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Eliminating Positively Charged Lysine ϵ-NH_3^+ Groups on the Surface of Carbonic Anhydrase Has No Significant Influence on Its Folding from Sodium Dodecyl Sulfate

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Abstract: This study compares the folding of two polypeptides—bovine carbonic anhydrase (BCA) and peracetylated BCA (BCA-Ac_{18})—having the same sequence of amino acids but differing by 18 formal units of charge, from a solution containing denaturing concentrations of sodium dodecyl sulfate (SDS). Acetylation of BCA with acetic anhydride converts all 18 lysine-ϵ-NH_3^+ groups to lysine-ϵ-$NHCOCH_3$ groups and generates BCA-Ac_{18}. Both BCA and BCA-Ac_{18} are catalytically active, and circular dichroism spectroscopy (CD) suggests that they have similar secondary and tertiary structures. SDS at concentrations above ~10 mM denatured both proteins. When the SDS was removed by dialysis, both proteins were regenerated in native form. This study suggests that large differences in the net charge of the polypeptide have no significant influence on the structure, the ability to refold, or the rate of refolding of this protein from solutions containing SDS. This study reinforces the idea that charged residues on the surface of BCA do not guide protein folding and raises the broader question of why proteins have charged residues on their surface, outside of the region of the active site.

Introduction

The mechanism of folding of polypeptide chains into functional proteins is an important and still unresolved problem.[1−4] While burial of hydrophobic residues is considered the major source of stability in a folded protein,[5] the contribution of electrostatic interactions is unclear and may differ from protein to protein.[6] In this study, we drastically increased the net charge on the surface of a model protein—bovine carbonic anhydrase (BCA, EC 4.2.1.1)—in order to probe the effects of surface charge on the ability of a protein to fold. Large differences (a factor of approximately 5) in the net charge of BCA had no significant influence on its refolded structure and only marginally observable effects on the rate at which it folds.

The interactions between charged groups in a protein can be classified as local (e.g., in salt bridges), where the two oppositely charged groups are in proximity (≤4 Å), or long-ranged, where the charged residues interact with multiple other charged residues over larger distances (>4 Å). Salt bridges buried in the low dielectric interior of a protein can contribute as much as 2−5 kcal/mol to protein stability.[7] The magnitude of the contribution to stability of salt bridges that are exposed on the surface of a protein is variable, with reports of both significant[8−10] and negligible effects.[11] Local electrostatic interactions may shift the equilibrium of protein folding toward the native state[12,13] and constrain backbone flexibility in the native state.[14,15] The effects of long-ranged, nonspecific electrostatic interactions are usually more subtle than local interactions. Many groups have used site-directed mutagenesis of single charged amino acids to uncharged analogues to show that reducing the overall charge on the protein has a small (<0.5 kcal/mol), but usually stabilizing, effect.[16−19]

Since it is difficult to modify large numbers of residues using site-directed mutagenesis, the only studies that have made large changes in the net charge of the protein have involved chemical modifications. Hollecker and Creighton chemically modified a

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number of proteins using succinic anhydride to convert the positive charge on the lysine residues to a negative charge.[20] They found that the stability toward denaturation of the modified protein by urea was similar to that of the unmodified proteins. Carbeck and co-workers used acetylation of lysine groups to increase the charge on α-lactalbumin by ~8 charges (from ~ −5.6 to ~ −13).[21] This change had a minimal effect on the stability of the protein toward thermal denaturation ($\Delta\Delta G_{\text{unfolded}-\text{folded}} \cong 2.5$ kcal/mol at 25 °C). In an attempt to remove *all* electrostatic interactions in ubiquitin, Loladze and Makhatadze used a combination of site-directed mutagenesis and chemical modification.[22] They first replaced all of the arginine residues with lysine residues by site-directed mutagenesis, carbomylated all of the lysine-ϵ-NH_3^+ groups, and then examined denaturation at pH 2; at this value of pH, all of the carboxylic acid residues are protonated and the net charge of the protein is close to zero. They found that the chemically modified ubiquitin (with all Arg mutated to Lys) was more stable than unmodified ubiquitin toward denaturation with urea ($\Delta\Delta G = 2.6$ kcal/mol) and concluded that surface charge−charge interactions are not essential for protein folding and stability.

Experimental Design. We wished to look at the effects of *large* perturbations to the long-range interactions of surface charges on the ability of a protein to fold into an active form by *increasing* the charge on the surface of a model protein, BCA. In general, we might expect that increasing the net charge on a protein should contribute unfavorably to its stability, due to increased electrostatic repulsion, and favorably to its solubility.[23]

The increase in net charge due to chemical modification is analogous to the increase in net charge upon changing the pH to a value above the pK_a of the Lys-ϵ-NH_3^+ groups. By either changing the pH or adding charge to the surface of a protein using chemical modifications, we expect a decrease in stability due to the electrostatic repulsion between surface-exposed ionized groups; this repulsion is minimized in the unfolded state. We hypothesized that by substantially increasing the net charge on the protein, without changing the pH, we might be able to explore the influence of electrostatic repulsion on stability.

We chose to focus on comparing the refolding of BCA and BCA-Ac_{18} after denaturation in SDS for two reasons. First, SDS−PAGE is one of the most widely used techniques in protein chemistry.[24,25] The operation of SDS in this technique has been extensively studied, but never fully understood. We thus, wished to study SDS for its relevance to proteomics. Second, SDS is a denaturant that is negatively charged, and we thought that the chances of seeing an interaction between SDS and proteins with different net charges were higher than with urea.

