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THE ELECTROCHEMISTRY OF PROTEINS AND RELATED SUBSTANCES PART III. BOVINE SERUM ALBUMIN

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ABSTRACT

The electroreduction of bovine serum albumin (BSA) in pH 7.4 solution was studied at the hanging mercury drop electrode by double potential step chronocoulometry and cyclic voltammetry, and at a mercury pool electrode by controlled potential coulometry. The proposed mechanism involves reduction of an adsorbed monolayer of BSA (maximum coverage 3.3 μC^{-2}); at short times 3 or 4 disulfide bonds are reduced, the product remains adsorbed and may be reoxidized. On the coulometric time scale (hours) 8 or 9 of the total 17 disulfide bonds are broken and an insoluble product which cannot be oxidized is formed.

INTRODUCTION

There have a been a number of studies of the adsorption and reduction of proteins on mercury electrodes (see [1—9] and references therein). The model which emerges from these studies involves strong adsorption of the protein molecule because of the interaction of the disulfide bonds with the mercury surface and subsequent reduction of these bonds at potentials in the vicinity of —0.5 to —0.7 V vs. SCE. Some questions of interest in connection with these electrochemical studies pertain to the number of disulfide bonds reduced and how this number relates to their availability or location in the molecule, the change in the structure or activity of the molecule (e.g., an enzyme) upon reduction, and the reformation of disulfide linkages upon oxidation of the reduced protein. In a previous paper [9] we described the reduction of insulin, a small protein of known structure containing three disulfide bonds. The results showed reduction of the adsorbed molecule with four electrons transferred (i.e. two disulfide linkages reduced); the adsorbed reduced product appeared to undergo a molecular reorientation on the electrode surface (with a rate constant of the order of 10^{-2} s^{-1}) such that upon reoxidation of the adsorbed species, a 2*e* reoxidation was observed. Following this oxidation, the reduction was a 2*e* reaction, and at steady state 2*e* reduction and oxidation reactions of adsorbed intermediates were found.

In this paper we discuss the behavior of bovine serum albumin (BSA), a molecule in the form of a single polypeptide chain of molecular weight 69,000, which is cross linked by 17 disulfide bonds [10]. The molecule also contains one free sulfhydryl group located on the molecular surface; 68% of it is in the free form and 32% of it is complexed with cysteine or glutathione [11]. The two-dimen-

sional structure of the molecule has been determined by Brown [12]. The polypeptide chain is brought into close proximity by two disulfide linkages in eight places; adjacent cysteine residues cannot cross link because the sulhydryl moieties are located *trans* to one another [12]. Other interactions, such as hydrogen bonding and Van der Waals forces, maintain the secondary and tertiary structure of the molecule, with about 50% of the species being in the alpha helix conformation [13]. There is some disagreement about the overall shape of the molecule. Multiple internal reflection studies by Harrick and Loeb [14] show that the molecule is spherical with a diameter of 60 Å. Nakagaki and Sano [15] reported the molecule to be a prolate spheroid with major axis, 2a = 140 Å, and minor axis, 2b = 70 Å using light scattering and viscosity measurements [15]. X-ray crystallographic data on BSA are not available.

