

Accelerated Articles

# Influence of the Zn(II) Cofactor on the Refolding of Bovine Carbonic Anhydrase after Denaturation with Sodium Dodecyl Sulfate

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This paper uses capillary electrophoresis to follow a globular metalloprotein—bovine carbonic anhydrase II (BCA, EC 4.2.1.1)—on unfolding upon treatment with sodium dodecyl sulfate (SDS) and refolding upon removal of SDS, both in the presence and the absence of its Zn(II) cofactor. This research demonstrates that the Zn(II) cofactor is not required for refolding into a natively like conformation, does not remain associated with the unfolded protein, and does not significantly change the rate of refolding. The presence of the Zn(II) cofactor, however, does increase the total amount of recovered protein by a factor of 2. Capillary electrophoresis could distinguish between native and denatured protein, based on the difference in electrophoretic mobility between the native protein and the aggregate of denatured protein and SDS. In addition, the active site was probed by observing binding of BCA to a charged arylsulfonamide using affinity capillary electrophoresis. These studies provide a foundation for future physical-organic studies using BCA as a model to examine interactions between proteins and SDS.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) is a technique widely used in biochemistry for the separation of proteins based on differences in their molecular weight.<sup>1–3</sup> The details of the interaction of proteins with SDS are

not well understood, and a better understanding of this interaction could lead to improved methods of analyzing proteins or to different uses of SDS–PAGE. Since many proteins contain a metal bound in the active site, we wished to study how a metal cofactor influences the interaction of a protein with SDS. We chose bovine carbonic anhydrase II (BCA, EC 4.2.1.1), which contains a Zn(II) cofactor, as a model protein for studying the influence of metal cofactors on the unfolding of proteins with SDS and their subsequent refolding.

**Carbonic Anhydrase as a Model Protein.** BCA is often used as a model for physical-organic studies of proteins and protein–ligand binding.<sup>4–9</sup> It is stable ( $T_m = 65\text{ °C}$ ); it consists of a single polypeptide chain with no disulfide bonds; it is available commercially and inexpensively. The mechanism of binding of BCA to arylsulfonamide inhibitors is well characterized.<sup>10</sup> Sulfonamides can be synthesized in great structural variety, and many are available commercially.

Specifically relevant to this paper, there is extensive literature on the denaturation of BCA with guanidine hydrochloride (GuHCl)<sup>11–15</sup> and, to a lesser extent, urea.<sup>16,17</sup> Surfactant-induced denaturation of BCA has not been investigated thoroughly. The

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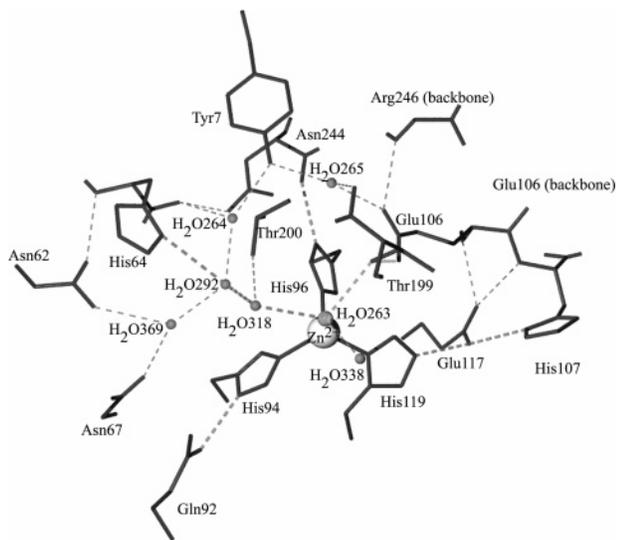
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**Figure 1.** Active site of human carbonic anhydrase (HCA II). The labeled residues are conserved between HCA II and BCA. Histidines 94, 96, and 119 are directly coordinated to the Zn(II) cofactor in a tetrahedral shape. The other histidine in the binding pocket, histidine 64, has a large amount of conformational flexibility ( $\sim 10 \text{ \AA}$ )<sup>25</sup> that allows it to act as a proton shuttle. Water molecules 292 and 318 act as a proton shuttle between the ZnOH<sup>+</sup> moiety and histidine 64. The hydroxide ion is shown as the sphere bound to the Zn<sup>2+</sup> cofactor; in the crystal structure, it is denoted H<sub>2</sub>O 263. Dashed lines represent hydrogen bonds.

only mechanistic study of the interaction of BCA with SDS showed that the complexes formed between BCA and SDS at high (3.5 mM) and low (0.875 mM) concentrations of SDS were different as monitored by circular dichroism (CD) spectroscopy, UV difference spectroscopy, and intrinsic viscosity.<sup>18</sup> Like most proteins, BCA binds SDS in a ratio of  $\sim 1.4$  g of SDS/g of protein.<sup>18</sup> We therefore believe that BCA is a suitable model protein for further studies of the interactions between proteins and SDS.

#### Metal-free and Metallovariants of Carbonic Anhydrase.

BCA has a Zn(II) cofactor that is essential for catalysis. Three histidine residues chelate the Zn(II) at the base of the active site in a tetrahedral geometry (Figure 1).<sup>19</sup> The dissociation constant for this Zn(II)–His<sub>3</sub> complex is 1.2 pM at pH 7.0 in 10 mM *N*-(carbamoylmethyl)-2-aminoethanesulfonic acid buffer.<sup>20</sup> The Zn(II) binds a hydroxide ion in the fourth position of its tetrahedral coordination sphere at physiological pH;<sup>19</sup> this hydroxide group

reacts with CO<sub>2</sub> to form HCO<sub>3</sub><sup>-</sup> in the reaction catalyzed by BCA. The pK<sub>a</sub> value for the protonation of the ZnOH<sup>+</sup> is  $\sim 7$ .<sup>21,22</sup> Arylsulfonamide inhibitors (as ArSO<sub>2</sub>NH<sup>-</sup>) displace this hydroxide ion, and the binding of these inhibitors requires a metal ion.<sup>23</sup>

Apo-BCA (that is, the metal-free species) is not enzymatically active and does not bind with high affinity to sulfonamide inhibitors. Addition of Zn(II) to a solution of apo-BCA gives holo-BCA (that is, the Zn(II)-containing species) quantitatively and restores full enzymatic and binding activities. In lieu of Zn(II), other metals can be incorporated into apo-BCA. The Co(II)–BCA metalloenzyme is active in catalyzing the hydration of CO<sub>2</sub>, but the Fe(II)-, Mn(II)-, and Ni(II)-BCA metalloproteins have minimal (<10%) esterase activity as monitored by the rate of hydrolysis of *p*-nitrophenyl acetate.<sup>24</sup> Given the requirement of the Zn(II) or Co(II) cofactor for activity of the native enzyme, binding activity is a measure of the presence of Zn(II) or Co(II) in the active site.

BCA need not be denatured to remove the Zn(II). A convenient procedure to make the apo-BCA is to dialyze against a chelating agent (e.g., ethylenediaminetetraacetic acid (EDTA) or dipicolinic acid (DPA)).<sup>25</sup> Whether the Zn(II) influences the denaturation of BCA can be determined by comparing the denaturation and renaturation of holo- and apo-BCA.

Yazgan and Henkens showed that folding of BCA after denaturation with GuHCl is rapid (<10 min) in the presence of Zn(II); in its absence, or if added late in the refolding process, folding required approximately twice as long to complete.<sup>26</sup> They also observed that renaturation occurred through a long-lived, molten-globule intermediate in both the presence and absence of Zn(II). Zn(II) also stabilizes BCA against denaturation with GuHCl.<sup>26,27</sup> For example, apo-BCA denatures at 1.2 M GuHCl, while BCA bound to its Zn(II) cofactor remains in its native state.<sup>28</sup> BCA folds and regains full activity upon addition of Zn(II) to a solution of apo-BCA in 1.2 M GuHCl.

