Redox Sensitive Cysteine Residues in Calbindin D_{28k} Are Structurally and Functionally Important†

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ABSTRACT: Human calbindin D_{28k} is a Ca^{2+} binding protein that has been implicated in the protection of cells against apoptosis. In this study, the structural and functional significance of the five cysteine residues present in this protein have been investigated through a series of cysteine-to-serine mutations. The mutants were studied under relevant physiological redox potentials in which conformational changes were monitored using ANS binding. Urea-induced denaturations, as monitored by intrinsic tryptophan fluorescence, were also carried out to compare their relative stability. It was shown that the two N-terminal cysteine residues undergo a redox-driven structural change consistent with disulfide bond formation. The other cysteine residues are not by themselves sufficient at inducing structural change, but they accentuate the disulfide-dependent conformational change in a redox-dependent manner. Mass spectrometry data show that the three C-terminal cysteine residues can be modified by glutathione. Furthermore, under oxidizing conditions, the data display additional species consistent with the conversion of cysteine thiols to sulfenic acids and disulfides to disulfide-*S*-monoxides. The biological function of calbindin D_{28k} appears to be tied to the redox state of the cysteine residues. The two N-terminal cysteine residues are required for activation of *myo*-inositol monophosphatase, and enzyme activation is enhanced under conditions in which these residues are oxidized. Last, oxidized calbindin D_{28k} binds Ca^{2+} with lower affinity than does the reduced protein.

Calbindin D_{28k}, a cytosolic Ca^{2+} binding protein, has been implicated to play a role during apoptosis in association with Alzheimer's disease and amyotrophic lateral sclerosis (*1*, *2*). Apoptosis is accompanied by a disruption in Ca^{2+} homeostasis (*6*), providing toxic levels of this second messenger. When calbindin D_{28k} is overexpressed in cells that are induced to undergo apoptosis, increased cell survival has been observed (*1*−*5*). Calbindin D_{28k} has been suggested to protect the cell by acting as a Ca^{2+} buffer (*6*); however, recent studies have brought the attention to the fact that the Ca^{2+} binding affinity displayed by calbindin D_{28k} is lower than for a typical Ca^{2+}-buffering protein (*7*). Calbindin D_{28k} has also been shown to undergo both Ca^{2+}- and pH-dependent structural changes (*8*) and to bind specific target proteins in a Ca^{2+}-dependent manner. One of the targets, caspase 3, is a central protein in one of the pathways leading to apoptosis (*4*) opening up the possibility that calbindin D_{28k} indeed protects against apoptosis in other ways than by acting as a Ca^{2+} buffer. Calbindin D_{28k} has recently also been shown to activate *myo*-inositol monophosphatase (*9*), an enzyme responsible for maintaining cellular inositol levels. Inositol has been reported to protect cells from apoptosis (*34*).

In addition to a large influx of Ca^{2+} into the cytosol, cells undergoing apoptosis also experience an increased oxidizing environment. The redox potential of the cytosol, which varies between −160 and −260 mV, changes during the life cycle of the cell (*10*). In proliferating cells, the redox potential is approximately −240 mV (*11*−*14*), whereas a more oxidizing environment (ca. −200 mV) is found in the cytosol of differentiating cells (*13*). In cells triggered for apoptosis, the redox potential can increase up to −170 mV (*12*, *13*). The redox state in a cell is determined by the presence of several redox pairs, including glutathione (GSSG/2GSH),[1] nicotinamide adeneine dinucleotide phosphate (NADP/NADPH), and thioredoxin ($TrxSS/Trx(SH)_2$). The intracellular glutathione concentration ranges from 1 to 11 mM, which is much higher than for other redox systems. Therefore, the glutathione system is regarded as the principal system determining the redox potential in the cell. The redox environment is overall reducing as the reduced form of glutathione predominates (for a review of cellular redox systems, see ref 15).

Redox regulation of cellular systems within the cytosol still remains a controversial issue despite many elegantly conducted studies. The best understood and most thoroughly studied redox regulating systems involve signal transduction in the mammalian central nervous system (*17*) and include

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[1] Abbreviations: wt, wild type; ANS, 8-anilinonaphthalene-1-sulfonic acid ammonium salt; DTT, 4-dithiothreitol; GSH and GSSG, reduced and oxidized glutathione; NADPH and NADP, reduced and oxidized nicotinamide adenine dinucleotide phosphate; $Trx(SH)_2$ and TrxSS, reduced and oxidized thioredoxin; *E. coli*, *Escherichia coli*; IMPase, *myo*-inositol-monophospatase.