There have been several studies concerned with the reduction of the disulfide bonds in serum albumins. Markus and Kharush [16] examined the reduction of the disulfide bonds of human serum albumin by β-mercaptoethylamine hydrochloride in the presence and in the absence of the denaturing agent sodium dodecyl sulfate (SDS). In the absence of SDS only one disulfide bond per molecule was reduced while in the presence of SDS, which causes unfolding of the protein molecule, all of the disulfide bonds reacted. These results suggest that most of the disulfide groups in the native BSA are inaccessible to the reducing agent. Kolthoff et al. [11] carried out amperometric titrations of the disulfide groups of native and denatured BSA with silver nitrate in the presence of sulfite at a rotating mercury pool electrode. In native BSA, no reactive disulfide was found; however at pH 9, in a solution containing 4 *M* guanidine hydrochloride and 0.05 *M* sodium bisulfite, all 17 disulfide bonds could be titrated. With 4 *M* guanidine hydrochloride alone at the mercury pool, the maximum number of reactive disulfide bonds was 11. Leach et al. [17] claimed that BSA was not electrochemically reducible directly on a mercury pool in the pH range 2.9 to 9.0 at potentials of −1.2 or −2.0 V vs. SCE. When they used β-mercaptoethanol as a mediator with the mercury pool held at potential −1.2 V vs. SCE, 15 disulfide bonds were reduced. Cecil and Weitzman [18] studied briefly the polarographic reduction of BSA at a dropping mercury electrode (DME). They observed a single wave at pH 1, with $E_{1/2}$ at −0.26 V vs. SCE; the current increased with concentration reaching a limiting value of 0.02 μÅ at 2.5 μ*M*. At pH 9.2 a very small wave was observed at −0.8 V at a concentration of 30 μ*M*. They suggested a value of $n = 10.5$ for the reduction using the Ilkovic equation and a diffusion coefficient of 4.49×10^7 cm^2s^{-1}. Kuznetsov and Shumakovich [19] concluded from their study of BSA adsorption at a hanging mercury drop electrode (HMDE) by a.c. polarography that BSA was irreversibly adsorbed and reversibly reduced and reoxidized. Behr et al. [20] found that adsorbed BSA at a HMDE blocked the reduction of cadmium ions at pH 3 but not at pH 7. They ascribed this behavior to a change in BSA charge with pH.

The use of d.c. polarography and the DME presents some problems in the study of BSA. The anodic processes following disulfide bond reduction cannot be studied easily using conventional polarographic techniques. The HMDE is also advantageous, because it has a smaller charging contribution (since the electrode area remains constant). Moreover the stationary drop of the HMDE can be left exposed to the very dilute protein solutions for sufficiently long times that ad-

sorption equilibration can occur; this is not possible with the DME. Finally the very useful technique of double potential step chronocoulometry [21] can be employed with the HMDE to measure faradaic current accurately even in the presence of appreciable charging current. We describe here cyclic voltammetric and double potential step chronocoulometric studies of BSA at the HMDE as well as controlled potential coulometric studies at a large mercury pool electrode, and propose a mechanism for the electrochemical behavior of BSA.

EXPERIMENTAL

The electrochemical cell for coulometry as well as electrodes, apparatus and techniques for cyclic voltammetry and coulometry have been described previously [7,9] and details of experimental procedures and complete listings of data are available [22]. The electrochemical cell used for most BSA studies, with the exception of the coulometric ones, was a 100 ml beaker with the top cut off, fitted with a Teflon cap. The electrode arrangement and procedures are the same as those previously described [7]. The double potential step chronocoulometry experiments were performed on an instrument similar to that of Anson and Payne [23] programmed with a Wavetek Model 114 function generator. Current-time traces were monitored with a Tektronix Model 564 oscilloscope. The instrument was interfaced to a PDP-12A (Digital Equipment Corp.) digital computer equipped with a real time clock and a 10 bit A-D converter. This apparatus has been previously described [24,25]. The BSA, from either Sigma (crystallized and lyophilized BSA) or Miles Pentex (crystallized bovine albumin) was used as obtained.