#### Refolding Kinetics of BCA after Denaturation with GuHCl.

Many groups have observed three time scales in the refolding of BCA after denaturation with GuHCl.<sup>11,12,15,29,30</sup> Semisotnov et al. showed that the fastest of the three stages ( $t_{1/2} \cong 0.4$  s) is a compaction of the protein chain and the formation of a molten-globule intermediate.<sup>15</sup> In this fast collapse, the hydrophobic clusters desolvate and a nativelylike hydrophobic core forms. The other two stages ( $t_{1/2} \cong 2$  and 10 min) have been attributed to proline isomerizations.<sup>30,31</sup> Proline isomerizations have been proposed as the rate-limiting step in the folding of many slow-

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folding proteins, including BCA.<sup>32–36</sup> If the isomerization is rate-determining, the protein should refold much faster when renatured soon after denaturation—that is, before the Pro bonds have time to randomize their orientation. Semisotnov et al. showed that BCA has this dependence on delay time.<sup>30</sup> In addition, Fransson et al. showed that addition of proline isomerase to the refolding reaction mixture abolishes the effect of delay in refolding and reduces the half-time of refolding from 9 to 4 min.<sup>31</sup> Based on these studies of denaturation of BCA in GuHCl, we believe that BCA is an excellent choice for studying the behavior of the Zn(II) cofactor on denaturation with SDS, and on subsequent renaturation.

**Using Capillary Electrophoresis (CE) To Monitor Interactions of Carbonic Anhydrase with SDS.** CE can distinguish between states of a protein that differ in their electrophoretic mobility. CE can also differentiate between subpopulations of active and inactive proteins of the same electrophoretic mobility in one sample, if a charged inhibitor is added to the electrophoresis buffer at concentrations at which it binds to the protein (affinity capillary electrophoresis, ACE).<sup>4,37,38</sup> Many biochemical assays cannot distinguish between active and inactive subpopulations of a protein. CE also distinguishes protein from protein–SDS complexes. The use of CE with UV absorbance as a detection technique is especially convenient in these studies because the amide bonds and the aromatic side chains of proteins absorb light at 214 nm, while SDS is transparent at this wavelength. Observation at this wavelength, thus, directly detects only protein (whether native or denatured) without additional labeling and without interference from the denaturant. CE with UV detection is, therefore, an excellent technique for studying the interaction of proteins with SDS because it allows selective examination of protein (regardless of form), even in the presence of high concentrations of SDS.

## RESULTS AND DISCUSSION

**Preparation of Low-Zinc Buffer.** Because the concentration of Zn(II) in buffer was an important parameter in these studies, we examined a number of methods of generating and characterizing buffers with the lowest achievable concentrations of this ion. We used atomic absorption (AA) spectroscopy to compare the total zinc concentration in many commercial sources of water and buffer salts and concentrates. The purest water that we tested was from our Millipore Synthesis ultrapure water system (Millipore, Billerica, MA). Water from this source had 14.3 nM total zinc. We diluted the 10× Tris–Gly concentrates (and component salts) to 1× Tris–Gly buffer before analysis because the organic impurities smoked when heated in the AA furnace and adversely affected the absorption measurements. We found that 1× of the concentrated stock from Sigma had the lowest zinc concentration

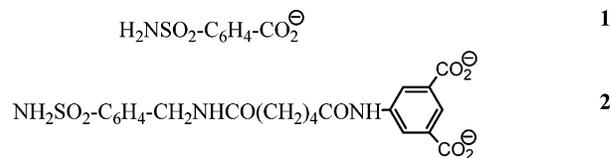
of any Tris–Gly buffer tested, with 539 nM zinc. We then treated the 10× Tris–Gly concentrate with Chelex-100, a chelating resin. This weakly acidic resin is a styrene–divinylbenzene copolymer containing iminodiacetate ions that strongly chelate divalent metal ions. After the first treatment with the chelating resin, the total zinc concentration in the buffer dropped to 115 nM, and a second treatment brought the zinc concentration down to 25 nM. Additional treatments did not appear to remove any additional zinc.

We did not use any glassware in these experiments because the molds used to make the glassware often contain zinc. We only used plasticware that had been thoroughly washed with 10 μM EDTA solution.

**Preparation and Characterization of Holo-BCA and Apo-BCA.** We purified BCA by affinity chromatography using 4-(aminomethyl)benzenesulfonamide immobilized on an *N*-hydroxysuccinimidyl-activated agarose resin, followed by ion exchange chromatography. BCA has two isozymes: one has an arginine at position 56; the other has a glutamine. Because the two amino acids differ by one unit of charge, we used ion exchange chromatography to remove the isozyme containing Glu56. We prepared a sample of apo-BCA by dialysis of the holo-BCA against DPA for 72 h.<sup>25</sup> We used DPA because it is nearly a factor of 20 faster than EDTA at removing Zn(II) from BCA.<sup>39</sup> DPA is thought to form a ternary complex with the Zn(II) and BCA to aid in the removal of the Zn(II), whereas EDTA simply sequesters free zinc once it dissociates from BCA.

Mass spectrometry (Figure 2) showed an apparent mass difference between apo- (observed MW 29 025 Da) and holo-BCA (observed MW 29 088 Da) of 63 ± 1 Da; this mass difference corresponds to the mass of Zn and suggests that, upon protonation during electrospray ionization (ESI), the holo form readily loses the Zn-bound OH group, or it is exchanged for an acetate (observed MW 29 148 Da). We used AA to measure the amount of residual zinc in the sample of apo-BCA. A sample of 500 nM apo-BCA contained a total Zn concentration of 20 nM. Because BCA·Zn(II) has a dissociation constant of ~1 pM, we assume that all of the measured zinc was bound by BCA. There was, therefore, less than 5% holo-BCA contamination in the apo-BCA sample.

We used ACE<sup>4,37,38</sup> (Figure 3) to demonstrate that holo-BCA can be distinguished from apo-BCA based on its ability to bind to a charged sulfonamide, 4-carboxybenzene-1-sulfonamide (**1**) or



5-[6-[[[4-(aminosulfonyl)phenyl]methyl]amino]-1,6-dioxohexyl]-amino-1,3-benzenedicarboxylic acid (**2**).

Apo-BCA does not bind sulfonamide inhibitor; holo-BCA binds to **1** with a dissociation constant of 0.42 ± 0.02 μM. Using ACE, we confirmed that our apo-BCA sample contained less than 2% holo-BCA.

Removal of the Zn(II) cofactor does not cause unfolding of the protein. The CD spectra of apo- and holo-BCA are similar

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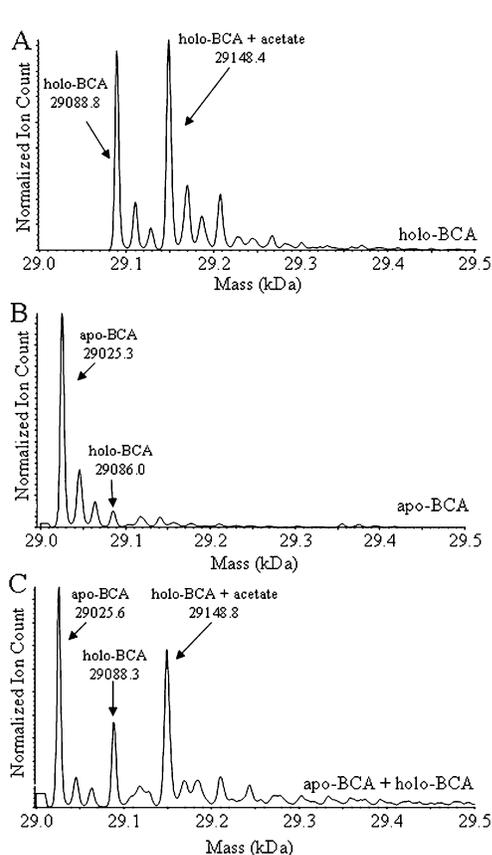
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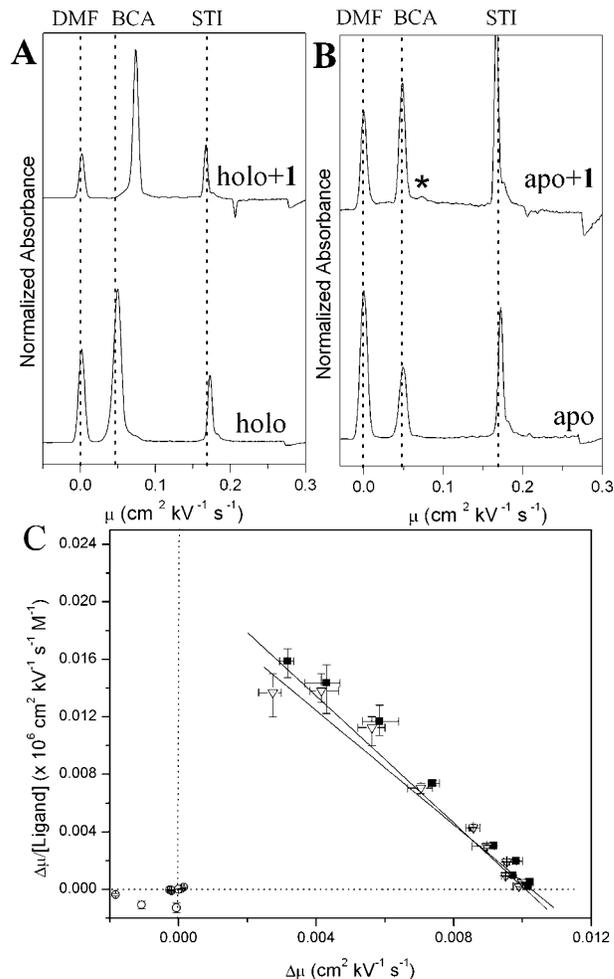
**Figure 2.** (A) Reconstructed mass spectrum of holo-BCA (observed mass  $29\,089.3 \pm 0.2$  Da; calculated mass  $29\,087.3$  Da). The additional major peak (mass  $29\,148.4 \pm 0.2$  Da) corresponds to holo-BCA bound to an acetate ion. Acetate is a known inhibitor of BCA and binds in the active site. (Hakansson, K.; Briand, C.; Zaitsev, V.; Xue, Y.; Liljas, A. *Acta Crystallogr., Sect. D* **1994**, *D50*, 101–104.) The additional unlabeled peaks correspond to sodium and water adducts of holo-BCA. (B) Reconstructed mass spectrum of apo-BCA (observed mass  $29\,025.7 \pm 0.2$  Da; calculated mass  $29\,024.3$  Da). The holo-BCA contamination in the apo-BCA sample is labeled. The area of this peak is less than 1% of the area of the major, apo-BCA peak. (C) Reconstructed mass spectrum of a 50:50 mixture of holo- and apo-BCA.