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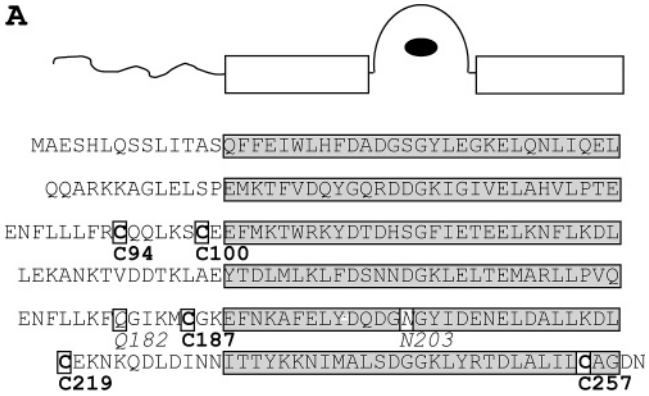

B

	94	100	182	187	203	219	257
QN-CCCCC(wt)	C	C	Q	C	N	C	C
ED-CCCCC	C	C	E	C	D	C	C
ED-SSSSS	S	S	E	S	D	S	S
ED-CCCCS	C	C	E	C	D	C	S
ED-SSCCS	S	S	E	C	D	C	S
ED-CCSSS	C	C	E	S	D	S	S
QD-CCSSS	C	C	Q	S	D	S	S
QD-CSSSC	C	S	Q	S	D	S	C
QD-SCSSC	S	C	Q	S	D	S	C

FIGURE 1: Cysteine residues in the amino acid sequence of human calbindin D_{28k}. (A) Secondary structure of the EF hand helix−loop−helix motif and Ca^{2+} binding site. The cysteine residues (bold) are located outside the helix−loop−helix motif (grey, boxed) except for C257, which is located at the end of the last helix. Possible deamidation sites are shown in italic. (B) Calbindin D_{28k} mutants used in this study. The nomenclature is based on the amino acid replacements made. For example, in ED-CCSSS the two deamidation sites glutamine and asparagine are replaced with glutamic acid and aspartic acid, and the three C-terminal cysteine residues are replaced with serine.

the transcription factors AP-1 and NF-κB (*16*), as well as specific cellular receptors like the ryanodine receptor (*18*). The redox regulatory effect is exerted via cysteine residues in which the thiol functional group of the side chain can be oxidized to generate a disulfide bond or a sulfenic acid (*19*). Cysteine residues can also undergo covalent modifications, including S-nitrosylation or S-glutathiolation by nitric oxide and glutathione, respectively (*20*). The reducing environment of the cytosol and the low cellular pH of 7.1 are unfavorable conditions for the oxidation of cysteine thiols (pK_a ca. 8.8); however, the reactivity of the thiol group is strongly influenced by the surrounding environment in which a lowering of the pK_a would favor oxidation. The pK_a of a cysteine residue can be perturbed by the presence of nearby charged residues as well as by its local conformation, as has been shown for a series of short peptides in which the pK_a varies from 7.4 to 9.1 (*21*). In naturally occurring proteins, pK_a values as low as 3.5 have been determined (*22*). Induction of a protein conformational change, which may take place during ligand binding, may also dramatically affect the pK_a of a cysteine residue by aligning it optimally for disulfide bond formation and providing a high effective concentration of the reacting groups.

Human calbindin D_{28k} contains five cysteine residues. The four N-terminal residues are conserved in calbindin D_{28k} from man, cow, mouse, and rat (*23*−*26*). The cysteine residues are all located in the linker regions connecting the proposed EF hand subdomains, except for the nonconserved fifth cysteine residue, which is located at the end of the C-terminal α-helix of EF hand 6 (Figure 1). In a recent mass spectrometry study, it was reported that the two N-terminal cysteine residues, C94 and C100, in rat calbindin D_{28k} engage in a disulfide bond (*27*), but no experimental details were provided. Using mass spectrometry, we have since shown that, under conditions excluding a reducing agent, a disulfide bridge can be formed between the corresponding residues in human calbindin D_{28k}.[2] The result is uncharacteristic of a cytosolic protein in which cysteine residues are typically found in the reduced form. Tao et al. (*32*) have also shown that all five cysteine residues react with cysteine-specific reagents. In this study, we have therefore undertaken a detailed investigation under physiologically relevant redox conditions to address the structural and functional roles of the five cysteine residues in human calbindin D_{28k}.

