(NH_3)_6]^{3+}$ obtained with bare HMDE (black) and DTT-HMDE prepared with 5 μM (green), 150 μM (red), and 5 mM DTT (gray). Peak S is related to the reduction of Hg−S bond formed by DTT at positive potentials. CV: scan rate, ν 0.5 V/s. Other details as in Figure 1a,b.

In CPS Experiments, Protein Denaturation at DTT-HMDE Is Prevented. A large difference between peak H heights of native and denatured BSA (Figure 1b) was observed in 0.2 M McIlvaine in a wide range of BSA concentrations (from about 70 to 300 nM BSA, Figure S3). These results suggest that the BSA surface denaturation (occurring on bare HMDE in 0.2 M McIlvaine, Figure 1a)[22] does not proceed at DTT-HMDE. This can be due (i) to increasing the distance of the adsorbed protein from the electrode surface (and decreasing the effect of the electric field on the adsorbed protein) and/or (ii) to different properties of the DTT layer (to which BSA is adsorbed) as compared to the metal mercury surface. High hydrophobicity and affinity of Hg to sulfur are involved in BSA adsorption on bare Hg surface.[32] Earlier we suggested that strong adsorption of BSA (due to these two factors) at the bare HMDE combined with repulsion of the protein from the negatively charged electrode may result in the protein denaturation.[22] Elimination of the BSA surface denaturation at DTT-HMDE described in this paper is thus in a good agreement with our earlier suggestion.[22]

Impermeability of the DTT SAM at HMDE. Some compounds, such as $[Ru(NH_3)_6]^{3+}$, were used as probes for testing the integrity of thiol SAMs at Hg electrodes.[35−37,41] The distinct $[Ru(NH_3)_6]^{3+}$ redox peaks disappeared after alkanethiol deposition.[41] Here, we probed the permeability of the DTT layer using $[Ru(NH_3)_6]^{3+}$. Cyclic voltammetric scans were limited to the range from +50 to −400 mV to prevent complications due to oxidation of mercury at more positive potentials and reduction of the Hg−S bond at negative potentials. Figure 3 shows a cyclic voltammogram of $[Ru(NH_3)_6]^{3+}$ at a bare HMDE and DTT-modified HMDE obtained with different concentrations of DTT at t_A 60 s. The SAM produced by 5 μM DTT decreased peak H of native BSA ~30-fold (Figure 3a), but permeability of this SAM for $[Ru(NH_3)_6]^{3+}$ was decreased only slightly (Figure 3b), suggesting that the DTT layer contained a number of defects, which were large enough to permit the $[Ru(NH_3)_6]^{3+}$ redox process without allowing contact of a significant number of

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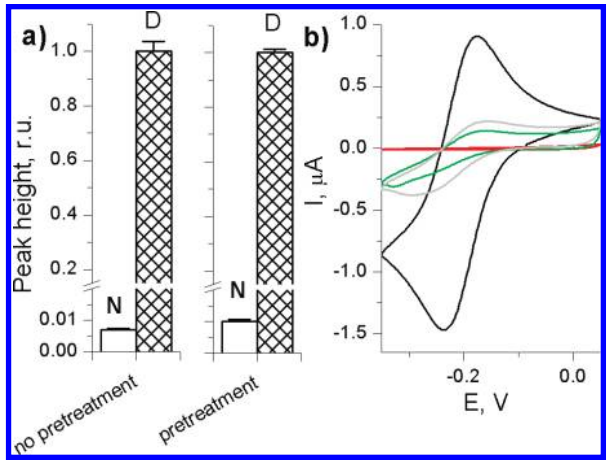

Figure 4. (a) Bar graph showing the effect of CPS pretreatment on relative peak H heights of native (N) and denatured (D) BSA at DTT-HMDE. DTT-HMDE was immersed in the cell with 100 nM BSA in the background electrolyte for t_A 60 s and the BSA-modified DTT-HMDE was pretreated using CPS from −0.1 to −1.7 V, followed immediately by peak H recording from −0.1 V. Results represent mean (± SD) obtained at least from 3 independent measurements. (b) Cyclic voltammograms of 1 mM $[Ru(NH_3)_6]^{3+}$ in the background electrolyte at bare HMDE (black) and DTT-HMDE without (red) and with CPS pretreatment from −0.1 to −1.5 V (gray) or to −1.7 V (green). Other details as in Figures 1 and 3.