(Figure 4). The far-UV CD spectra of both apo- and holo-BCA have negative bands at 180 and 210 nm and a positive band at 200 nm. The positions of these bands are consistent with a structure composed of  $\beta$ -sheets.<sup>24</sup> The structure of CA is dominated by a  $\beta$ -sheet with 10 strands that span the width of the protein. Estimation of the amount of secondary structure is known, however, to be problematic for BCA because the many tryptophan residues interfere in the far-UV region of the CD spectrum that is normally used to calculate secondary structure.<sup>40</sup> The similarity in both the far- and near-UV regions of the CD spectra of apo- and holo-BCA allows us to conclude, nonetheless, that apo-BCA retains its native structure in the absence of its Zn(II) cofactor.

#### Charge Compensation of BCA upon Removal of Zn(II).

We expected that removal of the  $(\text{ZnOH})^+$  from the active site of BCA would remove a positive charge from the protein at pH 8.4,

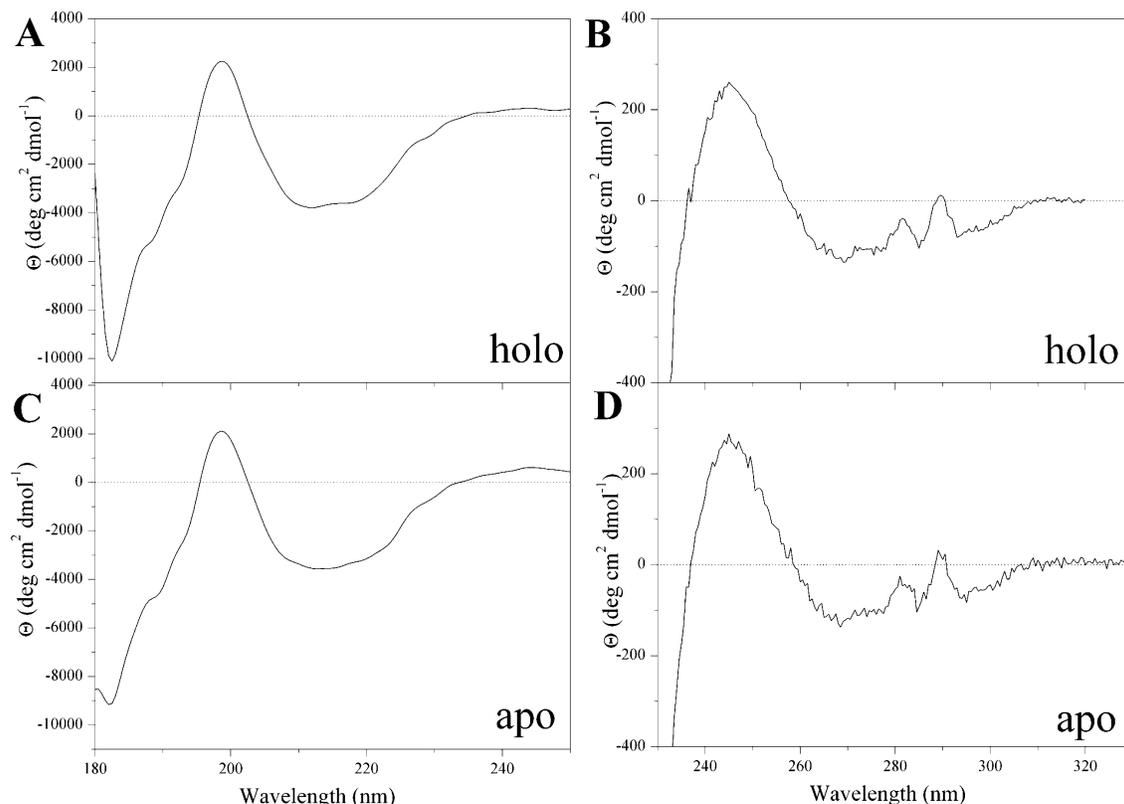
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**Figure 3.** Electropherograms of (A) holo-BCA and (B) with and without  $50\ \mu\text{M}$  **1** in the Tris–Gly running buffer. DMF was used as an electrically neutral marker to measure electroosmotic flow. Soybean trypsin inhibitor (STI) was used as a noninteracting internal protein standard. The holo-BCA contaminant in the apo-BCA sample is labeled with an (\*). (C) Overlay of Scatchard plots of holo-BCA (■) apo-BCA (○), and apo-BCA +  $10\ \mu\text{M}$  Zn(II) (as  $\text{ZnSO}_4$ ) (▽) treated with **1**. Error bars are the minimum and maximum values measured in four replicate measurements. The dissociation constants for **1** are  $0.44 \pm 0.02\ \mu\text{M}$  for holo-BCA and  $0.51 \pm 0.03\ \mu\text{M}$  for apo-BCA +  $10\ \mu\text{M}$   $\text{ZnSO}_4$ .

and we anticipated that apo- and holo-BCA would differ at this value of pH by one unit of charge. This difference would be observable by CE. We did not, however, observe the expected difference in mobility between apo- and holo-BCA; the electrophoretic mobilities of holo- and apo-BCA were indistinguishable in Tris–Gly buffer ( $25\ \text{mM}$  Tris,  $192\ \text{mM}$  glycine, pH 8.4). To account for the similarity in charge between apo- and holo-BCA, we suggest that one of the residues in the active site of apo-BCA protonates upon removal of  $\text{ZnOH}^+$  so that there is effectively no change in the net charge of the protein. Because the loss of Zn(II) did not cause any long-range structural changes (as measured by CD), we would expect its removal to affect only the amino acid residues in the active site.