EXPERIMENTAL PROCEDURES

Generation and Purification of Calbindin D_{28k} Cysteine Mutants. Figure 1 shows the calbindin D_{28k} cysteine mutant proteins generated in this project. Calbindin D_{28k} cysteine mutants were made by site-directed mutagenesis using the human calbindin D_{28k} gene cloned into the PetSac vector (a modified Pet3a plasmid with NdeI and SacI restriction sites). Residue Q182 was replaced with E and N203 with D to avoid deamidation (see below).[2] The gene containing the C257S mutation was made by PCR using the "start" primer and the "C257Sstop" primer, as shown in Table 1. To generate the other cysteine to serine replacements, overlapping PCR fragments, containing the appropriate cysteine to serine mutations, were made using the primers indicated in Table 1. To generate full-length calbindin D_{28k}, the overlapping fragments were then joined by PCR using the "start" primer and the "C257Sstop" primer. The resulting PCR fragments were cloned into the PetSac plasmid. DNA sequencing was performed for plasmid preparations originating from single colonies after transformation in the *Escherichia coli* (*E. coli*) strain ER2566. All PCR, DNA purification, and cloning steps were done using standard methods. The calbindin D_{28k} cysteine mutants were all expressed in *E. coli* strain BL21 De3 PLysS star, and purified as described for recombinant wild-type calbindin D_{28k} (*28*). Unless otherwise stated, the reported protein concentrations are calculated from the weight of lyophilized protein.

Terminology. Unreduced protein refers to conditions in which the redox potential has not been controlled, i.e., conditions under which no DTT or glutathione has been added.

Urea Denaturation of Calbindin D_{28k} Cysteine Mutants, As Monitored by CD and Fluorescence Spectroscopy. Calbindin D_{28k} mutants were dissolved at a concentration of 1.8 μM in buffer A, containing 0 or 10 M urea. The DTT concentration was 0 or 0.2 mM. In the glutathione experiments, the GSSG concentration was 5 mM. Urea solutions containing intermediate concentrations of urea, between 0 and 10 M, were mixed from the 0 and 10 M stock solutions. Each pipetted volume was weighed after addition to the Eppendorf tube and the final urea concentrations corrected. The samples were incubated overnight at room temperature. After incubation of the samples, the CD signals at 222 and

[2] Vanbelle, C., Halgand, F., Cedervall, T., Thulin, E., Åkerfeldt, K., Laprévote, O., and Linse, S., manuscript in preparation.

Table 1: Oligonucleotides Used To Generate Calbindin D_{28k} Cysteine Mutants[a]

function	forward primer	reverse primer
start	5′-GCTCATCATATGG CAGAATCCCACCTGCAG	
C257Sstop		5′-CCGTGGAGCTCCTACTAGTT ATCCCCAGCAGAGAGAATAAGAGC
C94S	5′-GCTGCTCTTCCG TCCCAGCAGCTGAAGTCC	5′-GGACTTCAGCTGCTG GGATCGGAAGAGCAGC
C100S	5′-GCAGCTGAAGTCC TCTGAGGAATTCATGAAGAC	5′-GTCTTCATGAATTCCTC AGAGGACTTCAGCTGC
C187S	5′GGGAATCAAAATGTCT GGGAAAGAGTTCAATAAGGC	5′-GCCTTATTGAACTCTTTCCC AGACATTTTGATTCCC
C219S	5′-CTGAAGGATCTG TCCGAGAAGAATAAACAGG	5′-CCTGTTTATTCTTCTC GGACAGATCCTTCAG

[a] Nucleotides responsible for the cysteine/serine mutations are underlined.

223 nm were recorded on a JASCO J-720 spectropolarimeter. The fluorescence emission, after excitation at 295 nm, was recorded between 310 and 400 nm on a Perkin-Elmer luminescence spectrometer LS 50 B. The CD and fluorescence emission measurements, carried out in duplicate, were made at 25 °C in a 1-cm quartz cuvette.