We chose BCA as the model protein for these studies because it is a particularly well-characterized globular protein and is often used as a model protein for physical−organic and biophysical studies of proteins and protein−ligand interactions.[26−29] BCA is stable, has a single polypeptide chain, and is readily available commercially. It has no cysteines and, therefore, no disulfide bonds to complicate denaturation experiments. Ten β-strands dominate the structure of BCA; they span the width of the folded protein.[30] The central β-strands are composed primarily of hydrophobic residues and, even at 6.2 M guanidine (GuHCl), are not fully accessible to solvent.[31,32] BCA has a stable molten-globule[31,33] intermediate in its folding pathway; the molten globule has a compact structure, significant exposure of hydrophobic residues to solvent, and fluctuating tertiary structure.[34] The rate-limiting steps in the folding of BCA are thought to be the isomerizations of two proline residues:[35,36] BCA has 18 proline residues, two of which are in the less stable cis-conformation in the native structure.[30]

We eliminated 66% (18 out of 27) of the positively charged residues on BCA by acetylating the 18 Lys-ϵ-NH_3^+ side chains on its surface;[37] the nine arginine groups are unchanged. Each modification neutralized the charge of a lysine residue, while changing its size only minimally. We chose chemical modification instead of site-directed mutagenesis in order to avoid the potential of other changes in the structure of the protein, as well as the technical difficulties of multiple rounds of mutation, and of poor expression of mutants or of formation of inclusion bodies.

Figure 1 shows the electrostatic potential at the surface of BCA and BCA-Ac_{18} (see Supporting Information for details of the calculation). The chemical modifications do not alter the potential near the active site because there are no lysine groups in or near the active site. The backside of the enzyme shows a much larger change in potential than the front of the enzyme and demonstrates that BCA is a good model system for studying the effects of *long-range* electrostatic interactions in proteins without large changes in the activity. The large amount of negative (red) surface in BCA-Ac_{18} is a reflection of the large net negative charge on this protein.

Of course, the removal of the charge on the lysine side chains not only perturbs the network of electrostatic interactions in the protein but also increases the hydrophobicity of these residues. (The Hansch π parameter,[38,39] that is, the hydrophobicity constant, for NH_3^+ is −2.12,[40] and for $NHCOCH_3$ is

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(37) The N-terminus of BCA is acetylated post-translationally and is, therefore, not modified by acetic anhydride.
(38) The hydrophobicity constant is a measure of the change in energy to transfer a compound from octanol to water with the change of functionality (e.g. from NH_3^+ to $NHCOCH_3$) to a parent molecule. A large number indicates that the additional functionality requires more energy to transfer the molecule to water.
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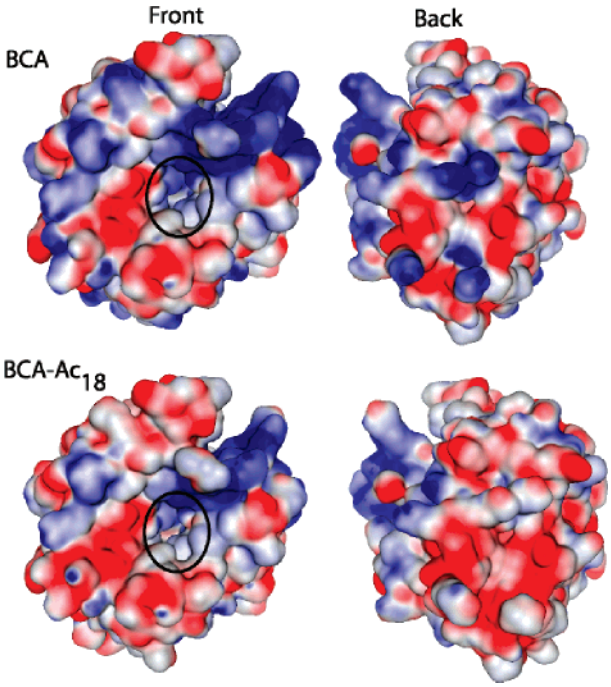

Figure 1. Calculations of electrostatic potential on the surface of BCA and BCA-Ac_{18}. Negative potentials at or below $-8\ k_bT$/e are colored red; positive potentials at or above $+8\ k_bT$/e are colored blue ($8\ k_bT$/e $\approx$ 220 mV). Regions that are colored white have a potential of $0k_bT$/e. The active site is circled in both proteins. The potential in the region around the active site is similar in both BCA and BCA-Ac_{18}; the back of the enzymes shows significant differences, with BCA-Ac_{18} having a large, negative potential.

-1.21.[5,41] Both groups are thus hydrophilic, but the $NHCOCH_3$ is less so.) We anticipated that this increase in hydrophobicity might allow the lysine-ϵ-$NHCOCH_3$ residues to partition into the hydrophobic core of the protein, and thus, perhaps, to hinder the folding of modified protein.

Results and Discussion

Preparation of BCA-Ac_{18}. We peracetylated the ϵ-amino lysine groups of BCA to >90% conversion to the peracetylated form (BCA-Ac_{18}).[37,42] The remaining 10% of the protein in the sample was BCA-Ac_{17}, with 17 out of the 18 lysine groups acetylated, as determined by peak areas in CE separation. Additional equivalents of acetic anhydride did not significantly increase the amount of perfunctionalized species. We believe that this conversion is sufficient to follow the behavior of the majority species, BCA-Ac_{18}, clearly.

