Brdíčka Currents Observed with Bovine Serum Albumin and Completely Reduced Bovine Serum Albumin in the Presence of Urea

(catalytic currents/urea effects)

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ABSTRACT In ammonia buffers of varying composition and pH, native bovine serum albumin and completely reduced bovine serum albumin, denoted by \( \Theta(SH)_{35} \), yield quite different Brdíčka current-voltage (i-E) curves, but they are identical in the presence of 5 mM calcium chloride. This means that in the presence of calcium, bovine serum albumin becomes completely reduced to \( \Theta(SH)_{35} \). In 8 M urea and ammonia buffers the Brdíčka i-E patterns of serum albumin and \( \Theta(SH)_{35} \) are identical even in the absence of calcium, the effect of calcium on the first wave being negligible while calcium slightly increases the second wave. The maximum polarographic effect is attained at urea concentrations of about 5-6 M. Quite generally, the appearance of a second Brdíčka wave is attributed to complexation of Co(II) with \( S^- \) and a group of ligands that is different on the second than on the first wave. The effect of calcium on Brdíčka currents of bovine serum albumin in the absence of urea is attributed to an orientation of the protein on the surface of the electrode such that all disulfide groups are reduced and with other ligands can complex with Co(II). Denaturation of bovine serum albumin in buffers with a pH less than 10.5, and which are 8 M in urea, is (polarographically) completely reversible if dilution is made within 15 minutes after preparation. Changes in Brdíčka i-E patterns upon longer aging at varying pH are attributed—in part at least—to dimerization of the denatured protein by interaction of \( S^- \) in one molecule with \( S-S \) in another.

In previous papers (1-4) Brdíčka currents observed under various conditions in different buffers with native bovine serum albumin (BSA) and presodium currents (5) with native and modified BSA have been reported and a chain mechanism for the qualitative interpretation of the effects of pH and the kind of buffer on Brdíčka currents has been proposed (3). Quite generally it was found that small concentrations of calcium (chloride) as well as magnesium greatly increase the total catalytic currents, or in buffers with two Brdíčka waves, the second one, and also affect the shape of current-voltage curves in the voltage range in which Brdíčka currents are observed (denoted as Brdíčka i-E curves). It appeared of interest to investigate the effect of urea on Brdíčka and presodium currents (5). For many years it has been known that urea in concentrations of 6-8 M is a denaturant of proteins, including BSA. Although the interpretation of the mechanism of this denaturation, and particularly the effect of urea on the structure of the protein, is incompletely understood (6, 7), there is little doubt that urea in larger concentrations brings about a partial unfolding of the compact structure of BSA. This unfolding is accompanied by a large increase of the reduced viscosity of solutions of BSA. In the present paper are reported the effects of urea in various concentrations on Brdíčka currents of BSA in different buffers in the absence and presence of small concentrations of calcium (chloride). In addition, some effects of 6 to 8 M urea have been investigated using a modified BSA in which all disulfide bonds had been broken by reduction to sulfhydryl groups. Considering that most BSA products contain close to 1 —SH and 17 —S— groups per molecule, this reduced BSA is denoted by the symbol \( \Theta(SH)_{35} \) (2).

EXPERIMENTAL

Chemicals. Three different commercial samples of BSA have been used (1-4), which yielded similar polarograms. All chemicals were C.P. products and the same, or of the same quality, as those used previously (1-5).

Apparatus and Technique. These were the same as described previously (1-4). Unless stated otherwise, the currents in the presence of larger concentrations of urea have been corrected for viscosity of solutions by multiplying the observed currents by 1.1, 1.2, or 1.3 for 3, 6, or 8 M urea solutions, respectively. Polarography and denaturation were carried out at 25°. Unless mentioned otherwise, the characteristics of the capillary at open circuit and 75 cm mercury pressure were: \( m = 1.57 \) mg/sec, \( t = 5.0 \) sec.