much larger BSA molecules with the bare mercury surface. At higher DTT concentrations, peak H of native BSA disappeared while peak H of denatured BSA practically did not change (Figure 3a). Under these conditions, almost no current was produced by $[Ru(NH_3)_6]^{3+}$ (Figure 3b) indicating formation of a densely packed DTT layer insulating the mercury surface (charged to potentials between +0.05 and −0.4 V) from contact with $[Ru(NH_3)_6]^{3+}$.

We measured peak H of native and denatured BSA as well as permeability of the DTT layer to $[Ru(NH_3)_6]^{3+}$ after running CPS analysis from −0.1 to −1.5 or −1.7 V. Such treatment resulted in a small increase of the permeability of the DTT SAM to $[Ru(NH_3)_6]^{3+}$ (Figure 4b), which was, however, much smaller than that obtained with DTT-HMDE prepared by immersion in lower (5 μM) DTT concentration (Figure 3b). The same treatment did not result in any significant change in the height of peak H (Figure 4a). We may thus conclude that exposure of DTT-HMDE to CPS analysis from −0.1 V to potentials close to the foot of peak H disturbs integrity of the tightly packed DTT layer making it partially permeable for $[Ru(NH_3)_6]^{3+}$ (Figure 4b), without allowing contact of a significant number of BSA molecules with the bare mercury electrode. We can thus imagine that, in the CPS experiment (Figure 4) at potentials of peak H, BSA remains adsorbed at the disturbed DTT layer. This layer protects the protein from denaturation but does not prevent the communication between the protein and the electrode necessary for the electrocatalysis.

Contact of BSA with DTT-HMDE Does Not Result in Significant Protein Reduction. Chemical reduction of disulfide bonds in native BSA resulted in a large increase of peak H at bare HMDE.[19] Figure 1b shows that at DTT-HMDE the chemically reduced BSA also produced a large peak suggesting denaturation of the protein after breaking the disulfide groups. On the other hand, adsorption of native BSA on DTT-HMDE for t_A 60 s did not result in appearance of peak H (Figure 1b), showing that no significant BSA reduction took place at the DTT-HMDE surface. Thus, the DTT-HMDE can be used not only for studies of proteins in their reduced states, but also for disulfide-containing proteins (such as blood proteins) just to prevent their contact with the bare mercury surface. Nevertheless, we expect that DTT-HMDE will be particularly useful for the analysis of intracellular proteins in their reduced state.