RESULTS

Cyclic voltammetry of BSA

A typical cyclic voltammogram (c.v.) for 0.1 *M* phosphate buffer, pH 7.4, containing 20 μM BSA at a HMDE, with a fresh mercury drop (area, 0.0267 cm^2 in all experiments) is shown in Fig. 1; very similar results are obtained for higher BSA concentrations. A c.v. of the buffer alone at this scan rate (v) showed a flat charging current of about 0.2 μA until background discharge at −1.5 V vs. SCE. Addition of BSA clearly depressed the charging current (i.e. decreases double layer capacity) and causes background reduction to occur at −1.35 V. A plot of the cathodic peak current (i_{pc}) of the symmetrical reduction wave (E_{pc} = −0.630 V) against v is linear with zero intercept for scan rates of 0.02 to 0.5 V s^{-1} (typical results at 0.4 m*M* are given in Table 1). These results suggest this wave can be attributed to reduction of adsorbed BSA. Upon scan reversal following this wave a single oxidation wave occurs (E_{pc} = −0.60 V). A plot of the anodic peak current of the symmetric oxidation wave vs. v is also linear with zero intercept for scan rates of 0.02 to 0.5 V s^{-1}. Upon repetition of the c.v. trace, the peak currents and peak potentials of the adsorption wave are virtually the same as those of the first trace (Fig. 1) except for what appears to be a slight change in the double layer capacitance. In a solution of this concentration the electrode attains adsorption equilibrium rather rapidly upon squeezing out

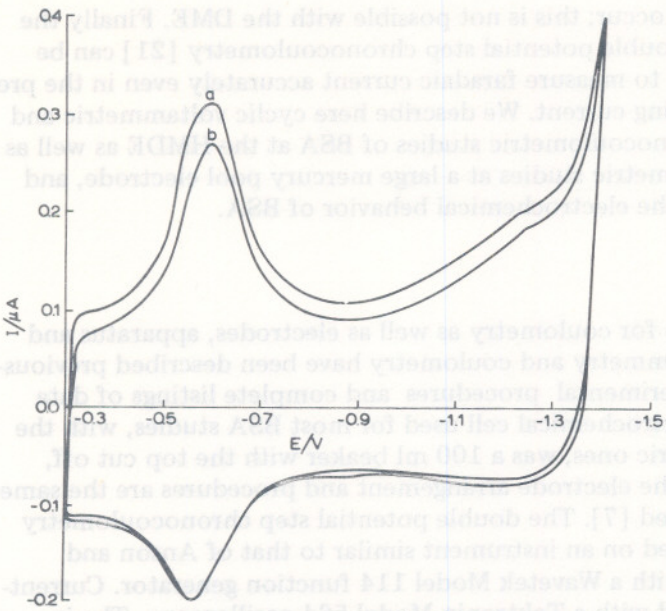


Fig. 1. Cyclic voltammogram of 20 μM BSA solution. pH 7.4, v = 0.2 V s^{-1}. (a) First scan; (b) second scan.

of a fresh drop and the integrated reduction current (Q_c) was 3.4 μC cm^{-2}, Q_a/Q_c was 0.89, and the ratio of $i_{pa}/_{pc}$ was 0.9. The ratio of i_{pa}/i_{pc} of slightly less than one perhaps suggests some desorption of the product but the apparent change and large contribution of the double layer charging made accurate measure of corrected peak currents and areas difficult. However the nearness of this ratio to unity and the fact that the steady state peaks are very close to those of the initial scans suggest that both reactant and product are strongly adsorbed. In contrast to the behavior of insulin [9] and cystine [7] no wave attributable to non-adsorbed BSA diffusing to the electrode following the adsorption wave was observed.

Since the peak currents and integrated peak areas were almost the same for

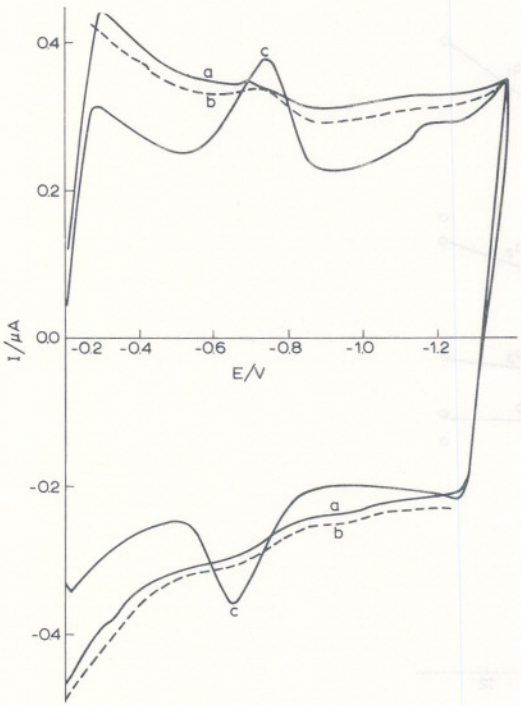
Table 1

Cyclic voltammetric peak currents for a 0.4 mM BSA solution (pH 7.4 phosphate buffer) at HMDE