RESULTS

Native BSA: Experiments in 8 M urea

Ammonia Buffers. The shape of the Brdíčka i-E curves of BSA and \( \Theta(SH)_{35} \) in the absence and presence of 6-8 M urea greatly depends upon the concentrations of ammonia and ammonium chloride. Fig. 1 presents one example of the shape in a buffer 0.1 M in ammonia and 0.1 M in ammonium chloride and 8 M in urea. In 8 M urea Brdíčka currents exhibit two maxima, the second wave becoming larger and the maximum more accentuated in the presence of 5 mM calcium chloride (curves 4a and 4b). The calcium effect is much smaller than that observed with undenatured (native) BSA. The experiments in Fig. 1 were carried out with Co(NH$_2$)$_2$Cl$_2$, denoted as Co(III), as catalyst. They were repeated in 8 M urea, with Co(II) (CoCl$_2$) as catalyst. In 8 M urea the shapes of the i-E curves were the same and the magnitude of the currents some 10-20% smaller with Co(II) than with the same concentration of Co(III). At the same ammonium chlor-
ride concentration as in Fig. 1, but in 1 M ammonia, the shape of the Brdička i-E curves is quite different from that in 0.1 M ammonia. (Fig. 2) The current exhibits one pronounced maximum, and 5 mM calcium in 6-8 M urea has practically no effect on the shape and on the current. With cobalt(II) the same pattern was observed as with Co(III), the currents again being some 10-20% smaller than with Co(III). The difference between the effects of calcium upon Brdička currents with native BSA and BSA in 8 M urea is particularly striking in a buffer 0.02 M in ammonium chloride and 1 M in ammonia, the calcium effect being very large in the absence of urea. The effect of 8 M urea on Brdička currents of BSA has also been determined in borax buffers containing 0.025 M borax (pH 9.2; apparent pH in the presence of 8 M urea was 9.8), 0.025 M borax and 0.1 M boric acid (pH 8.5), and 0.025 M borax and 0.3 M boric acid (pH 7.7). Qualitatively the effects are similar in the three buffers, the currents decreasing with decreasing pH. One example is given in Fig. 3 in 0.025 M borax. In the absence of calcium chloride the two Brdička waves are greatly increased when the buffers are made 8 M in urea, but in the presence of 5 mM calcium chloride the first and second Brdička currents are about the same as those in the absence of urea. With Co(II) (Fig. 4) instead of Co(III), the pattern of i-E Brdička currents is quite different, the Co(II) being present as a much less stable

\[ \text{Fig. 1. Brdička waves in buffer 0.1 M in ammonia and 0.1 M in ammonium (chloride). Capillary: } m = 1.743 \text{ \(mg/sec\), } t = 4.47 \text{ sec. Concentration of protein 0.25 \(\mu\text{M, of Co(III)} = 0.5 \text{ mM. Curve 1, Co(III); curve 2a, native BSA; 2b, same as 2a + 5 mM CaCl}_2; \text{ curve 3a, } \theta(SH)_{35}; \text{ curve 3b, same as 3a + 5 mM CaCl}_2; \text{ curve 4a, BSA or } \theta(SH)_{35} \text{ in 8 M urea; curve 4b, same as 4a + 5 mM CaCl}_2. \text{ All currents are corrected for viscosity.} \]

\[ \text{Fig. 2. Buffer 1 M in NH}_3 \text{ and 0.1 M in NH}_4\text{Cl. Other conditions as in Fig. 1. Curve 1, Co(III); curve 2a, 0.25 \(\mu\text{M native BSA; curve 2b, same as 2a + 5 mM CaCl}_2; \text{ curve 3a, } \theta(SH)_{35}; \text{ curve 3b, same as 3a + 5 mM CaCl}_2; \text{ curve 4a, BSA or } \theta(SH)_{35} \text{ in 8 M urea; curve 4b, same as 4a + 5 mM CaCl}_2. \]
greater ammonia buffers (2, 3). Buffer in 1 M complex, urea. tion of less than 1 are that in Co(III) or Co(II) 0.5 mM. In this medium the Brdicka currents are characterized by a well-defined rounded maximum. The effect of urea is already noticeable at a concentration of less than 1 M (curve 3a, Fig. 5), especially in the presence of 5 mM calcium (curve 3b, Fig. 5). At urea concentrations greater than 4 M the effect of calcium is different from that in the absence of urea. This difference between the effects of calcium in the absence and presence of different concentrations of urea in a buffer 0.1 M in both NH$_3$ and NH$_4$Cl is illustrated in Fig. 6. It appears that at urea concentrations greater than about 5 M the orientation of the protein on the surface of the electrode remains the same. In a buffer only 0.02 M in NH$_4$Cl and 1 M NH$_3$ and 8 M in urea, 0.5 mM in Co(III), and 0.26 mM in BSA, the shape of the polarogram is like that in Fig. 1, but the currents at the two maxima are some six times smaller than in Fig. 1. In the presence of 5 mM calcium only one peak current is observed, which is some 20 times larger than that at the second peak in the absence of calcium. In the presence of 1 M urea the Brdicka i-E curve is similar to that in the absence of urea, but the peak current is about one-half of that in the absence of urea, while a large increase in peak current is observed in the presence of 5 mM calcium. The curves in the presence of 4 and 8 M urea resemble those in Fig. 5, and the effect of calcium then becomes virtually negligible.