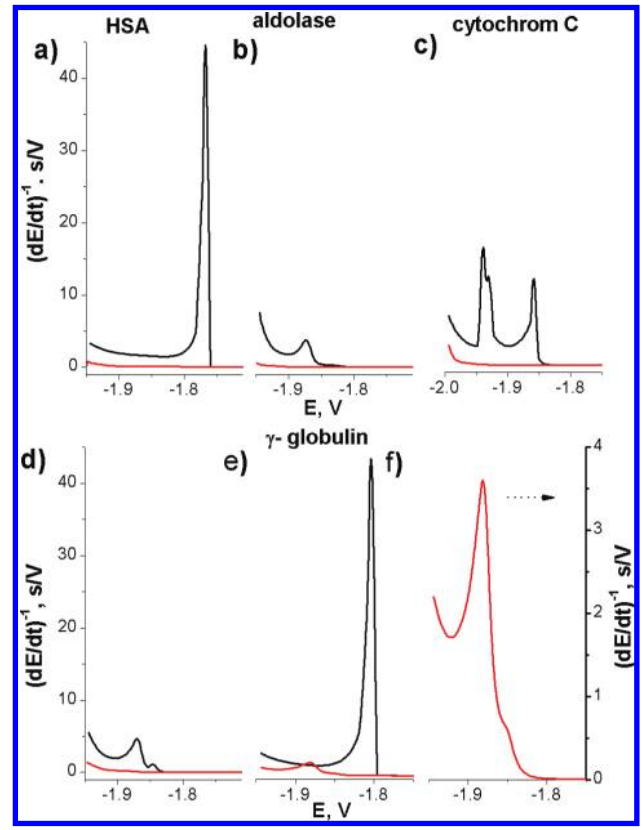

Figure 5. Peak H of 200 nM (a) HSA, (b) rabbit aldolase from rabbit muscle, (c) cytochrome C from bovine heart, and (d−f) gamma globulin from bovine blood at bare (black) and DTT-HMDE (red) in 0.2 M Na-phosphate, pH 7; (a−d) I_{str} −70 μA and (e and f) −50 μA; conventional (*in situ*) CPS. Other details as in Figure 1b.

Glutathione-*S*-transferase at DTT-HMDE and Solid Amalgam Electrode. Among a large number of proteins whose biological activity is related to their reduced state, we chose glutathione-*S*-transferase (GST) (for more details see Supporting Information). We used adsorptive transfer (*ex situ*) stripping[18] in which 5 μM urea-denatured GST with 5 mM DTT were coadsorbed at DTT-HMDE followed by transfer of the GST-DTT-modified electrode into blank background electrolyte. Denatured GST produced two peaks, H1 at −1.76 V and H2 at −1.81 V, in contrast to native GST which yielded much smaller single peak H at −1.80 V (Figure 1c). A large difference between the responses of denatured and native GST was obtained also when DTT-HMDE was replaced by DTT-p-AgSAE[39] (Figure S4, for more details see Supporting Information). Similar results were obtained also with other reduced proteins and BSA (Figure S5). The ability of the thiol-modified solid amalgam electrode to yield peak H (capable of sensing the protein structure) opens the door for utilizing peak H in sensors for highly parallel analysis in proteomics and biomedicine.

Peak H of Different Proteins at Bare and DTT-Modified Mercury Electrodes. We have analyzed a number of different proteins at bare HMDE and we observed peak H with all tested proteins.[18] Here, we show CPS responses of 200 nM BSA, human serum albumin (HSA), aldolase, cytochrome c, and γ-globulin in 0.2 M sodium phosphate, pH 7, both at bare and DTT-modified HMDE (Figure 5). Using I_{str} −70 μA, these

proteins yielded peak H at bare HMDE but not at DTT-HMDE (Figure 5a−d). Generally, less negative cathodic I_{str} (or lower current density) offers better sensitivity of the protein determination, while more negative stripping currents are important for preventing changes in protein structures occurring at negatively charged bare mercury electrodes. Using I_{str} −50 μA, native γ-globulin yielded a small peak H at DTT-HMDE (Figure 5f) as compared to much larger peak produced at bare HMDE (Figure 5e). Using higher sensitivity of the instrument, a well measurable peak was obtained even at DTT-HMDE (Figure 5f). Thus, both DTT-modified and bare Hg-containing electrodes can be used in protein research, depending on the aim of the analysis and experimental conditions. Our results show that different proteins in their native state can produce CPS signals both at bare and DTT-modified HMDE. At DTT-HMDE, native protein molecules are better protected from denaturation at the electrode surface and their CPS peaks can be therefore absent (Figure 5a−d) or rather small under conditions best suited for the protein structure analysis. Moderate decrease of the current density may help to obtain higher peaks (e.g., Figure 5e,f) without losing the sensitivity for changes in the protein structure. Greatly reduced current densities were successfully applied in the CPS analysis of a peptide containing only a single catalytically active amino acid residue.[42]