BCA has 18 lysine groups, but the change in net charge from BCA to BCA-Ac_{18} is smaller than 18 units due to charge regulation—the adjustment of protonation states of other ionizable residues in a manner that reduces the total change in charge.[43,44] Using the Linderstrom−Lang model of cooperativity in proton binding,[45] we calculated the charge on BCA-Ac_{18} to be -19 at pH 8.4 and an ionic strength of 10 mM; the charge on native BCA is -2.9 at this pH.[46] The net change in charge is thus approximately -16.

Briefly, the Linderstrom−Lang model assumes that a protein is a sphere with uniform electrostatic potential on its surface. This potential determines the local concentration of hydrogen ions near the surface via a Boltzmann distribution. The ionization state of every titratable group can be estimated using the local proton concentration, rather than the bulk value, and the ionization constant, $pK_{a,i}$, of that group.[47] The net charge on the protein—the summation of all negative and positive charges—was in turn used to recalculate the surface potential via the Debye−Huckel equation. This process was repeated until the values become constant; the net charge was thus calculated iteratively.

Denaturation and Renaturation of BCA and BCA-Ac_{18}. We denatured BCA and BCA-Ac_{18} in a buffered (25 mM Tris−192 mM Gly, pH 8.3) solution of 10 mM SDS. Using microcalorimetry,[48,49] we measured the critical micelle concentration (cmc) to be 4.3 mM in this medium (Supporting Information). The solutions of denatured proteins were diluted 5-fold to reduce the SDS concentration below the cmc. The diluted solutions were then dialyzed against Tris-Gly buffer containing 10-μM $ZnSO_4$ for 48 h to remove SDS; during this dialysis, the proteins refolded.[50]

We used capillary electrophoresis (CE) to monitor the denaturation of BCA and BCA-Ac_{18}. As the protein associated with negatively charged SDS molecules, its electrophoretic mobility increased (Figure 2). Upon renaturation, the mobility returned to its original value.

Measurement of Recovered Protein Activity and Structure. The amount of recovered protein after renaturation was measured by comparing the absorbance at 280 nm before denaturation and after renaturation. Recovery of total protein was quantitative (>99.5%, as measured by absorbance at 214 nm) for both BCA and BCA-Ac_{18}.

We assayed the amount of protein that was recovered and that had the original electrophoretic mobility—and hence charge and size—by measuring the area of the peak with the mobility of the starting material in the electropherograms. The recovery of protein with the original mobility was experimentally indistinguishable for BCA ($83 \pm 5\%$) and BCA-Ac_{18} ($78 \pm 5\%$).[51] We did not observe the other 20% of the protein by CE. We attribute this loss of protein to aggregation, or perhaps, to refolding into a range of conformations with different mobilities; aggregates and/or misfolded protein might appear in CE as broad peaks indistinguishable from noise or baseline.

To determine if the proteins had refolded to the same state as before denaturation, we measured both the activities (binding of inhibitors and esterase activity) of the refolded proteins and

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(46) This calculation was done using the linearized Poisson−Boltzmann equation and is valid for potentials below 25 mV. The potential around the BCA-Ac_{18} exceeds 25 mV and, therefore, this calculation is only an approximation.
(47) The pK_a values for the titratable groups used were 12.5 for arginine, 10.3 for tyrosine and lysine, 7.0 for the hydroxide bound to the Zn(II) cofactor, 6.2 for histidine, 4.5 for glutamic acid, 3.2 for the C-terminus, and 2.5 for aspartic acid.
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(50) The addition of the Zn(II) ion, the cofactor of BCA, to the refolding buffer increases the yield of refolded protein and causes all of the refolded protein to be in the active, Zn(II)-containing form.
(51) The error reported in the amount of recovered protein is the difference in the largest and smallest values measured from four replicate measurements.