We hypothesize that this similarity in charge is due to a protonation in the active site (or, in principle, elsewhere in the protein) upon loss of the  $(\text{ZnOH})^+$ . Accordingly, that proton should be removable at or above the  $\text{pK}_a$  of that ionizable group;



**Figure 4.** CD spectra of holo-BCA in the (A) far- and (B) near-UV spectral regions and apo-BCA in the (C) far- and (D) near-UV spectral region.

increasing the pH should allow us to resolve apo- and holo-BCA. To test this hypothesis, we varied the pH of the Tris–Gly buffer used for capillary electrophoresis between pH 7.0 and 10.5 (Figure 5). Apo- and holo-BCA are resolvable near pH 7. The  $pK_a$  of the  $(ZnOH_2)^{2+}$  moiety is  $\sim 7$ ;<sup>21,22</sup> this cofactor is only present in holo-BCA. Therefore, it is reasonable to expect that holo-BCA could bind an additional proton below pH 7 and become more positively charged than apo-BCA. We observed this expected difference in mobility between apo- and holo-BCA at pH 7 (Figure 5). Above pH 9.5, the peak in the electropherogram also splits, and when the pH is increased above 10.25, the holo-BCA and apo-BCA are clearly resolvable. This splitting is evidence that apo-BCA has a titratable proton with a  $pK_a$  near 10 that is not present in holo-BCA.

We cannot conclusively identify a residue in the active site with a  $pK_a$  near 10. Because the active site is composed of a network of charges, however, a change in the degree of protonation of one group can influence the degree of protonation of the other ionizable groups in the active site. This process of cooperativity in proton binding (or “charge regulation”) is commonly observed in proteins.<sup>41–43</sup> There are many basic residues in the active site whose ionization state could be affected by the lack of  $(ZnOH)^+$ . Three histidines normally chelate the Zn(II), and there is an additional histidine in the active site, His64, which behaves as a proton shuttle.<sup>44,45</sup> His64 may have a  $pK_a$  value that is dependent on the metal cofactor.<sup>21</sup> All of the histidine residues are hydrogen

bonded to electron-withdrawing groups that could elevate the  $pK_a$ 's of the histidines, and any of these histidines could have an abnormally elevated  $pK_a$ . In addition, it is possible that two (or more) histidines could share a proton and the  $pK_a$  of this group would likely be much higher than 7.

#### Denaturation and Renaturation of BCA and Apo-BCA.

BCA (10  $\mu$ M) was denatured in Tris–Gly buffer (pH 8.4) containing 10 mM SDS. We observed only the denatured peak in the electropherogram within 30 min after addition of the SDS. There was no observable change in the peak shape and distribution by CE after 4 h, so the protein was left for 24 h to ensure complete denaturation. BCA was renatured by diluting a solution of denatured BCA 10-fold in Tris–Gly buffer (to reduce the SDS concentration below its critical micelle concentration) and then dialyzing against Tris–Gly buffer for 48 h (Figure 6).

We measured the amount of protein recovered after renaturation using absorbance at 280 nm.<sup>46</sup> We measured the amount of folded BCA that we recovered by comparing peak areas in the electropherograms before denaturation and after renaturation. When the holo-BCA sample was renatured in Tris–Gly buffer with 10  $\mu$ M  $ZnSO_4$  (in order to generate only holo-BCA), the recovery

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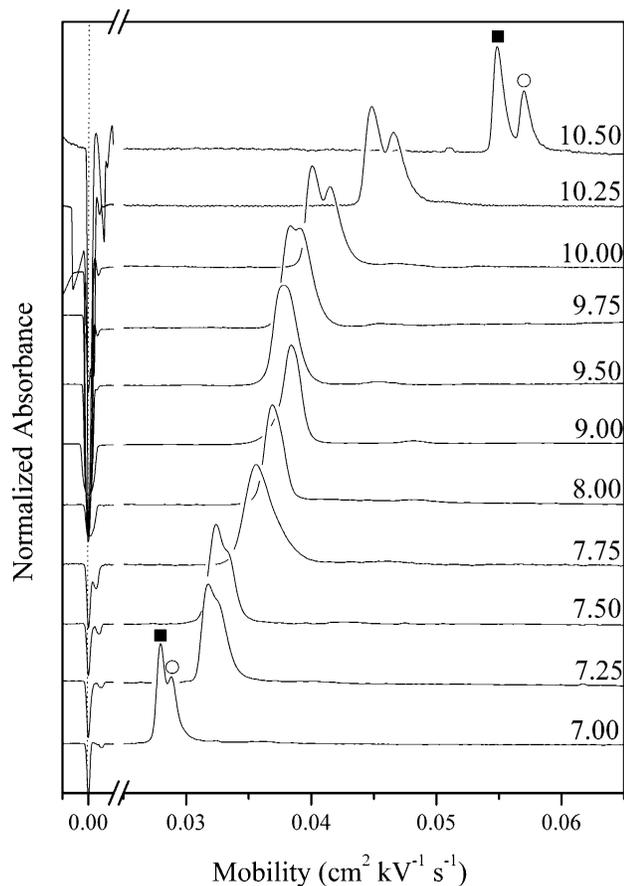
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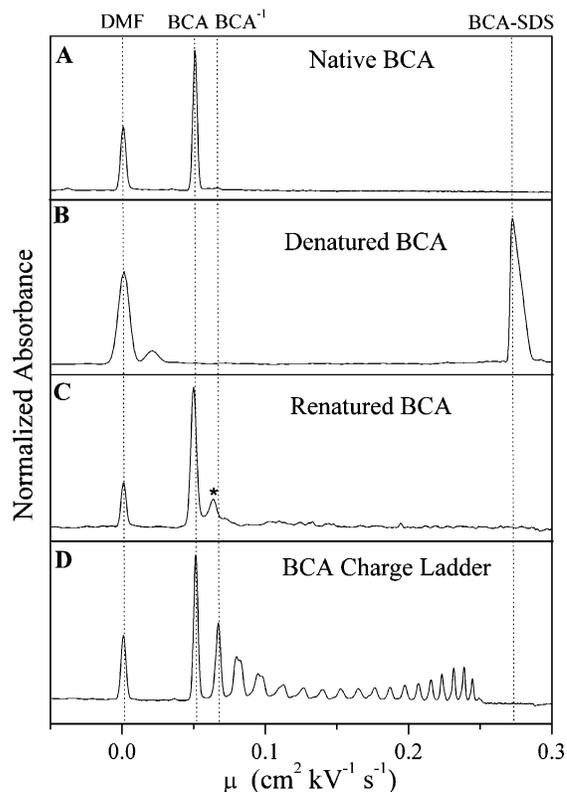
(46) Blanking of the UV–visible instrument was not reproducible at 214 nm because the absorbance of the Tris–Gly buffer interfered with the measurement. Therefore, measurements of total protein concentration using absorbance were done at 280 nm. The absorbance of the tris ions does not affect CE experiments because the tris is at a constant concentration (25 mM) in all solutions.



**Figure 5.** Electropherograms of a mixture of 60% holo-BCA (■) and 40% apo-BCA (○) in Tris-Gly buffer with the pH adjusted between 7.00 and 10.50. The protein sample contained 3  $\mu\text{M}$  holo-BCA and 2  $\mu\text{M}$  apo-BCA in water at pH 7. The negative peak at zero mobility results from a buffer mismatch between the sample, in water, and the buffer (Tris-Gly). At pH 10.5, the peaks corresponding to holo- and apo-BCA are clearly resolved. The apo-BCA peak has a higher mobility and, thus, a more negative charge. At pH 7, there is also partial separation of the two proteins; again, the apo-BCA is more negatively charged.

of protein was quantitative (>99%), as measured by absorbance at 280 nm. The area of the BCA peak observed by CE (and therefore, the recovery of folded protein) was only 80% of that of the starting material. There were no other peaks observable by CE. This result suggests that 20% of the protein had not regained its original fold and was not observed by CE, possibly due to a range of partially folded conformations or aggregation. We then used CE to quantify the amount of *active* protein as measured by sulfonamide binding. All (>98%) of the refolded holo-BCA detected by CE shifted to a higher mobility on addition of **1** at 50  $\mu\text{M}$ ; this shift indicates that all of the refolded holo-BCA observed by CE bound inhibitor.

In a similar experiment using apo-BCA, the protein was renatured in Tris-Gly buffer with 10  $\mu\text{M}$  EDTA in order to sequester Zn(II) and to generate only apo-BCA. We recovered only 45% of the total protein, as measured by UV absorbance at 280 nm. This result suggests that apo-BCA may be more prone than holo-BCA to aggregation or adhesion to the dialysis membrane. The area of the BCA peak observed by CE for the apo-BCA sample was ~35% of that of the starting material (or 80% of the recovered protein). Since the recovery measured by CE was



**Figure 6.** Capillary electropherograms of (A) native BCA, (B) BCA denatured with 10 mM SDS, and (C) BCA after renaturation. A charge ladder made by acetylation of lysine residues (D) is included to show that the secondary peak appears to increase the charge by ~0.9 unit. DMF was used as a neutral marker to measure electroosmotic flow. The (\*) indicates the new peak observed in the renatured electropherogram, but not the native BCA electropherogram. This peak is discussed further in the text.