Scan rate	$i_{pc}{}^a/\mu A$	$i_{pa}{}^b/\mu A$	i_{pa}/i_{pc}
500	0.44	0.353	0.80
200	0.17	0.15	0.88
100	0.08	0.077	0.96
50	0.040	0.033	0.83
20	0.018	0.018	1.00

[a] E_{pc} = −0.630 V at all v.
[b] E_{pa} = −0.600 V at all v.

20 μM and 0.4 mM BSA, saturation coverage of the electrode appears to occur at these concentrations. Lower concentrations were investigated to determine the adsorption isotherm. In experiments at sub-μM concentrations a fresh mercury drop was squeezed out into the quiet BSA solution and adsorption equilibrium was established by continuous cycling of the potential until steady state behavior was achieved. Typical very low concentration steady state voltammograms are shown in Fig. 2. No reduction wave is observed for a 10^{-9} M BSA solution. For a 10^{-8} M solution a small decrease in the charging current and a small reversible wave appeared. For 4×10^{-8} M BSA a larger charging current decrease took place and a well defined BSA wave was found at steady state, with E_{pc} at —0.760 and E_{pa} at —0.700 V vs. SCE. For 6×10^{-8} M BSA a well-defined double reduction wave occurred on the first scan; at steady state only one reduction wave at —0.630 V and an oxidation wave at —0.60 V were found. Saturation of the mercury surface by adsorbed BSA occurred at about this concentration, as can be observed from the integrated steady state reduction currents of these waves shown in Fig. 3. The sharp break in the integrated current at 3.0 μC cm^{-2} occurs at 6×10^{-8} M BSA, the same concentration at which the shift in E_{pc} from —0.760 V to —0.630 was found. When the electrode was pre-equilibrated with the solution by holding a fresh drop at —0.3 V in a stirred solution for ten minutes before recording a c.v., somewhat larger values of Q_c were found for the



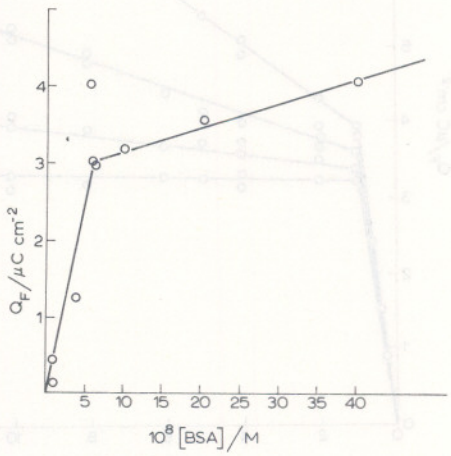


Fig. 2. Steady state cyclic voltammograms of pH 7.4 buffer at 0.5 V s^{-1} containing: (a) 10^{-9} M, (b) 10^{-8} M, (c) 4×10^{-8} M BSA.

Fig. 3. Integrated steady state c.v. cathodic current as a function of BSA concentration.

first scans (Fig. 4). The steady state values were similar to those obtained by cycling without the pre-equilibration step, however, with values of 3.3 to 4.4 μC cm^{-2} for a BSA concentration range of 0.1 to 1.6 μM. Within this concentration range the steady state Q_a values were constant at 3.3 μC cm^{-2}.