Effect of Concentration of BSA. In buffers that are 0.1 M in both NH$_3$ and NH$_4$Cl and 6–8 M urea two waves with rounded maxima are observed at a BSA concentration of 0.25

Effect of Concentration of Urea. This effect was determined in various ammonia buffers. An example is given in Fig. 5 in a buffer 1 M in ammonia and 0.1 M in ammonium chloride in which the BSA concentration was 0.25 μM and that of Co(III) or Co(II) 0.5 mM. In this medium the Brdicka currents are characterized by a well-defined rounded maximum. The effect of urea is already noticeable at a concentration of less than 1 M (curve 3a, Fig. 5), especially in the presence of 5 mM calcium (curve 3b, Fig. 5). At urea concentrations greater than 4 M the effect of calcium is different from that in the absence of urea. This difference between the effects of calcium in the absence and presence of different concentrations of urea in a buffer 0.1 M in both NH$_3$ and NH$_4$Cl is illustrated in Fig. 6. It appears that at urea concentrations greater than about 5 M the orientation of the protein on the surface of the electrode remains the same. In a buffer only 0.02 M in NH$_4$Cl and 1 M NH$_3$ and 8 M in urea, 0.5 mM in Co(III), and 0.26 mM in BSA, the shape of the polarogram is like that in Fig. 1, but the currents at the two maxima are some six times smaller than in Fig. 1. In the presence of 5 mM calcium only one peak current is observed, which is some 20 times larger than that at the second peak in the absence of calcium. In the presence of 1 M urea the Brdicka i-E curve is similar to that in the absence of urea, but the peak current is about one-half of that in the absence of urea, while a large increase in peak current is observed in the presence of 5 mM calcium. The curves in the presence of 4 and 8 M urea resemble those in Fig. 5, and the effect of calcium then becomes virtually negligible.

FIG. 5. Effect of concentration of urea in buffer 1 M in NH$_3$ and 0.1 M in NH$_4$Cl. Other conditions as in Fig. 1. Curve 1, 0.5 mM Co(III); curve 2a, 0.25 μM native BSA; curve 2b, same as 2a + 5 mM CaCl$_2$; curves 3a and 3b, same as 2a and 2b, respectively, in 1 M urea; curves 4a and 4b, same as 2a and 2b, respectively, in 4 M urea; curves 5a and 5b, in 6 M urea; curves 6a and 6b, in 8 M urea.

FIG. 6. Effect of urea concentration in buffer 0.1 M in both NH$_3$ and NH$_4$Cl. Other conditions as in Fig. 1. Curve 1, 0.5 mM Co(III); curve 2a, same as 1 + 0.25 μM BSA; curve 2b, same as 2a + 5 mM CaCl$_2$; curve 3a, same as 2a + 1 M urea; curve 3b, same as 3a + Ca; curve 4a, same as 2a + 4 M urea; curve 4b, same as 4a + Ca; curve 5a, same as 2a with 6 M urea; curve 5b, same as 5a + Ca.