~20% less than that measured by UV absorbance, the same proportion of apo-BCA and holo-BCA samples had not regained the original fold. None of the samples of apo-BCA that we refolded in EDTA bound sulfonamide. The absence of Zn(II) from the center of the hydrophobic core of BCA may allow this region of the protein to be less ordered than with the Zn(II) cofactor and, thus, allow aggregation of a partially folded intermediate. Hammarstrom et al. showed that HCA II aggregated during renaturation with heat and GuHCl.<sup>47</sup> They also showed that this aggregation only occurred when the protein was in the molten-globule intermediate and not when the protein was in the native or denatured state. In addition, the structure of the aggregates was specific and involved only the central  $\beta$ -sheet of HCA II.

#### High-Mobility Product of Denaturation/Renaturation.

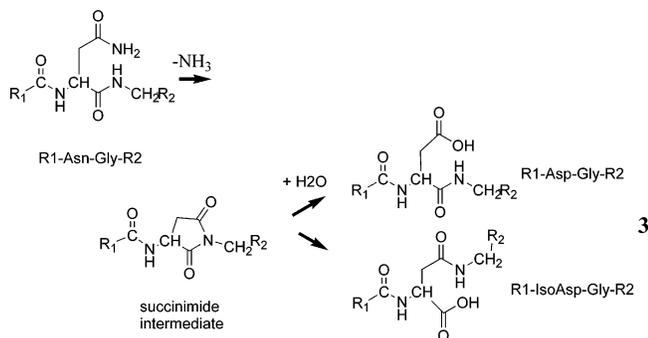
Upon renaturation of both apo- and holo-BCA, we observed a new peak in the electropherogram (Figure 6C). Using CE, we were able to determine that, in the presence of Zn(II), the new peak binds sulfonamide with high affinity ( $K_d < 10 \mu\text{M}$ ). By comparison of the mobility of the new peak with the mobilities of the rungs of a charge ladder,<sup>48</sup> we infer that the new species is BCA with ~0.9 additional negative charges. We suspect that this new species is due to the addition of a negative charge, but that through charge

(47) Hammarstrom, P.; Persson, M.; Freskgard, P.-O.; Martensson, L.-G.; Andersson, D.; Jonsson, B.-H.; Carlsson, U. *J. Biol. Chem.* **1999**, *274*, 32897–32903.

regulation, the  $pK_a$  values of other residues are changed so that the net change in charge is less than a full charge.

The new peak also appears in the same relative amounts in samples that we denatured in GuHCl and subsequently renatured (results not shown); the new species is thus not native protein that has somehow retained a molecule of SDS or some derivative of SDS. The area of the new peak does not seem to increase with the amount of time the protein spends in the denatured state or with multiple cycles of denaturation and renaturation. The interpretation of yields is complicated, however, by the fact that the area of the native protein peak decreases with these treatments. It is thus possible that the yield of this new peak does increase with time or with cycles of denaturation but that this increase is masked by net loss of protein.

We hypothesized that the new species is the product of deamidation of an asparagine to an aspartic acid residue. This reaction 3 usually proceeds through a cyclic imide intermediate



at neutral pH and, for steric reasons, is 30–50 times faster when the asparagine is followed sequentially by a glycine residue.<sup>49–51</sup>

The rate of this reaction also depends on the conformation of the polypeptide backbone, and folded proteins are usually too rigid to permit buried asparagine residues to form succinimides.<sup>49,53</sup> There are three asparagines that are followed by a glycine residue in the primary sequence of BCA, at positions 10, 23, and 61.<sup>52</sup>

We used three proteases, trypsin, endoprotease LysC, and endoprotease AspN, to digest both BCA and BCA that had been refolded. We then analyzed the peptide fragments from each protein sample by reversed-phase chromatography coupled to mass spectrometry (data not shown). If any asparagine is deamidated, the peptide fragment containing that residue should either be 17 Da lighter, if the asparagine remains as the succinimide intermediate, or 1 Da heavier, if the succinimide intermediate is hydrolyzed to either aspartic acid or isoaspartic acid.

We observed a peptide containing Asn10 that also contained the succinimide intermediate (–17 Da) in both BCA and refolded BCA samples. In both protein samples, we compared the ion count

of the peptide containing the succinimide intermediate with the ion count of the original peptide. In both the BCA and refolded BCA samples, the amount of deamidated Asn10 is approximately the same. Although we cannot rule out a small amount of deamidation of residue 10 due to denaturation, it appears that most of the deamidation of this residue is caused by either the protease or mass spectrometry procedures or is present in the original sample.

The other two Asn–Gly sequences in BCA showed evidence of that the process of denaturation with SDS followed by renaturation caused additional deamidation. The digests of refolded BCA with both endoprotease LysC and endoprotease AspN showed that peptides containing Asn23 had peptides with +1 Da; the AspN digest also contained a peptide with a mass of –17 Da. None of these changes were observed in the digests of BCA. The changes in mass provide clear evidence that Asn23 is deamidated during the denaturation process.

The trypsin and AspN digests of refolded BCA have peptides containing Asn61 with a mass of an additional 1 Da. The analysis of asparagine 61 is somewhat complicated, however, by the fact that these peptides also contains two other asparagines (Asn60 and Asn66) that could, in principle, also be deamidated. An extra proteolytic cleavage fragment in the AspN digest of refolded BCA provides strong evidence that Asn61 is deamidated. AspN cleaves before aspartic acid residues (but not asparagine residues), and there is an additional cleavage site before residue 61 in the sample of refolded BCA; this extra cleavage site indicates that, in some of the refolded BCA sample, Asn61 has been deamidated to Asp61. We did not observe any peptides that would indicate new cleavage sites at residues 60 or 66.

We conclude, therefore, that the additional, higher mobility peak in the electropherograms of refolded BCA corresponds to forms of BCA that have been deamidated at either Asn23 or Asn61. No single asparagine residue seems particularly susceptible to deamidation in the denatured state, but rather, the additional flexibility in the denatured protein allows any of the asparagine–glycine sequences in BCA to become deamidated. Wearne and Creighton found that the deamidation of ribonuclease A was increased more than 30-fold when denatured with heat,<sup>53</sup> so it seems plausible that the rate of deamidation of BCA is also increased in the denatured state.

**Influence of Zn(II) on the Refolding of BCA.** To assess whether the Zn(II) cofactor influences the rate or product of refolding, we compared the behavior of apo- and holo-BCA during denaturation and renaturation with SDS. To assay whether the refolded proteins could bind inhibitor, we used a benzenesulfonamide, **2**, which has two negative charges at pH 8.4. The additional charge of inhibitor **2** relative to inhibitor **1** allows the BCA bound to the inhibitor to be distinguished from the peak due to the presumed deamidation of BCA. Denaturation and renaturation of both holo- and apo-BCA in Tris–Gly buffer containing 25 nM Zn(II), the residual amount of Zn(II) present in the buffer, yielded ~50% of the recovered protein as holo-BCA, as observed by the shift in mobility of 50% of the protein upon addition of a charged inhibitor (Figure 7A and B). We infer that the denatured polypeptide chain does not retain Zn(II). We presume that the observation that some of the protein in the refolded sample binds inhibitor reflects the ability of apo-BCA to scavenge residual Zn-

(48) A charge ladder is a set of derivatives of a protein produced by the partial acetylation of its Lys  $\epsilon$ -NH<sub>3</sub><sup>+</sup> groups (Colton et al., *J. Am. Chem. Soc.* **1997**, *119*, 12701–12709). CE resolves these derivatives into distinct peaks (“rungs”) that differ from each other by approximately one charge.