Cyclic voltammetry was also carried out at pH values of 6.13, 8.5, and 10.4. At pH values of 6.13 and 8.5 the pair of waves were seen, the waves shifting to more negative potentials with increasing pH (E_{pc} shifted 52 mV/pH unit from pH 6.13 to 7.4 and 90 mV/pH unit from pH 7.4 to 8.5). The waves decrease in height and broaden as the pH increases so that at pH 10.4 the waves are barely discernable from the double layer charging process.

Double potential step chronocoulometry

In an effort to obtain a better estimate of the total faradaic charge for reduction of BSA in the presence of appreciable non-faradaic charging, chronocoulometric experiments were undertaken. This technique has been used very successfully for the determination of the amount of adsorbed reactant in the presence of diffusing reactant and double layer charging (see [21,25] and references therein). For the case under consideration here, however, the situation is somewhat dif-

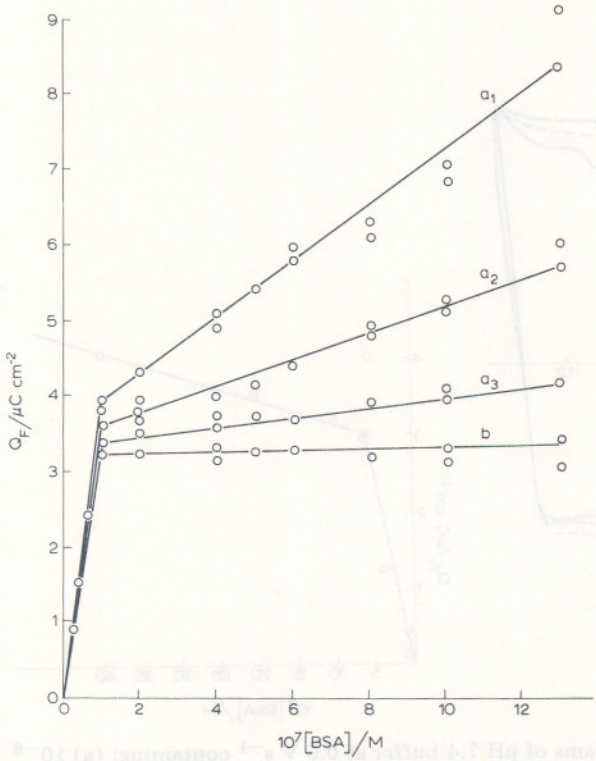


Fig. 4. Integrated currents of cyclic voltammograms for HMDE equilibrated with BSA solutions for 10 min with stirring (v = 0.5 V s^{-1}). (a1) First reduction scan; (a2) second reduction scan; (a3) steady state reduction scan; (b) oxidation scans.

ferent, since no diffusing species is involved and we desire separation of double layer charging from the electrochemical reactions of adsorbed species. If the double layer capacitance, C_{dl}, can be assumed constant with potential and with changing coverage of the oxidized and reduced forms over the range of the reduction wave, then the non-faradaic charging current is

$$Q_{dl} = C_{dl}(E_j - E_i) \quad (1)$$

for the potential step from E_i, the initial potential, before the reduction wave to E_j on the wave. If E_j is more negative than the peak potential, E_{pc} then all of the adsorbed species can be assumed to be reduced. The total charge passed during the potential step is then:

$$Q = Q_{dl} + Q_{ads} = C_{dl}(E_j - E_i) + nF\Gamma_s \quad (2)$$

where Γ_s is the amount of adsorbed species. Thus a plot of Q vs. $E_j - E_i$ yields an intercept of $nF\Gamma_s$ and a slope C_{dl}. In the absence of adsorbed reactant, or for steps from E_i to potentials before the reduction wave, a Q vs. $E_j - E_i$ line with zero intercept is expected. Typical results for the forward step are shown in Fig. 5. In all experiments E_i = 0 V, E_j was varied in 0.1 V steps, and Q was determined by integrating the current for 100 ms, with data obtained with the digital data acquisition system [25]. The fresh drop of the HMDE was equilibrated with the stirred solution containing 0.85 μM BSA for two minutes before the experiment. The results of analysis of the chronocoulometry experiments are summarized in Table 2. The intercept of the linear portion of the Q vs. E_j plot for potentials beyond E_p is 3.3 μC cm^{-2}. The C_{dl} determined from the slope of this portion of the curve (Fig. 5c) is essentially the same as that for potential steps before the foot of the wave (Fig. 5b), suggesting that the change in C_{dl} during the reduction is small. The C_{dl} value in the presence of BSA is smaller than that of the supporting electrolyte solution alone, as was also indicated from the cyclic voltammetry. Similar results were obtained by integration of the reverse potential step. The intercept of the Q_b vs. E_j line for potentials beyond E_{pc}, corrected for the slight change in C_{dl}, was 3.0 μC cm^{-2}.

Coulometric reduction of BSA

The coulometric reduction of 18 ml of a pH 7.4 (0.1 M phosphate buffer) solution containing 70 μM BSA was carried out at a large area (7.6 cm^2) mer-

Table 2
Double potential step chronocoulometry results

C_{dl} (with BSA) /μF cm^{-2}	C_{dl} (with reduced BSA)/μF cm^{-2}	Q_F/ μC cm^{-2}	Q_B/ μC cm^{-2}	C_{dl} (supporting electrolyte)/ μF cm^{-2}
Native BSA				
15.6	15.4	3.30	3.0	22.6
Urea denatured BSA				
21	20.7	3.30	—	22.0