ANS Binding by Calbindin D_{28k} Cysteine Mutants. Lyophilized calbindin D_{28k} mutants were dissolved in buffer A (10 mM $KHPO_4$, pH 7.5, 0.15 M KCl, and 0.5 mM EDTA), containing 0 or 1 mM DTT, to a final protein concentration of 13 μM. The samples were incubated overnight (for a minimum of 12 h) at room temperature (20−24 °C). The following day, ANS, in buffer A, was added to one protein sample to a final ANS concentration of 60 μM, immediately prior to recording the fluorescence emission. The intensity of the fluorescence emission was recorded at 25 °C in a 1-cm quartz cuvette between 400 and 600 nm, after excitation at 385 nm, on a Perkin-Elmer luminescence spectrometer LS 50 B. The experiments were carried out in triplicate, except for those on wt calbindin D_{28k}, which were run in duplicate. The fluorescent signals were normalized assuming the signal for ED-SSSSS to be equal between experiments.

To measure ANS binding under specific redox potentials, glutathione solutions containing 10 mM GSH or GSSG were made in buffer A. The pH was adjusted to 7.5 by KOH. To achieve a redox potential between −300 and −100 mV, different volumes of 10 mM GSH and 10 mM GSSG were mixed. Lyophilized calbindin D_{28k} mutants were dissolved in buffer A and then added to the glutathione mixtures to a final protein concentration of 13 μM. The final glutathione concentration was 5 mM. The correct GSH/GSSG ratio needed to make each redox potential was calculated from the Nernst equation (see below). To render the buffers free from oxygen, they were degassed with N_2 (for a minimum of 10 min) prior to the experiments. The protein mixtures were then incubated overnight at room temperature in 1.5-mL Eppendorf tubes in which the air on top of the solution had been purged with N_2 and the tube subsequently sealed with Parafilm. ANS was added to one sample at the time to a final concentration of 60 μM and the emission intensity was recorded, as described above. The experiments were carried out three or four times.

Preparation of Glutathione Buffers with Different Redox Potentials. Buffers of different redox potentials were made by making solutions containing different ratios of GSH and GSSG. The total glutathione concentrations were kept at 5 mM. The redox potential were calculated from the Nernst equation (1):

$$E_{hc} = E° - (59.1/2) \log ([GSH]^2/[GSSG])\ \text{mV, at 25 °C, pH 7.0} \quad (1)$$

in which $E°$ was adjusted for a buffer pH value of 7.5 by applying eq 2:

$$E_{pH7.5} = E_{pH7.0} + [(7.5 - 7.0)(-59.1)] \quad (2)$$

in which $E_{pH7.0} = -240$mV.

CD Spectroscopy of Calbindin D_{28k} Cysteine Mutants. Calbindin D_{28k} mutants were dissolved in buffer B (20 mM $KHPO_4$, pH 7.5, 0.15 M KCl, 0.5 mM EDTA) and 0 or 1 mM DTT to a final protein concentration of 13 μM. The samples were incubated overnight at room temperature. After incubation of the sample, the CD signal was scanned between 260 and 190 nm in a 1-mm quartz cuvette at 25 °C using a 62DS Aviv circular dichroism spectropolarimeter.

Sample Preparation for Mass Spectrometry Analysis. Calbindin D_{28k} cysteine mutants (13 μM) were stored and shipped in buffer A, containing a 5 mM mixture of reduced and oxidized glutathione to make a redox potential of −170 mV. Alternatively, the samples were freeze-dried before shipment. The samples were analyzed by electrospray ionization mass spectrometry at the Keck Facility, Yale University, CT. Immediately prior to analysis, the samples were desalted using ZipTip desalting columns.

Ca^{2+} Binding by Calbindin D_{28k} Cysteine Mutants. Ca^{2+}-free buffer was made as described (*29*). Removal of Ca^{2+} from the protein stock solutions was achieved with EDTA treatment, followed by gel filtration through saturated NaCl to remove the EDTA (*28*). ^{1}H NMR spectroscopy was used to identify EDTA-free fractions, which were pooled and freeze-dried. Protein solutions at a concentration of approximately 50 μM were made in 2 mM Tris-HCl, pH 7.5. Quin-2 chelator solutions of between 50 and 60 μM were made in 2 mM Tris-HCl, pH 7.5. The buffer chelator and Ca^{2+} concentrations were determined as described (*29*). The protein and chelator solutions were mixed in a one-to-one ratio to make the final concentration of both substances between 20 and 30 μM. Chelator-protein solutions were titrated with 3 μL aliquots of a 1.5 mM $CaCl_2$ solution. The titration was monitored as a change in absorbance at 263 nm. The data were analyzed with Caligator software (*30*) using a model with a chelator of known Ca^{2+} affinity and four macroscopic Ca^{2+} binding constants for the protein.