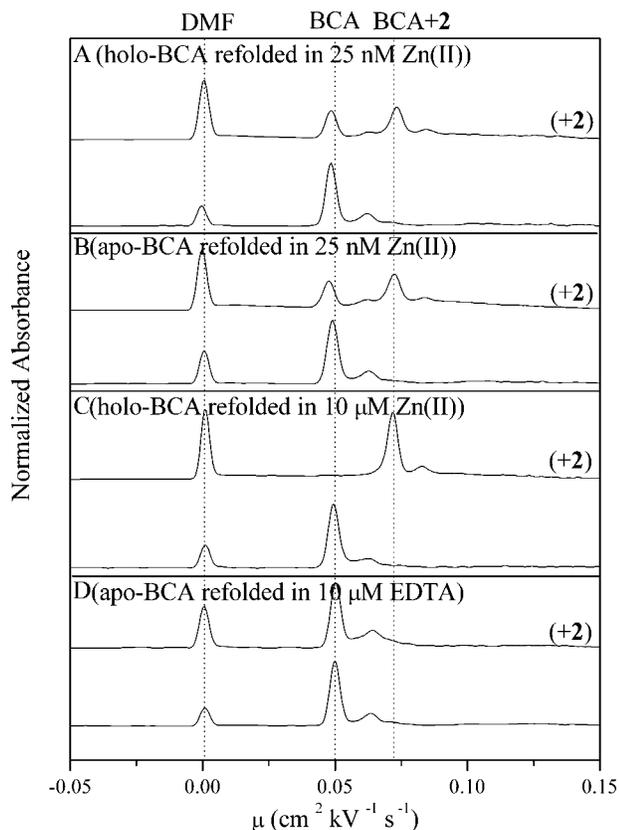
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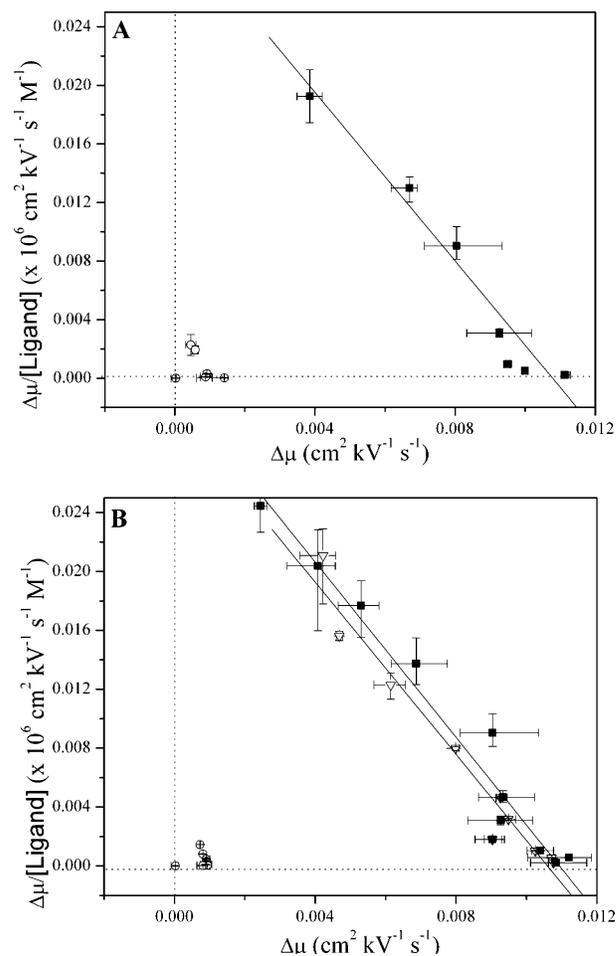


**Figure 7.** Electropherograms of (A) refolded holo-CA, (B) refolded apo-CA, (C) refolded holo-CA in 10  $\mu\text{M}$   $\text{ZnSO}_4$ , and (D) refolded apo-CA in 10  $\mu\text{M}$  EDTA, without and with 50  $\mu\text{M}$  **2**. DMF was used as a neutral marker to measure electroosmotic flow.

(II) from the buffer. We used 100  $\mu\text{L}$  of 10  $\mu\text{M}$  BCA dialyzed against 4 L of treated buffer. At these concentrations, we expected that all of the BCA could have bound Zn(II). It is possible that because the plasticware we used for the dialysis was washed with EDTA, the concentration of free Zn(II) was lower than 25 nM.

Denaturation and renaturation of holo- or apo-BCA in the presence of 10  $\mu\text{M}$   $\text{ZnSO}_4$  generates >98% of the refolded protein in a form that binds the inhibitor **2** (Figure 7C). This result confirms that apo-BCA takes up Zn(II) from the buffer during renaturation. Denaturation and renaturation of holo- or apo-BCA in 10  $\mu\text{M}$  EDTA gives protein that does not bind inhibitor, with the mobility of the refolded peak matching that of the protein before denaturation. This result suggests that EDTA is effective at sequestering all of the Zn(II) and leaves only apo-BCA as the refolded product. Upon addition of excess  $\text{ZnSO}_4$  to the protein sample renatured in EDTA, all of the protein in the resulting sample binds inhibitor (Figure 8). To demonstrate that the refolded samples had regained the original conformation, we used CD to show that BCA, apo-BCA, renatured BCA, and renatured apo-BCA are structurally indistinguishable by this technique (Figure 9). The fact that refolding regenerates apo-BCA that is indistinguishable from the starting material, both by ACE and by CD, suggests that Zn(II) is not necessary for refolding of BCA. The absence of the cofactor, however, decreases the yield of recovered protein, presumably due to nonspecific adsorption of the protein to surfaces.

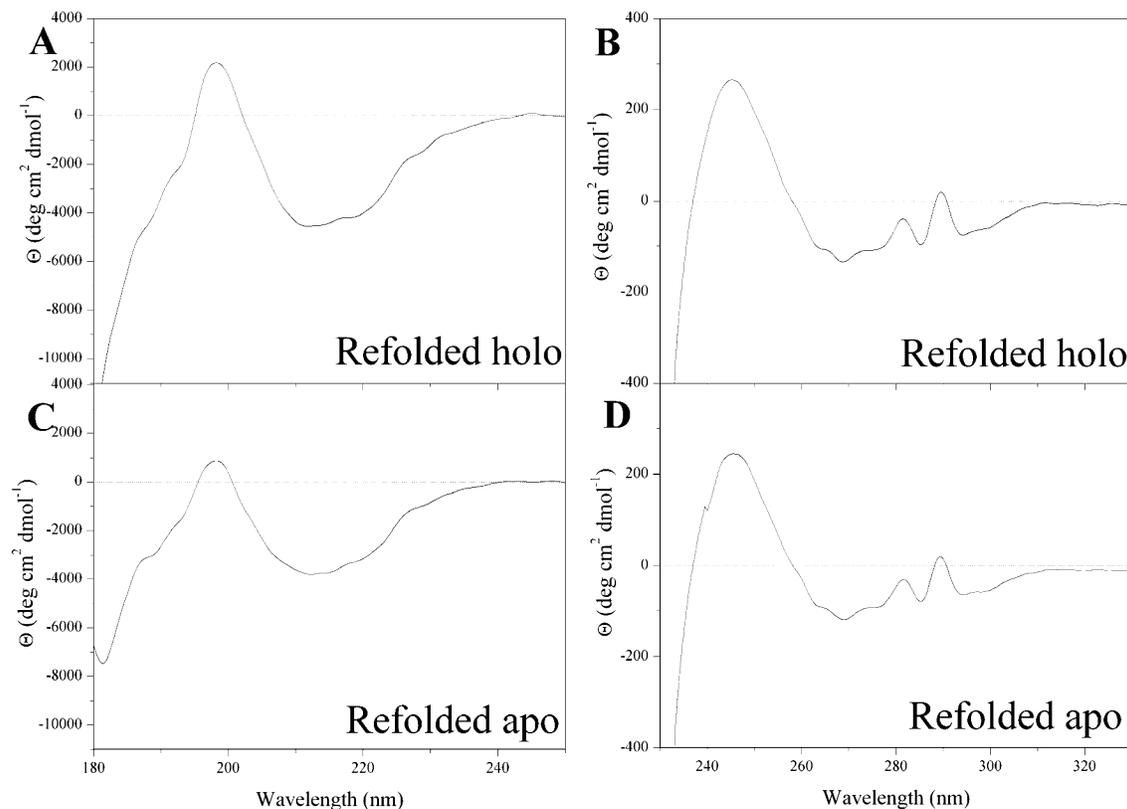
**Kinetics of Refolding of Holo- and Apo-BCA.** Following Semisotnov,<sup>30</sup> we measured the kinetics of refolding by diluting