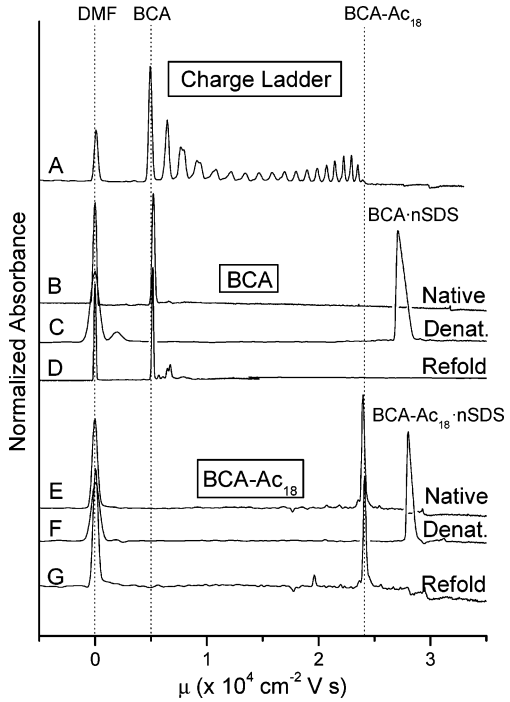

Figure 2. Electropherograms showing BCA (B) and BCA-Ac_{18} (E) before denaturation, after denaturation in 10 mM SDS (C, F), and after refolding (D, G). An electropherogram of the charge ladder of BCA (Colton et al. *J. Am. Chem. Soc.* **1997**, *119*, 12701−12709) (A) provides a reference for the change of mobility of BCA as a function of the charge of BCA. The mobility of the renatured peak matches that of the protein before denaturation for both BCA and BCA-Ac_{18}. For both BCA and BCA-Ac_{18}, the aggregate of protein with SDS has higher mobility than the protein in the absence of SDS. Dimethyl formamide (DMF) served as an electrically neutral marker to measure the rate of electroosmotic flow. An additional peak in D at $\mu = 0.6$ cm^2 V^{-1} s^{-1} corresponds to an active form of BCA with approximately one additional negative charge (Gudiksen et al. *Anal. Chem.* **2004**, *76*, 7151−7161); we hypothesize that this peak corresponds to BCA in which an asparagine, probably either Asp23 or -61, has been deamidated to an aspartic acid.

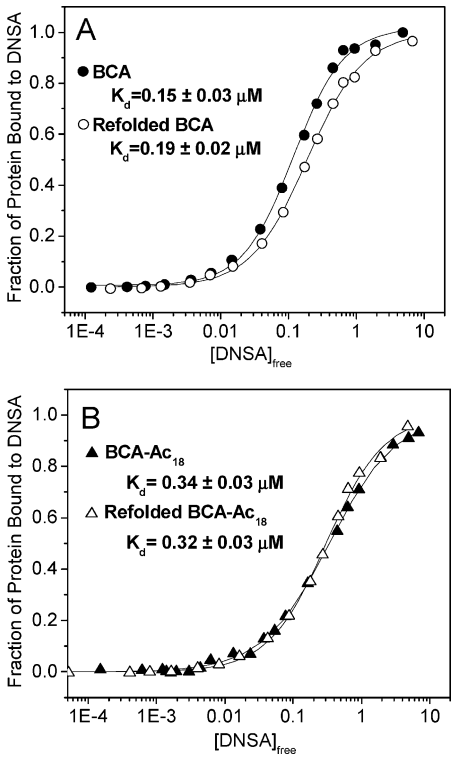

Figure 3. Characterization of BCA and BCA-Ac_{18} before denaturation and after renaturation. Binding of DNSA to (A) BCA and (B) BCA-Ac_{18}, measured by fluorescence spectroscopy.

their tertiary structure (by circular dichroism spectroscopy, CD). We measured the binding activity using a dansyl amide (DNSA) binding assay.[52] DNSA, when free in solution, has negligible fluorescence (quantum yield 0.055, emission wavelength 580 nm). When bound to BCA, the quantum yield is enhanced by a factor of 15 (quantum yield 0.84, emission wavelength 468 nm).[52] To monitor binding, we measured the intensity of fluorescence as a function of the concentration of DNSA. The modification of the lysine groups on BCA does not significantly change the activity of the protein (Figure 3): the dissociation constant (K_d) for the BCA·DNSA complex was 0.15 ± 0.03 μM (Figure 3A); K_d for the BCA-Ac_{18}·DNSA complex was 0.34 ± 0.03 μM (Figure 3B).[53] The dissociation constants for the refolded proteins (and thus, we infer, their active site structures) for both BCA (K_d = 0.19 ± 0.02 μM) and BCA-Ac_{18} (K_d = 0.32 ± 0.03 μM) are restored upon renaturation.

In addition to the binding of inhibitors, we assayed the undenatured and refolded proteins for their enzymatic activity. BCA catalyses the hydrolysis of *p*-nitrophenylacetate to *p*-nitrophenoxide and acetate.[54,55] The apparent second-order rate constant for this reaction, k_{cat}/K_m, was determined by measuring the rate of reaction as a function of the substrate concentration, using a protein concentration of 1.5 μM estimated by the intensity of UV absorbance at 280 nm (ϵ_{280} = 57 000 M^{-1} cm^{-1}).[52] BCA has a k_{cat}/K_m of 500 ± 50 M^{-1} s^{-1}; BCA-Ac_{18} has a k_{cat}/K_m of 430 ± 40 M^{-1} s^{-1}. We also determined the observed values of k_{cat}/K_m for the refolded proteins: 400 ± 100 M^{-1} s^{-1} for BCA, and 390 ± 50 M^{-1} s^{-1} for BCA-Ac_{18}[56] on the basis of the total protein concentration in solution. The apparent values of k_{cat}/K_m for the refolded material were 81% of that of the starting material for BCA and 91% of that of the starting material for BCA-Ac_{18}; these values of rates are consistent with our estimate by CE that ∼80% of the refolded protein was in the active form.

Further evidence that the refolded proteins had regained their original fold comes from CD. The CD spectrum is a sensitive measure of both the secondary and tertiary structure of a protein. CD indicated that refolded BCA and refolded BCA-Ac_{18} had structures very similar to the proteins from which they were derived (Figure 4).

Because both polypeptides refolded to structures having activities and inhibitor-binding properties indistinguishable from those before denaturation, we conclude that, for this protein, the charges distributed on the surface do not influence the ability to fold into the original state.

Rates of Refolding. We also investigated the *kinetics* of refolding of BCA and BCA-Ac_{18} from SDS solution by monitoring the quenching of tryptophan residues by acrylamide following rapid (<30 s) dilution. A solution containing 30 μM denatured protein and 10 mM SDS was diluted 100-fold into a

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(56) The error reported in the activity assay is the difference in the largest and smallest values measured from three replicate measurements.