Comparison of CPS with Established Optical Methods. Our previous studies suggested that CPS peak H has a number of advantages[18,19,24,29] as compared to established methods of protein analysis. These advantages include very high sensitivity of protein determination and small volume requirements (few microliters of nanomolar protein are usually sufficient) as well as high sensitivity to changes in protein structures.[18,22,23] Here, we traced the initial stage of BSA denaturation by 2 M GdmCl at 37 °C using adsorptive transfer CPS (*ex situ*) with DTT-HMDE. Figure 1b inset shows significant increases of BSA peak H height after 30, 60, and 120 s of the GdmCl treatments. After 60 s, peak H area corresponded to about 13% of that of fully denatured BSA measured under the same conditions. These changes in peak H might be related to different orientation of partially denatured BSA molecules at the electrode surface. We also measured changes in tyrosine/tryptophan fluorescence, which showed a small decrease in the spectrum at 340 nm after 60 s treatment (see Figure S6). If we assume that both methods are equally sensitive to structural changes in the initial stage of BSA denaturation, we can see the advantage of CPS in the protein volume requirement (5 μL of 400 nM BSA compared to 2 mL of 500 nM BSA for fluorescence).

The proposed CPS method can thus complement the established methods. In addition, proteins interact with various surfaces *in vivo* and chemically modified electrode surfaces may represent simple and convenient models for such interactions. Electrochemical instrumentation is simpler and less expensive, requires lower energy, and is amenable to parallelization using protein chips, and the instrumentation is relatively inexpensive. Arrays of solid amalgam electrodes were recently constructed.[43,44]

Conclusion

This paper shows for the first time that the HMDE and solid amalgam electrodes can be modified using a DTT SAM to avoid protein contact with the bare mercury surface, but without losing the ability of proteins to catalyze hydrogen evolution. Very large differences in peak H heights between native and denatured BSA were observed (Figure 1b). These results suggested that native proteins did not denature as a result of their interaction with the DTT-HMDE. In solution, hydrophobic amino acid side chains are buried in native BSA (as in other natively folded proteins) and some catalytically active proton donor groups (e.g., in lysine, arginine and cysteine residues) can be inaccessible in the native protein. A large difference in catalytic peak H of folded versus unfolded (denatured) proteins can be thus expected unless the protein is rapidly denatured at the electrode surface. Our recent results suggest that BSA and other proteins do not denature as a result of their adsorption (close to neutral pH) at the bare Hg surface close to potential of zero charge.[22,35] Their denaturation may however occur when the potential is shifted to negative values. Such denaturation at the bare Hg electrode can be prevented by using CPS and adjusting proper ionic conditions[18,22,35] or by formation of thiol (such as DTT) layer at the electrode.

Compared to established label-free methods of measuring protein denaturation, such as fluorescence and circular dichroism, working frequently with micromolar protein concentrations, peak H is either more sensitive or comparable, but in the CPS, microliter volumes of protein solution can be used. Our previous work with bare mercury electrodes[24] showed that early changes preceding aggregation of α-synuclein (involved in Parkinson's disease) detected by peak H were in accordance with the dynamic light scattering but not detectable by fluorescence measurements. Specific binding of riboflavin to the riboflavin-binding protein[45] was detected using peak H. These results support the notion that electrochemical analysis is simpler and more sensitive than most of the established methods of protein analyses; in addition peak H possesses great resolution power for changes in protein structures. New technique proposed in this paper complements established methods of protein analysis and can be particularly useful in testing the effect of various drugs on structures of wild-type and mutant proteins. We believe that combination of electrocatalysis and thiol-modified mercury-containing electrodes opens the door for application of electrochemistry in protein research and particularly in biomedicine and proteomics.

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Supporting Information Available: The contents of Supporting Information includes the following: (1) electrochemistry of DTT and application of DTT as a protein reducing agent, (2) chronopotentiograms of DTT and denatured BSA, and (3) dependence of peak H height on the concentration of native and denatured BSA at bare HMDE and DTT-HMDE, (4) chronopotentiograms of glutathione-*S*-transferase (GST) and BSA at polished solid silver amalgam electrode, (5) fluorescence spectra of initial stage of BSA denaturation. This material is available free of charge via the Internet at http://pubs.acs.org.

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