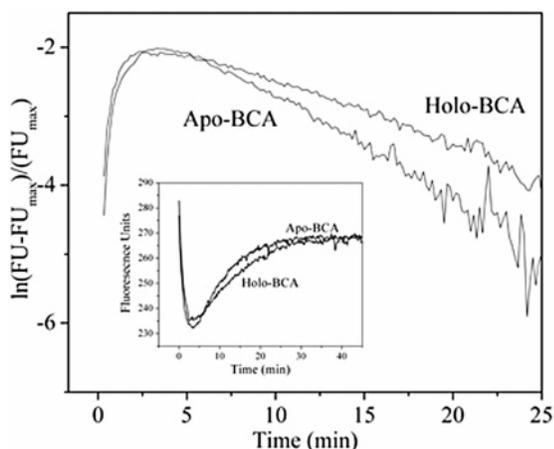
**Figure 8.** (A) Scatchard analysis of the nonbinding population and the binding population ( $\blacksquare$ ,  $K_d = 0.35 \pm 0.05 \mu\text{M}$ ) of holo-CA of after refolding in Tris–Gly without additional Zn(II). (B) Scatchard analysis of holo-CA refolded in the presence of 10  $\mu\text{M}$   $\text{ZnSO}_4$  ( $\blacksquare$ ,  $K_d = 0.34 \pm 0.03 \mu\text{M}$ ), apo-CA refolded in the presence of 10  $\mu\text{M}$  EDTA (○), and apo-CA refolded in the presence of 10  $\mu\text{M}$  EDTA after addition of 50  $\mu\text{M}$   $\text{ZnSO}_4$  ( $\nabla$ ,  $K_d = 0.34 \pm 0.04 \mu\text{M}$ ). Error bars are the minimum and maximum measured values of four replicate measurements.

an aliquot of denatured holo- or apo-BCA containing 10 mM SDS by 100-fold in Tris–Gly buffer containing 0.2 M acrylamide and either 10  $\mu\text{M}$   $\text{ZnSO}_4$  or 50  $\mu\text{M}$  EDTA, respectively. The acrylamide is an effective quencher of tryptophan fluorescence. Since quenching is strongly dependent on distance between fluorophores, the tryptophan fluorescence would be quenched when the protein is denatured and the tryptophan residues are exposed to solvent. As BCA folds and the tryptophan residues are buried, the distance between quencher and fluorophore increases and the observed fluorescence increases. We observe an initial decrease in fluorescence and a subsequent increase (Figure 10). We cannot explain why there is an initial decrease in tryptophan fluorescence, but the time scale of this decrease does not change when we changed the acrylamide concentration from 0.2 to 0.04 M (data not shown). The two time scales for the renaturation of BCA are  $t_1 = 1.1 \pm 0.3$  min for the initial decrease in fluorescence and  $t_2 = 9.6 \pm 0.9$  min for the slower increase for holo-BCA refolded in 10  $\mu\text{M}$   $\text{ZnSO}_4$ , and  $t_1 = 1.6 \pm 0.2$  min and  $t_2 = 6.5 \pm 1.4$  min for apo-BCA refolded in 50  $\mu\text{M}$  EDTA.

These two time scales correspond to the two longer time scales (of  $\sim 2$  and  $\sim 10$  min) observed in the renaturation of BCA from



**Figure 9.** CD spectra of holo-CA after refolding in Tris–Gly buffer in the (A) far- and (B) near-UV spectral regions and apo-CA after refolding in Tris–Gly in the (C) far- and (D) near-UV spectral region.



**Figure 10.** Kinetics of refolding of holo-BCA in the presence of 10  $\mu\text{M}$   $\text{ZnSO}_4$  and apo-BCA in the presence of 50  $\mu\text{M}$  EDTA, both with 0.2 M acrylamide to quench the fluorescence of tryptophan residues in the denatured state. On the logarithmic scale, there are two distinct phases to the refolding. The inset graph shows the raw fluorescence data (ex 280 nm, em 340 nm) with an initial decrease followed by a slow increase.

GuHCl. We suggest, therefore, that the two time scales observed in renaturation from SDS are, as in renaturation from GuHCl, due to isomerizations of two proline residues. With this method, we are unable to observe any change in fluorescence with a time scale of less than  $\sim 1$  min and are thus unable to observe the fastest process in the renaturation of BCA—the formation of the molten globule.<sup>15</sup> The time scales of refolding that we detect are very similar between holo- and apo-BCA. Instead of the 2-fold slower

refolding of apo-BCA that Yazgan and Henkens reported,<sup>26</sup> we observe a slight increase in the rate of refolding of apo-BCA, although the difference between apo- and holo-BCA is not significantly greater than the error in the measurements. We would not expect the Zn(II) cofactor to have an effect on proline isomerization, and the result that apo- and holo-BCA have similar rates of refolding supports the hypothesis that the rate-determining step in BCA folding is the isomerization of proline residues. Because it is located in the center of the hydrophobic core, Zn(II) may have an effect on the rate of collapse of the denatured chain into the molten-globule intermediate, but with this method, we are unable to measure any kinetics on the fast time scale that would be necessary to observe a difference between apo- and holo-BCA.

## CONCLUSIONS

This work demonstrates that CE is a useful technique for studies of denaturation and renaturation of BCA, and, perhaps, of other proteins, with SDS. CE readily distinguishes between BCA in its native form and BCA denatured with SDS. It also distinguishes the more subtle difference between subpopulations of protein that have the same mobility but different activity, by showing a shift in mobility upon binding of a charged inhibitor. This characteristic should be useful for analyzing complex mixtures of proteins and for quantifying the fraction of a sample of protein that is active in binding a ligand as inhibitor.

Conversely, CE can resolve two populations of protein that have indistinguishable activity in binding but differ by a single unit of charge, for example, native BCA and BCA with a presumed deamidation (Figure 7). These two populations could not be easily

identified by traditional biochemical assays. This high sensitivity to small changes in charge makes CE generally useful for studying (i) the interactions of proteins with singly or doubly charged metal ions, surfactants, and ligands, (ii) the effects of pH on ionizable groups on a protein, and (iii) isozymes with similar activity but different amino acid sequence. CE is thus an excellent complement to ion exchange chromatography, gel electrophoresis, and isoelectric focusing techniques. As with these techniques, CE may not be very useful for studying proteins containing many functional groups that ionize over a small pH range or molecules that degrade with changes in pH.

This work also used CE to demonstrate several important characteristics of the interaction of CA with its Zn(II) cofactor: (i) Zn(II) does not remain associated with the polypeptide chain of BCA when denatured in SDS; (ii) Zn(II) is not required for refolding of BCA into its native conformation; (iii) Zn(II) does not affect the rate of refolding; and (iv) Zn(II) increases the recovery of active protein after renaturation by a factor of 2. The last characteristics suggest that Zn(II) may stabilize an early intermediate in the folding pathway of BCA and reduce aggregation of unfolded proteins; its presence in the buffer may improve laboratory preparations or manipulations (e.g., chromatography) of BCA.

This work provides the basis for further, detailed studies of the interaction of BCA with SDS.

## EXPERIMENTAL SECTION

**Sources of Chemicals and Reagents.** All chemicals were reagent grade unless stated otherwise. Dipicolinic acid, 4-(aminomethyl)benzenesulfonamide, NHS-activated agarose, 10× Tris–Gly concentrate, Chelex-100, cesium iodide, ammonium acetate, ammonium bicarbonate, and BCA were all purchased from Sigma-Aldrich (St. Louis, MO). Dialysis cassettes (weight cutoffs of 10 kDa) were purchased from Pierce (Rockford, IL). Q-Sepharose resin was purchased from Amersham Biosciences (Uppsala, Sweden). The HPLC grade water, 2-propanol, and acetonitrile used for mass spectrometry were from Burdick and Jackson (Muskegon, MI). SDS was purchased from J.T. Baker (Phillipsburg, NJ). SDS (Baker Chemical Co., 4095-04) was recrystallized in hot ethanol three times,<sup>54</sup> then dried, and stored at –20 °C until use. SDS was discarded or repurified after 2 months. Tris–Gly buffer was made by diluting 100 mL of the 10× concentrate with 900 mL of freshly distilled, deionized water and filtered with a 0.22- $\mu$ m filter (Pall, Ann Arbor, MI) prior to use.