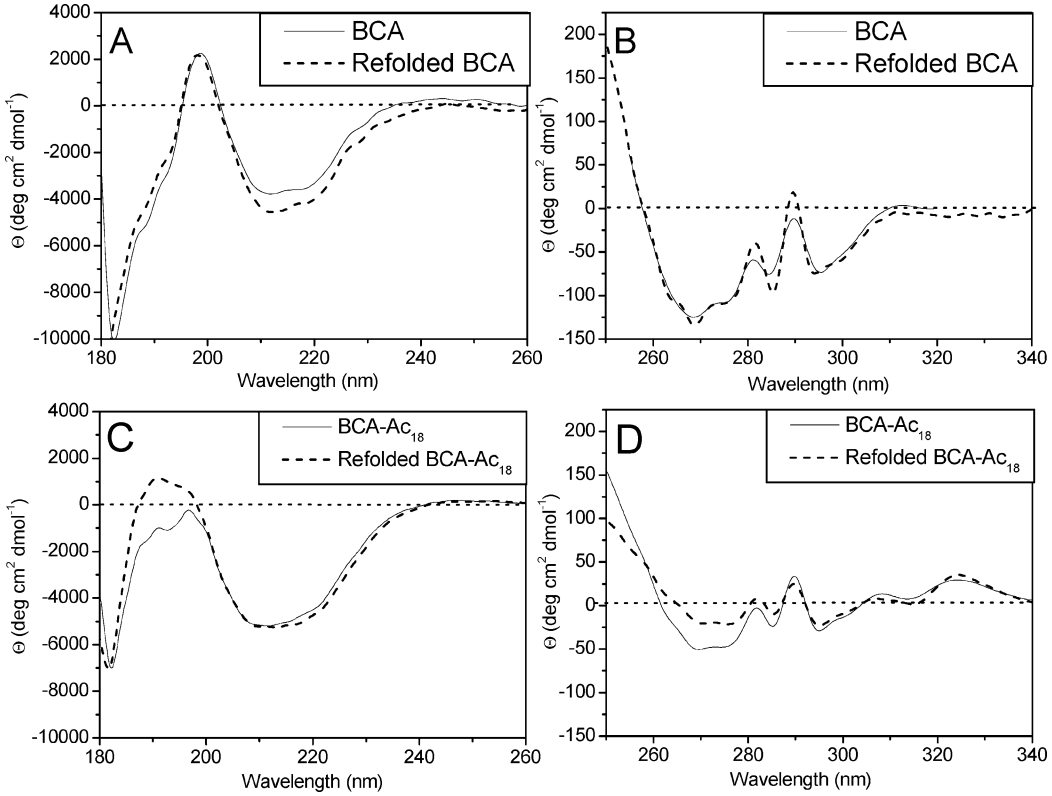

Figure 4. CD spectra of BCA in the (A) far and (B) near UV regions and BCA-Ac_{18} in the (C) far and (D) near UV regions.

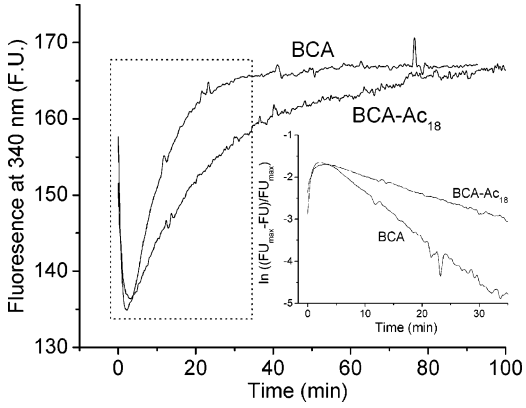

Figure 5. Kinetics of refolding of BCA and BCA-Ac_{18} upon rapid (<30 s) dilution in the presence 0.2 M acrylamide. Tryptophan fluorescence was excited at 280 nm and the emission was measured at 340 nm. The inset shows the data plotted on a logarithmic scale to demonstrate the two time-scales, an initial decrease followed by an increase in fluorescence, in the renaturation. Only the data in the dotted box (before 35 min) was used for the inset.

solution containing Tris-Gly buffer with 10 μM $ZnSO_4$ and 0.2 M acrylamide. 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 or BCA-Ac_{18} folds and the tryptophan residues are buried, the distance between quencher and fluorophore increases and the observed fluorescence increases. We observed an initial decrease in fluorescence and a subsequent increase (Figure 5) during the refolding of both BCA and BCA-Ac_{18}. We speculate that the initial decrease in tryptophan fluorescence and subsequent increase could reflect the transition of moving from a hydrophobic (acrylamide excluded) environment, with the tryptophans in interaction with SDS molecules, to a hydrophilic environment, with the tryptophans exposed to solvent, and, finally, to an internally folded state of the protein with the tryptophans buried (and thus also excluded from quenching by acrylamide). The time-scale of this decrease did not change when we changed the acrylamide concentration from 0.2 to 0.04 M (data not shown). The two observed time-scales in the refolding are $t_{1/2} = 1.2 \pm 0.4$ min, for the decrease in fluorescence, and $t_{1/2} = 11 \pm 1$ min, for the subsequent increase in fluorescence for BCA, and $t_{1/2} = 1.0 \pm 0.3$ and 21 ± 2 min, for BCA-Ac_{18}.[58] The shorter time-scale was indistinguishable for BCA and BCA-Ac_{18}; the longer of the two time-scales was similar for BCA and BCA-AC_{18}, but may differ by as much as a factor of 2. Because of the mixing time for the dilution of the protein, we were unable to measure any processes occurring on a time-scale of less than ~20 s.