Before the analysis, the protein concentration was determined by acid hydrolysis and amino acid analysis. Two sets of data were obtained and averaged.

Activation of IMPase by Calbindin D_{28k} Cysteine Mutants. The activity of IMPase was measured for samples prepared by mixing calbindin D_{28k}, substrate, and enzyme solutions. Lyophilized wt calbindin D_{28k} or mutants were dissolved at a concentration of 37.5 μM in buffer C (50 mM Hepes-KOH, pH 7.5, 150 mM KCl, 2 mM $MgCl_2$, and 0.2 mM EGTA), or in buffer C with 1 mM DTT. In the experiments with glutathione containing buffers, mixtures of 10 mM GSH and GSSG were made. Lyophilized protein was dissolved in buffer C and mixed with the glutathione solutions to yield a final protein concentration of 37.5 μM. The final glutathione concentration was 5 mM and the calculated redox potentials were −250 or −170 mV. In the experiments with glutathione, all buffers were oxygen depleted by passing N_2 through the solution before use. The samples were placed under a blanket of N_2 and the vials sealed with Parafilm and incubated overnight for a minimum of 12 h at room temperature. The IMPase was purified as described (*9*) and frozen in aliquots. For the activation experiments, a new aliquot of the IMPase stock solution was used each time to ensure that the enzyme activity was the same. After incubation of calbindin D_{28k} samples or buffer blanks, 50 μL of 80 μM *myo*-inositol-1-phosphate was added to 100 μL of protein solution. Last, the reaction was started by adding 100 μL of the IMPase solution. The samples were then incubated for an additional 2−4 h at 37 °C. The reaction was quenched by adding concentrated HCl to a final concentration of 1.2 M and the samples were centrifuged at 13 000 rpm. The supernatant (200 μL) was mixed with a solution of 1.05% ammonium molybdate, 1.25 M HCl, and 0.15% Malachite green (800 μL) and incubated for 30 min, and the absorbance was recorded at 660 nm. The assay quantifies the amount of released phosphate (*31*). Nine to fifteen sets of data were obtained except in the experiments using 1 mM DTT, in which three sets of data were used.

RESULTS

Calbindin D_{28k} Mutant Nomenclature and Design. Eight different mutants were designed to investigate the role of the five cysteine residues present in human calbindin D_{28k} (Figure 1). To exhaustively explore all possible permutations of one, two, three, four, and five cysteine replacements would require 31 different mutants; however, conclusive results concerning the role of different residues could be gained using a smaller set, and the choice of mutants was based on the following considerations. First, a mutant was made in which all cysteine residues were replaced with serine (ED-SSSSS). As discussed in the introduction, a disulfide bond has been observed between residues C94 and C100 in rat (*27*) as well as in human calbindin D_{28k}.[2] Mutants were therefore made that contained one (QD-SCSSC and QD-CSSSC) or both of these residues (QD-CCSSS and ED-CCSSS). Second, of the species explored so far, the fifth, C257, residue is only present in human calbindin D_{28k}. A mutant lacking this residue was thus made, ED-CCCCS. The third cysteine residue, C187, has been termed "hyperreactive" as it has been shown to be more reactive toward thiolation compared to the other residues (*33*). We therefore set out to explore its reactivity in the context of the other cysteine residues. A combination of mutants was evaluated in which this residue is present together with successively fewer cysteine residues, as in ED-CCCCC, ED-CCCCS, and ED-SSCCS. The behavior of these mutants did not warrant the investigation of additional combinations. As we have shown,[2] calbindin D_{28k} undergoes a spontaneous deamidation reaction. At the time, we suspected residues Q182 and N203 because they are each followed by a glycine residue in the amino acid sequence.[2] To avoid working with heterogeneous protein samples, these residues were therefore both mutated to the corresponding carboxylic acid residues, E182 and D203, giving rise to the mutant nomenclature "ED", as in ED-SSSSS and ED-CCCCC. The mutant ED-CCCCC was also made as a control for the wt protein, QN-CCCCC, to study if these extra substitutions had any effects on the behavior of the cysteine residues. Concomitantly with these studies, we learned that N203 is the site of deamidation and therefore Q182 was retained in the case of QD-CCSSS, QD-CSSSC, and QD-SCSSC.