**Purification of CA.** BCA was purified in two column steps. The first step was purification using affinity chromatography of an immobilized benzenesulfonamide; the procedure was similar to that of Khalifah et al.<sup>55</sup> The second step used anion exchange to remove the BCAII variant with glutamine at position 56.

**Affinity Chromatography.** A sulfonamide column for affinity purification of BCA was prepared by dissolving 4-(aminomethyl)-benzenesulfonamide (Aldrich, A6180-2) in 50% (v/v) acetone/water to a final concentration of 1 mM and adjusting the pH to 8.0. A 5-mL aliquot of *N*-hydroxysuccinimide-activated agarose beads was added, and the pH was maintained at 8.0 by addition

of 1 N NaOH for 2 h. The pH was then raised to 10.5 with 1 N NaOH to hydrolyze any unreacted NHS ester. The resin was washed with two column volumes (cv) of 50% (v/v) acetone/water, followed by two cv of water and two cv of Tris–Gly buffer. BCAII (Sigma, C-3934, Lot 22K1469) was dissolved in TG buffer (12.5 mg/mL). The protein mixture was added to the resin and shaken for 16 h. The sulfonamide-labeled beads were poured into a column ( $I_d = 25$  mm,  $L = 30$  cm) and washed with four cv of TG buffer to remove unbound protein. BCA was eluted with 0.4 M KSCN in TG buffer. All fractions that contained protein ( $A_{214\text{ nm}} > 0.2$ ) were pooled and concentrated using a filter concentrator (Centricon, molecular mass cutoff 10 kDa, Millipore Corp.). The concentrated protein was loaded onto a NAP column (Amersham Biosciences) to remove the KSCN.

**Anion Exchange Chromatography.** A Q-Sepharose column (Amersham Biosciences) was washed with three cv of water and three cv of Tris–SO<sub>4</sub> buffer at pH 8.3. The collected protein from the NAP column was loaded onto the Q-Sepharose column. The desired isoform of BCA did not bind to the column and was collected in the first cv of Tris–SO<sub>4</sub> wash. The fractions were assayed by CE, and those showing only the desired BCA isoforms were mixed, concentrated, and desalted. The pure BCA (10 mL) was dialyzed overnight against 4 L of water in a dialysis cassette. The protein was lyophilized and stored at –20 °C until use.

**Capillary Electrophoresis Experiments.** Capillary electrophoresis experiments were carried out in a Beckman PACE-MDQ system, using a capillary of inner diameter of 50  $\mu$ m of total length of 110.2 cm, 100 cm to the detector. Tris–Gly was used as the running buffer, and the applied voltage was 30 kV. Each sample contained 0.65 mM dimethylformamide (DMF) as an electrically neutral marker for electroosmotic flow.

**Removal of Zn(II) from BCA.** BCA (2 nmol) was dialyzed against 0.1 M dipicolinic acid in Tris–Gly buffer (1 L) for 3 days at a room temperature, with buffer exchange every 12 h. The dipicolinic acid was removed before analysis by dialyzing 1 mL of protein in a dialysis cassette, against 1 L of Tris–Gly buffer, with four changes of buffer.

**Treatment of Tris–Gly Buffer To Remove Zn.** Only plasticware that had been rinsed with 1 mM EDTA and dried was used. We added 100 g of chelating resin (Chelex-100) to 500 mL of the TG 10× concentrate. We stirred the solution overnight and filtered out the resin using a plastic membrane. When assayed by AA, we found that the treated buffer had ~25 nM Zn.

**Mass Spectrometry.** ESI mass spectra were acquired using a quadrupole orthogonal time-of-flight instrument (QoTOF) (Applied Biosystems/MDS Sciex, API QSTAR Pulsar i LC/MS/MS system; Toronto, ON, Canada). A Protana NanoES source (Proxeon Biosystems, Odense, Denmark) was employed using uncoated glass tips (World Precision Instruments, Kwik-Fil borosilicate capillaries, Sarasota, FL), pulled in-house to ~1  $\mu$ m i.d. using a micropipet puller (Sutter Instrument Co., model P-97, Novato, CA). Spray was produced with a voltage of 1200–1300 V applied via a platinum wire inserted into the glass tip in contact with the sample solution. Spectra of re-folded apo- and holo-bovine carbonic anhydrase were acquired in positive polarity over the range  $m/z$  2000–3500. This range was calibrated using 2 mg/mL cesium iodide in an aqueous solvent containing 50% isopropyl alcohol. Proteins were prepared at a concentration of 5–10  $\mu$ M in 10 mM

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aqueous ammonium acetate buffer (pH 8). The accuracy of protein mass measurements was  $\sim\pm 15$  ppm (standard deviation, based on five measurements).

**Enzymatic Digestion of Proteins.** Samples of BCA and refolded BCA (20  $\mu\text{M}$  in water) were diluted in 50 mM ammonium bicarbonate, pH 8.5. The proteases LysC, AspN, and trypsin (Roche, Mannheim, Germany) were added to separate aliquots at an enzyme-to-substrate ratio of 1:50 and incubated overnight at 37°C.

#### Liquid Chromatography/Mass Spectrometry of Peptides.

A Waters CapLC with an autosampler was used to perform chromatography. The samples of peptides from enzymatic digestions were diluted to a concentration of 1 pmol/ $\mu\text{L}$  with 0.1% aqueous formic acid, and 1  $\mu\text{L}$  of the peptide digest was loaded via a trapping column (Waters Optipak, Symmetry300 C18, 5  $\mu\text{M}$ ). The sample was washed for 4 min at a flow rate of 10  $\mu\text{L}/\text{min}$  with 3% acetonitrile, 0.1% formic acid using the auxiliary pump. The gradient used was 3% buffer B (97% acetonitrile, 3% water, 0.1% formic acid) to 60% buffer B over 60 min, 60–80% buffer B over 2 min, 80–90% buffer B over 1 min, and 90–3% buffer B over 2 min followed by 17 min column reequilibration between samples. The flow rate was  $\sim 400$  nL/min, split down from an instrument flow rate of 5  $\mu\text{L}/\text{min}$ . Eluent was sprayed at 2000 V from a distally coated tip (New Objective, Woburn, MA) into a QoTOF MS, as described above, using intelligent data acquisition (IDA). Ions that exceeded 10 counts in MS were subjected to MS/MS. Preset collision energies were used, based on the charge state and  $m/z$  of the selected ion ( $\sim 18$ –60 V). The spectra were calibrated externally using the MS/MS spectrum of GluFibrinopeptide.

**Atomic Absorption Spectroscopy.** AA experiments were performed on a Perkin-Elmer AAnalyst 300 spectrometer using a graphite furnace with a Zn lamp.

**Denaturation and Renaturation of BCA.** The protein (10  $\mu\text{M}$ , apo- or holo- BCA) was denatured with 10 mM SDS in TG buffer for 24 h at ambient temperature (25 °C). The denatured protein was renatured by dialysis in a dialysis cassette (MW cutoff

of 10 kDa, Pierce) at  $\sim 25$  °C against 1.5 L of TG buffer for 48 h, with buffer exchanges every 12 h.

**Circular Dichroism Experiments.** BCA was dialyzed against 1 mM sodium phosphate buffer (pH 7.5) for 12 h at ambient temperature. CD measurements were performed on a Jasco instrument in a quartz cuvette with a 1-mm path length. For far-UV measurements (180–260 nm), protein concentrations of 5–7  $\mu\text{M}$  protein were used; for near-UV measurements (250–340 nm), protein concentrations of 50–70  $\mu\text{M}$  protein were used. Each sample was scanned 30 times at 20 nm/min with step sizes of 0.2 nm.

**Kinetics of Refolding Experiments.** A solution of denatured protein (holo- or apo-BCA, 50  $\mu\text{M}$ ) in Tris–Gly buffer with 10 mM SDS was diluted 100-fold into a solution containing Tris–Gly buffer with 0.2 M acrylamide and either 10  $\mu\text{M}$  ZnSO<sub>4</sub> or 50  $\mu\text{M}$  EDTA in a quartz cuvette. The fluorescence of the tryptophan residues was measured as a function of time using a Perkin-Elmer LS 50B Luminescence Spectrometer. We used an excitation wavelength of 280 nm and an emission wavelength of 340 nm, both with 10-nm slit widths.

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