Many groups have observed three time-scales in the refolding of BCA after denaturation with GuHCl.[33,35,59−61] Semisotnov et al. showed that the fastest of the three stages ($t_{1/2} \cong 0.04$ s) is a compaction of the protein chain and the formation of a molten-globule intermediate.[61] Jonasson and co-workers found that the hydrophobic core of BCA forms on a time-scale of a few milliseconds.[62] In this fast collapse, the hydrophobic clusters

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Table 1. Charges and Charged Residues on Soluble CA Isoforms

label	isoform	organism	charge[a]	pI[b]	molecular mass (Da)	positive residues[c]	negative residues[d]	total residues	enzyme accession number
A	CA 1	flaveria	−2.3	5.70	35 906	34.5	37.0	330	P46512
B	CA 2	bovine	−1.5	6.58	28 980	28.1	29.0	261	P00921
C	CA 1	mouse	−1.4	6.47	28 482	22.2	24.0	260	P13634
D	HCA XIII	human	−1.4	6.45	29 734	28.2	30.0	262	Q8N1Q1
E	CA 2	mouse	−1.2	6.52	29 228	27.4	29.0	259	P00920
F	CA 2	chicken	−1.1	6.55	29 156	26.5	28.0	259	P07630
G	HCA VI	human	−0.8	6.65	35 601	27.8	29.0	308	P23280
H	CA 1	horse	−0.8	6.59	28 613	27.0	28.0	255	P00917
I	HCA I	human	−0.7	6.63	29 019	26.1	27.0	260	P00915
J	CA 1	gorilla	−0.7	6.63	29 030	26.1	27.0	260	Q7M316
K	CA 2	flaveria	−0.1	6.21	20 801	19.5	19.0	190	P46513
L	CA 1	macaque	0.0	6.88	29 087	27.0	27.0	260	P35217
M	HCA II	human	0.2	6.86	29 394	32.2	32.0	259	P00918
N	HCA III	human	0.2	6.94	29 767	31.2	31.0	259	P07451
O	CA V	mouse	0.2	6.86	36 977	38.2	38.0	317	Q9QZA0
P	CA 3	mouse	0.3	6.97	29 574	31.3	31.0	259	P16015
Q	HCA VII	human	0.4	6.92	29 961	27.4	27.0	264	P43166
R	CA 1	rhesus	0.9	7.34	29 085	27.0	26.0	260	P00916
S	HCA V	human	1.0	7.18	35 059	28.1	27.0	305	P35218
T	CA 1	opossum	1.2	7.27	29 442	29.4	28.0	262	Q8HY33
U	CA 3	horse	1.8	7.84	29 707	33.1	31.0	259	P07450

[a] The charge on the protein was calculated at the pH of the organism (pH 6.5 for flaveria, pH 7.4 for all others) using a Linderstrom−Lang model. The details of the calculation are provided in the text. [b] The isoelectric point (pI) was calculated using the EXPASY isoelectric point calculator: http://us.expasy.org/tools/pi_tool.html. [c] The number of positively charged residues is the number of lysine + arginine + X_i × histidine residues, where X_i is the fractional charge of the histidine residues at the pH of the calculation ($X_i = 0.5$ at pH 6.5 and 0.1 at pH 7.4). [d] The number of negatively charged residues is the number of aspartic acid + glutamic acid residues.

desolvate and a nativelike hydrophobic core forms. The other two stages ($t_{1/2} \cong 2$ and 10 min) have been attributed to isomerizations of two proline groups.[35, 36]

Because we cannot observe processes faster than ∼20 s, it is possible that BCA and BCA-Ac_{18} have different rates of collapse into a molten-globule intermediate. The two time-scales that we observe match those reported in the literature when BCA is refolded after denaturation with GuHCl.[33,35,36,59−61] After renaturation from GuHCl, the formation of the molten globule is orders of magnitude faster—hundreds of milliseconds—than the time-scale we measure. We, therefore, think it is unlikely that the rate-limiting step measured here is the exclusion of water from the hydrophobic core of the molecule. We thus attribute the two time-scales we measured to isomerizations of two proline groups. The modification of the lysine residues of BCA appears to have only a minimal effect on the rate of proline isomerizations. Since we cannot observe SDS directly, it is possible that the dissociation of SDS from a protein·SDS complex also occurs on this time-scale.

Analysis and Conclusions

This work demonstrates that a change in charge of ~ -16 units on the surface of BCA neither prevents refolding into a catalytically active state nor significantly changes the kinetics of refolding after denaturation with SDS. Changing the charge also does not significantly alter the activity of the protein, as measured by the esterase activity and the binding of inhibitor. Acetylated lysines—which have higher hydrophobicity than the charged, unmodified lysines—are not incorporated into the core upon folding. This result may imply that the hydrophobic collapse is due to a few specific residues in the primary sequence and lysines, acetylated or not, do not participate. We, however, do not wish to draw inferences about hydrophobic collapse from this result, or to generalize it to other proteins. CA is only one protein, and it may be a special case, since it is particularly stable. We emphasize that we do not directly observe the hydrophobic collapse in our measurements of the kinetics of refolding because it is faster than the time resolution of our instrument. The lysine residues, therefore, might, in principle, affect the kinetics of the hydrophobic collapse; we simply are unable to examine this process. Nonetheless, the acetylation of lysine residues does not prevent the folding of BCA, and we conclude that exclusion of charges from the interior of the protein plays little or no role in the ability of this particular protein to fold.