μM. The first wave remains unchanged in the presence of 5 mM calcium, but the second one increases, while the minimum current observed after the second maximum is larger in the presence of calcium than in its absence. The second wave in the presence of calcium becomes considerably more pronounced with increasing BSA concentration, as is seen in Fig. 7. In 1 μM BSA and presence of calcium the second wave still exhibits a rounded maximum, but in 10 μM BSA the second wave exhibits no maximum, but a well-defined limiting current. In a buffer 0.08 M both in ammonia and ammonium chloride and 6 M in urea and 0.4 M in Co(III), and at concentrations of BSA of 0.14, 0.27, and 0.41 μM, the following total Brdicka currents (corrected for viscosity) were found: 3.6, 6.7, and 8.3 μA respectively; and in the buffer 0.41 μM in BSA, values of 9.7 and 10.2 μA were found in the presence of 5 and 7.5 mM of calcium.

Reversibility of Denaturation (Renaturation). A great number of experiments has been carried out in which 1% BSA was aged for various periods of time in buffers 8 M in urea and of pH varying between 10.3 and 7.0. After a given period of time at room temperature, the solutions were diluted in an ammonia buffer to a BSA concentration of 0.25 μM and polarographed in the presence of 0.5 mM Co(III). In all instances the Brdicka currents after aging in 8 M urea for 15 min or less were identical with those of BSA not pretreated with urea. However, upon longer periods of aging in 8 M urea, changes in the shape of Brdicka i-E curves were found, which occurred more rapidly the higher the pH was during the aging. A typical example of the effect of aging is presented in Fig. 8. After the protein was aged for 1 hr in a buffer 0.1 M in both NH$_3$ and NH$_4$Cl and 8 M in urea, and diluted in the urea-free buffer, the minimum in curve 2a disappeared; the current at the peak in curve 2a then increased slightly until it merged with the presodium current (not shown in Fig. 8). However, when the buffer was made 5 mM in calcium, the curve was identical with that of untreated BSA (curve 2b). After 24 hr of aging in 8 M urea and dilution in urea-free buffer, a second wave was well defined (curve 3a), the effect
of 5 mM calcium now being almost negligibly small (curve 3b). As a matter of fact, after 24 hr of aging and dilution in urea-free buffer (curve 3a), the polargram was very similar to that of untreated BSA in the buffer with 5 mM calcium (curve 2b). On the other hand, virtually no effect of 24 hr of aging in 8 M urea was observed when the polargrams were run in a buffer 8 M in urea (curves 4a and 4b), curves 4a and 4b in Fig. 8 being almost identical with those of untreated BSA in 8 M urea (curves 4a and 4b in Fig. 1). The effect of aging in buffers 8 M in urea upon the shape of polargrams in urea-free buffers was found to decrease with decreasing pH. The effect after aging for 24 hr in a phosphate buffer of pH 7, which was 8 M in urea, was about the same as that after 1 hr in the buffer 0.1 M in both NH₄Cl and NH₃. Silver nitrate in a concentration of 0.2 mM was found to retard, but not to prevent, the aging in ammonia buffers 8 M in urea.

Experiments have also been carried out in which polargrams were run after the aging with Co(II) instead of Co(III) as catalysts. When the polargrams were run in buffers 1 M in NH₄ and 0.1 M in NH₄Cl, the polargrams were identical with those with Co(III). At smaller NH₃ concentrations of 0.1 and 0.02 M, the patterns of the i-E curves after 24-hr aging were similar to those with Co(III), but the currents were considerably smaller.

After aging for 24 hr of 1% BSA solutions in urea-free ammonia buffers of pH 10.3-8.3, and dilution in urea-free buffers, the polargrams were identical with those obtained before aging.

From various figures in this paper and a score of other polargrams, it appears that 8 M urea affects neither the potential at which presodium currents start (5) nor the shape of the presodium current-voltage curves.

**Comparison of BSA with $\Phi(SH)_{25}$**. From Figs. 1 and 2 it is seen that untreated BSA and $\Phi(SH)_{25}$ yield quite different shapes of Brdicka current-potential curves in urea-free ammonia buffers but identical curves in the presence of 5 mM calcium chloride, no effect of calcium being observed with $\Phi(SH)_{25}$. On the other hand, in 8 M urea the Brdicka i-E pattern of BSA is not affected by calcium and is identical with that of $\Phi(SH)_{25}$ at the same urea concentration.