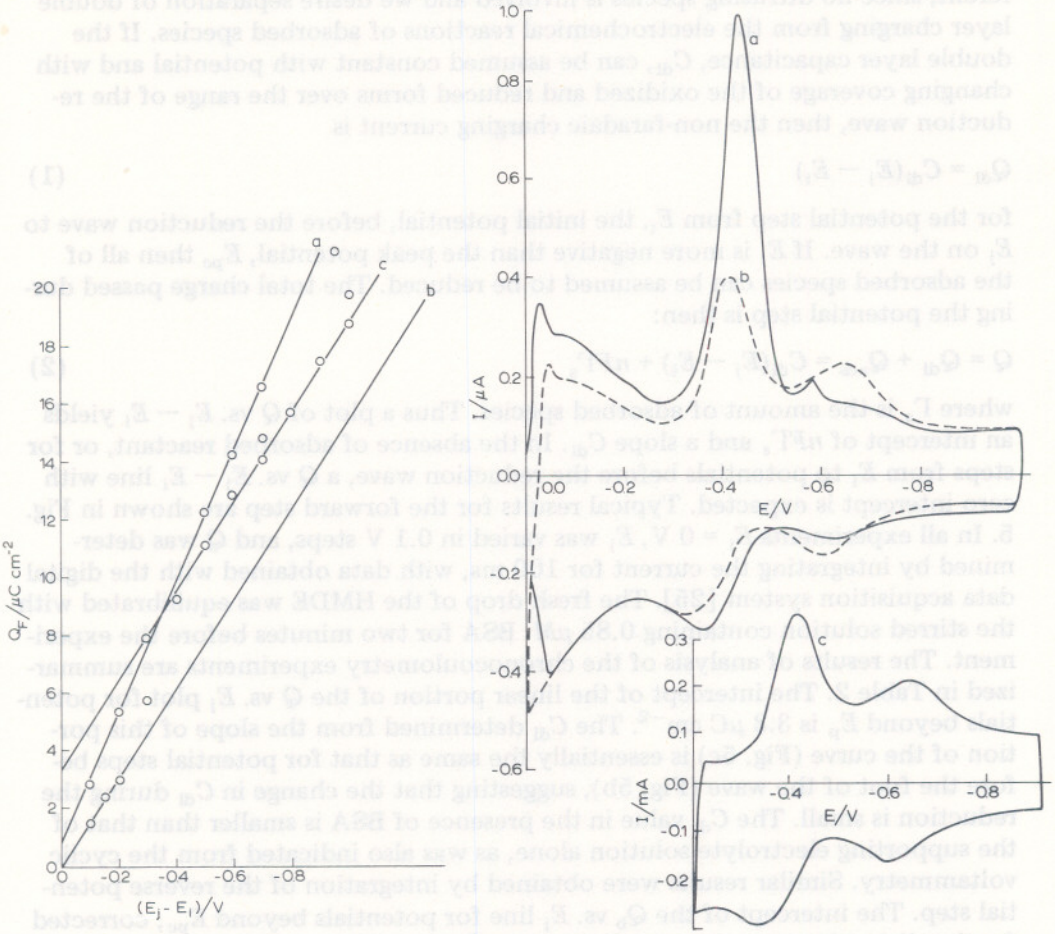


Fig. 5. Chronocoulometry results. E_i = 0 V. (a) 0.1 *M* phosphate buffer alone and with 0.85 μM BSA (b) for E_j before foot of wave and (c) for E_j beyond E_{pc}.

Fig. 6. Cyclic voltammetry results following coulometric reduction of BSA at —0.75 V at v = 0.2 V s^{-1}. At HMDE (a) first scan and (b) steady state and (c) at mercury pool.

cury pool electrode controlled at a potential of —0.75 V vs. SCE. The current decayed rapidly for the first ca. 10 minutes until an amount of charge equivalent to an n_{app} (electrons per molecule of BSA) of 2 was passed. From this time on the current was small and the decay was much slower; from 6 to 12 hours were required for the current to decay to the background level, at which time an overall $n_{app} \cong 18$ was found. The product of the reduction was an insoluble white polymer which adhered strongly to the electrode surface. Spectrophotometric measurements of the solution following the reduction showed some dissolved species, presumably a reduced form of BSA, in solution. Assuming that the extinction coefficient of the reduced species at 280 nm is about the same as that of unreduced BSA, its concentration was about 10^{-6} *M*. Following the coulometric reduction, cyclic voltammetric investigation of the product solution was

carried out both at a HMDE immersed in the solution above the mercury pool and at the pool itself; typical results are shown in Fig. 6. At the HMDE in addition to the wave pair originally found with BSA (E_{pc} = —0.63 V, E_{pa} = —0.60 V) a new couple at E_{pc} = —0.42 V and E_{pa} = —0.35 V appears. The i_{pc} for this wave drops at steady state to less than half its initial value. This couple is located near the potentials found for the oxidation of cysteine [7] and could be attributed to oxidation of —SH groups (to $Hg(RS)_2$) which are formed on reduction of BSA disulfide bonds. The peak currents for all of the waves, both on first scans and at steady state vary directly with v, so that all can be ascribed to adsorbed species. The behavior at the mercury pool electrode (Fig. 6c) was essentially the same as that at the HMDE. These waves represent the oxidation and reduction of adsorbed species coming from the small amount of soluble reduced BSA. The bulk of the reduced BSA existed as the polymeric material which could not be coulometrically oxidized (at 0 V).

Coulometric reduction of BSA at —0.75 V in a solution of pH 3.0 was similar to that at pH 7.4. Again rapid reduction occurred for n_{app} = 2 and then the current fell and decayed slowly. At this pH an n_{app} of ca. 25 was ultimately attained after about 9 hours. The polymer product did not adhere as strongly to the mercury pool electrode at this pH, and the same new couple was observed in a c.v. at the HMDE following reduction.

To test for any direct reaction between BSA and mercury at open circuit, a BSA solution identical to that used in the coulometry experiments was stirred over a mercury pool for 8 hours. No insoluble product resulted and no mercuric ion was detected by a spot test of the solution. A c.v. was taken of the solution at a HMDE every hour; no new couple at —0.4 V appeared. Thus no direct reaction of BSA with mercury or denaturation of BSA on stirring over the mercury pool occurs.