If charge outside of the active site does not influence the secondary and tertiary structure of BCA (and by extension, of at least some other proteins) and does not determine the rate or product of folding, what *is* the role of charged residues on its surface (and on the surface of other proteins)? We can, at this point, only speculate. Charges on the surface of a protein may be important for preventing aggregation[63] or for increasing solubility.[64] Charges can affect substrate binding and reactivity,[65,66] protein−protein interactions,[67] and secretion[68] or localization of specific proteins. In addition, the osmotic pressure of a cell is affected by the sum of the charges of all of its proteins.

To address the question of whether the net charge of the carbonic anhydrase (CA) isoforms is conserved, we analyzed 20 isoforms of CA that are catalytically active and not membrane bound (Table 1). We calculated the charges on these proteins using the charge regulation calculation described in the

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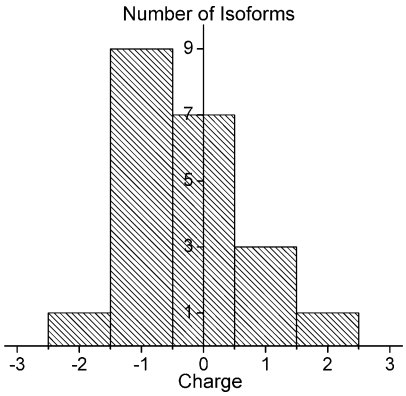

Figure 6. Histogram of calculated charges on CA isoforms.

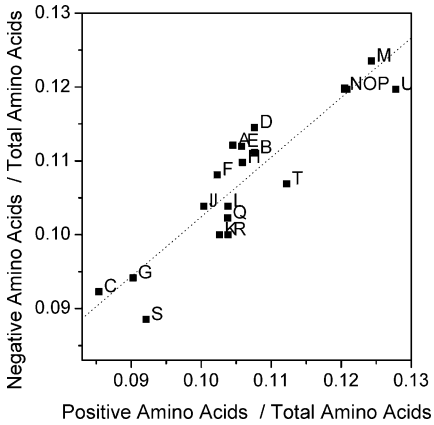

Figure 7. A plot of the number of negative amino acids divided by the total number of amino acids versus the number of positive amino acids divided by the total number of amino acids for the 20 CA isoforms listed in Table 1, identified by the label to the right of each data point. The line of best fit has a slope of 0.81 and a correlation coefficient of 0.92.

Results section that we used to calculate the charge on BCA and BCA-Ac_{18}. We performed this calculation at the pH of the organism or, for mammals, the pH of blood. Figure 6 shows a histogram of the charges found on the CA isoforms. The charge on these CA isoforms varied from +1.8 to −2.3, with 90% of them between +1.5 and −1.5. The charge on BCAII in blood (pH 7.4) is nearly a full charge less than that in Tris-Gly buffer (pH 8.4) because the eight titratable histidine residues and the hydroxide bound to the Zn(II) cofactor are protonated to a larger extent at the lower pH.

If there were evolutionary pressure to maintain the charge on CA between +1 and −2, changes in the number of positively charged amino acids in the protein would be balanced by corresponding changes in the number of negatively charged amino acids. In particular, a mutation that increased or decreased the charge on CA would be more likely to be followed by a mutation that restores the charge to the original value than one that left the new charge unaltered. We wanted to see whether an increase or decrease in the number of *negatively* charged amino acids in a CA isoform correlated with an increase or decrease in the number of *positively* charged amino acids. Specifically, we hypothesized that if there were pressure to maintain the net charge on CA, there might exist isoforms with a high percentage of charged amino acids and isoforms with a low percentage of charged amino acids, but both would have a similar net charge.