**DISCUSSION**

From a structural viewpoint, one of the most interesting results of this study is that native BSA and $\Phi(SH)_{25}$ yield quite different Brdicka i-E patterns, a second Brdicka wave not being observed with BSA in ammonia buffers 0.1 M in NH₄ and 0.1 M in NH₄Cl, while this wave is well developed in $\Phi(SH)_{25}$ (Fig. 1). In the presence of 5 mM calcium this i-E pattern of $\Phi(SH)_{25}$ is not changed, while that of BSA becomes identical with that of $\Phi(SH)_{25}$ (Fig. 1). On the other hand, in 8 M urea and the absence of calcium the polargrams of BSA are identical with those of $\Phi(SH)_{25}$, and calcium slightly increases the height of the second wave on both (Figs. 1 and 2). BSA is considered to be a compact molecule by intra- and interhelical hydrogen bonds between $C=O$ and $HN$ groups and also by $S=S$ bonds between helices. In $\Phi(SH)_{25}$ all $S=S$ bonds are broken, and this makes partial unfolding possible. This is accompanied by a marked increase of the reduced viscosity. This viscosity of a 1% BSA solution in a buffer 0.1 M in both ammonia and ammonium chloride is 0.04, and that of a 1% $\Phi(SH)_{25}$ solution 0.24. Kaumann (6) assumes that the partial unfolding of BSA in 8 M urea occurs as a result of breaking of all intramolecular hydrogen bonds by hydrogen bonding with urea of the $C=O$ and $HN$ groups. This interpretation may need considerable modification (7), considering that water undoubtedly is a stronger hydrogen bond donor than urea (and a weaker hydrogen bond acceptor). Undoubtedly, water and urea will hydrogen-bond
with each other, and the structure of water in the concentrated urea solutions is quite different from that in dilute aqueous solutions. The partial unfolding of BSA is accompanied by an increase of the reduced viscosity in an ammonia buffer of pH 9.2 from 0.04 in 1% native BSA to 0.26 in 8 M urea. Further unfolding by breaking all S-S bonds gives rise to a further large increase of the reduced viscosity to 0.50 in 1% \( \Phi(SH)_3 \) in 8 M urea. Whatever the exact physical picture of the unfolding may be, it definitely gives rise to a different orientation of the protein on the surface of dropping mercury. The appearance of a second wave in some buffers in the absence of urea and a distinct one in the presence of 6 to 8 M urea (except in 1 M urea) is puzzling. Considering that \( \Phi(SH)_3 \) is completely reduced BSA, the occurrence of a second wave is attributed to ligands which together with S- complexes with Co(II), the ligands being different on the first wave than on the second wave. It is possible that with native BSA all disulfide bonds are reduced on the first wave, but that the orientation of the reduced protein on the surface of the electrode is such that no reduction of Co(II) complexes responsible for the second wave occurs, or, in certain buffers, only to a small extent. The effect of calcium is then attributed to changing the orientation of the reduced molecule in such a way that those Co(II) complexes that are responsible for the appearance of the second wave can now be reduced to Co(0). On the other hand, in buffers that are 8 M in urea, the completely reduced BSA on the surface of the electrode becomes identical with \( \Phi(SH)_3 \) and yields an identical Brdicka i-E curve pattern as \( \Phi(SH)_3 \). This identity is attained already at a urea concentration of 5-6 M (Figs. 5 and 6). Again, the appearance of a second wave is accounted for in the same way as in the absence of urea.

From polarographic evidence it is concluded that the “denaturation” of 1% solutions of BSA in 8 M urea is completely reversible in buffers of pH less than 10 if diluted some 100 times soon after preparation. After longer standing, the “renaturation” is incomplete which, in part at least, must be attributed to dimerization or polymerization by interaction between \( \text{-S-} \) in one molecule and \( \text{-S-S-} \) in another (6).

\[
\text{-S-} + \text{S-} \rightarrow \text{S-S-}
\]

This dimerization is accompanied by an increase of the reduced viscosity. The reduced viscosity of a freshly prepared 1% BSA solution in a buffer 0.1 M in ammonium chloride, 1 M in ammonia, and 8 M in urea increased from 0.27 to 0.37 after 24 hr.

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