Denatured BSA

The same series of experiments, i.e., cyclic voltammetry, coulometry and double potential step chronocoulometry were performed with solutions in which the BSA was denatured by the addition of 8 *M* urea. The results of the c.v. experiments did not differ significantly from those of native BSA. The results of double potential step chronocoulometry in the presence of urea are given in Table 2. The coverage of BSA (3.3 μC cm^{-2}) is the same as for native BSA but the value for the double layer capacitance (21 μF cm^{-2}) was slightly larger and more similar to that for supporting electrolyte. Coulometric reduction of BSA at —0.75 V in a pH 7.4 solution containing 8 *M* urea yielded n_{app} values of 18 to 19.

DISCUSSION

A mechanism for the electrochemical behavior of BSA which is generally consistent with our experimental findings and past results can be proposed. The first reduction wave (E_{pc} = —0.630 V) is clearly an adsorption wave by the scan rate and concentration dependence. The chronocoulometric experiments yielded an integrated reduction current of 3.3 μC cm^{-2}, in very close agreement with the

maximum coverage values obtained from cyclic voltammograms. The oxidation wave (E_{pa} = —0.60 V) also had the symmetrical shape and the v and c dependence of an adsorption wave, with a coverage of the adsorbed species of 3.0 μC cm^{-2}.

Assuming the 3.3 μC^{-2} represents monolayer coverage by BSA, the number of electrons transferred per molecule (n) on the voltammetric time scale can be determined, if the number of BSA molecules on the surface can be estimated. This is much more difficult for BSA than for insulin, because the molecular size and conformation is not known and small differences in estimates of the effective diameter of adsorbed BSA result in significant differences in n. Thus if BSA is a sphere of diameter, d, of Å [14], and simply taking the coverage as $4/\pi d^2$ molecules cm^{-2}, an n of 6 is obtained. For a close-packed arrangement, where coverage is $1/d^2$, $n \approx 7$. On the other hand, if BSA is taken as a prolate spheroid with axes of 70 Å and 140 Å [15], then depending upon whether the molecule is oriented with its major axis parallel to or perpendicular to the electrode surface, n values of 8 to 20 are obtained. The results thus suggest that at least 3 or 4 disulfide bonds are rapidly reduced during voltammetry, and that oxidation of the adsorbed reduced form to reform the disulfides (or alternatively to form a species such as $Hg(RS)_2$) occurs on reversal. In coulometry the rapid reduction found initially may similarly represent partial reduction of several disulfide bonds, followed by a slower structural change of the molecule and further reduc- until finally n_{app} = 18 with a total of 9 disulfide bonds broken. An alternative explanation may be proposed for the coulometry results, namely that the initial reduction represents that material adsorbed on the electrode and the slow step represents replacement of adsorbed reduced BSA with dissolved molecules from the solution. However monolayer coverage of the mercury pool would account for only 25 μC of charge, while the initial reduction (to n_{app} = 2) involved 0.24 C. Moreover the mercury pool was stirred quite vigorously and fresh mercury was continually exposed to the solution throughout the electrolysis.

The lack of change in the cyclic voltammograms upon repeated scanning indicates that both oxidized and reduced forms of BSA are strongly adsorbed and that the electron transfer process is probably chemically reversible. If the oxidation process was not the reverse of the initial reduction process, the second and subsequent cathodic scans should show changes in E_{pc} and i_{pc}; such behavior is observed with cystine [7]. Thus a reasonable model for the electrochemical process involves adsorption of BSA with strong interaction of several of the exposed disulfide bonds with the mercury surface. Reduction of these leads to —SH groups with the molecule remaining on the surface with its structure maintained by the internal disulfide bonds and intramolecular hydrogen bonding. The —SH groups must remain in close proximity on the short time scale so that disulfide bonds can reform upon oxidation. This model is also consistent with the recent results on the electroreduction of urease adsorbed on a mercury electrode [8] where enzymatic activity could be destroyed and regenerated upon reduction and reoxidation.

ACKNOWLEDGMENTS

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