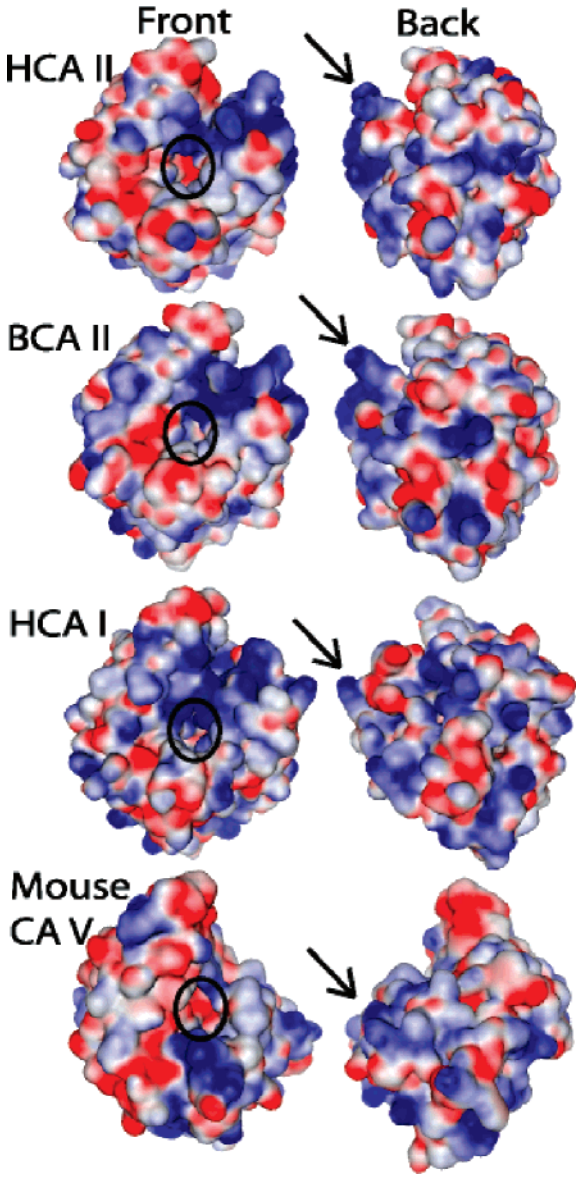

Figure 8. Depictions of calculated electrostatic potentials on the surface of the four CA isoforms in the Protein Data Bank. The colors and scales match those of Figure 1. Protein data bank id numbers are 1ca2 for HCA II, 1v9e for BCA II, 1crm for HCA I, and 1urt for mouse CA V. The active site is circled in each isoform for reference. The backbone has an extremely similar conformation in all four isoforms. The blue, positive patch on the side of the enzymes, shown with an arrow, appears to be conserved across all four isoforms.

To test this hypothesis, we normalized the number of positively and negatively charged amino acids by the total number of amino acids for each protein in Table 1. Figure 7 shows a plot of the normalized number of negative residues versus the normalized number of positive residues. A protein with a high proportion of amino acids that are negatively charged also has a high proportion of amino acids that are positively charged.[69] The slope of the data is less than unity, so each positive charge is balanced by only 0.8 negative charges.

This correlation implies that there is some evolutionary pressure to maintain the charge on CA. The net charge is

(69) A more detailed analysis than presented here, on a larger data set, would be needed to ascertain whether a mutation that increases or decreases the charge on CA is more likely to be followed by a mutation that restores the charge to the original value than one that leaves the new charge unaltered.

preserved, while the total number of charges varies. Even though the total number of charges (positive + negative) ranges from 36 (for CA 2 from flaveria) to 75 (for CA V from mouse), the net charge only varies by ~4.5 charges, between 1.5 and −2.9. The reason for this small difference in net charge remains unclear. The net charge, thus, may be more important from an evolutionary perspective to carbonic anhydrases than the details of the distribution of electrostatic potential on the surface.

We further examined the location of the charges on the surface of the CA isoforms to determine if the location of the charges was conserved. We analyzed the four isoforms of CA that have known crystal structures: human carbonic anhydrases I and II, bovine carbonic anhydrase II, and mouse carbonic anhydrase V. Because these isoforms have a net charges of −0.2, −1.9, −1.1, and −0.2 at pH 7.4 and a total number of charges of 63, 56, 52, and 75, respectively, they are representative of the isoforms detailed in Table 1.

We used the MOE program[70] to calculate the electrostatic potential on the surface of the proteins (Figure 8). There are regions on the surface of the proteins that appear to be roughly conserved between isoforms (e.g. the blue, positive patch on the side of each isoform marked with an arrow), but the overall distribution of charges appears to be significantly different for each isoform. We do not know if this patch of positive charges on the side of the CA isoforms has a function; we know only that it appears in all four isoforms with known crystal structures. It appears that there is not an overall distribution of charges that has been conserved to optimize CA; instead, only the net charge is conserved between isoforms.

Sear calculated the charge on all of the proteins in the *Escherichia coli* genome[71] and found that the distribution of charges on proteins was roughly symmetric and centered near zero, with a standard deviation of 8.[72] The soluble isoforms of CA fall in the center of this distribution. Perhaps if the charge on proteins is near zero, the cell can more easily regulate its osmotic pressure than if it were required to balance a large number of highly charged proteins with a large number of small ions. A net charge on a protein near zero, however, may induce aggregation, so a balance between these two factors may cause many proteins in a cell to have a small charge.

We find that the net charge on CA isoforms is more conserved than either the number of charges or the distribution of the charges on the surface. Because CA is not known to interact with other proteins, and because its substrates are relatively simple (CO_2, H_2O, and HCO_3^-), it seems unlikely that there is a need to orient reactants or products using electrostatics. It is, however, unclear if the conservation of charge is specific to CA or if the net charge on all proteins is maintained near zero. We have demonstrated that, for BCA II, the charges are not important for folding or activity in vitro.

BCA is not a perfectly representative protein (probably no protein is perfectly representative): it is exceptionally stable, has a high percentage of amino acids in β-sheets, and has a catalytically active His_3−Zn(II)-OH site. Still, the results in this paper demonstrate that there *is* at least one, not atypical, globular protein—BCA—for which charge is not important in folding. This observation is almost certainly not true for all proteins, and further experiments will be necessary to determine the generality of these results.

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Supporting Information Available: Experimental protocols for the acetylation of BCA, the calculations of the surface charges, the DNSA binding and enzymatic assays, the CE and CD procedures, and the microcalorimetry data for the cmc determination of SDS in TG buffer. This material is free of charge via the Internet at http://pubs.acs.org.

JA043804D

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