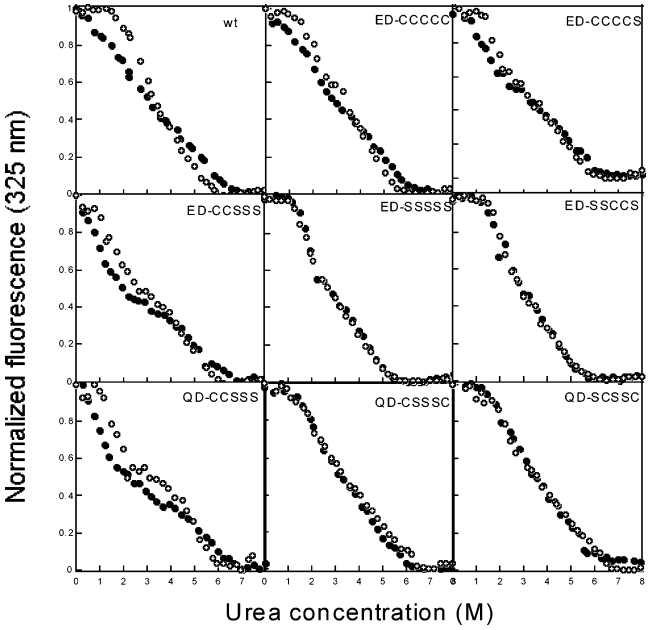

FIGURE 2: Urea-induced unfolding of calbindin D_{28k} mutants. Intrinsic tryptophan fluorescence emission at 325 nm as a function of increasing urea concentration (0−10 M) for 1.8 μM calbindin D_{28k} wt or mutant in 10 mM $KHPO_4$, pH 7.5, 0.15 M KCl, 0.5 mM EDTA at 25 °C. Unreduced (no DTT, closed circles) and reduced (0.2 mM DTT, open circles) samples of calbindin D_{28k} wt or mutants (as indicated in each panel) were incubated overnight before the fluorescence emission was recorded.

Urea-Induced Unfolding of Calbindin D_{28k} As Monitored by Intrinsic Tryptophan Fluorescence. The effect of redox potential on the stability of the calbindin D_{28k} mutants was evaluated by urea-induced denaturation, with and without DTT. The samples containing DTT are referred to as reduced and samples without DTT as unreduced. Denaturation was followed by monitoring the intrinsic (tryptophan) fluorescence emission at 325 nm. The pattern for unfolding clearly differ between the unreduced proteins (Figure 2). Most of the proteins display denaturation curves with more than one transition, indicative of complex behavior with one or more intermediate states. As shown by the absence of a plateau at the lower urea concentrations, unreduced wt calbindin D_{28k} lacks a distinctly folded state under the conditions tested.

However, in 0.2 mM DTT, wt calbindin D_{28k} displays a plateau between 0 and 2 M urea and denatures with a clear transition between the folded and the unfolded state (Figure 2). The denaturation profile also shows that the reduced protein is more stable than the oxidized form. ED-CCCCC, which contains Q182E and N203D mutations, shows a similar unfolding pattern to wt calbindin D_{28k} both with and without DTT. The denaturation of ED-SSSSS, on the other hand, which lacks all cysteine residues, displays a clear transition between the folded and unfolded state both with and without DTT, very much like the reduced wt calbindin D_{28k} (Figure 2). In the absence of DTT, ED-CCCCS unfolds in a manner similar to wt calbindin D_{28k} and ED-CCCCC, whereas ED-SSCCS unfolds similarly to ED-SSSSS. This excludes C187, C219, and C257 as being the residues responsible for the redox-dependent difference in unfolding of wt calbindin D_{28k}. However, for ED-CCSSS and QD-CCSSS, which both behave the same, there is a clear difference in unfolding in the presence and absence of DTT, implying that residues C94 and C100 are involved. The situation is more complex, however. When considering the unreduced forms, the unfolding of ED-CCSSS and QD-CCSSS differ from the unfolding pattern of wt calbindin D_{28k}, ED-CCCCC, and ED-CCCCS. This suggests that although residues C187 and C219 by themselves do not induce a redox-dependent change in unfolding, they modulate the stability of the protein when residues C94 and C100 are present. Finally, QD-CSSSC and QD-SCSSC, which contain one of C94 or C100, do not display any difference in unfolding between a reducing and a more oxidizing environment. Hence, the redox-dependent behavior requires both C94 and C100, again indicating the potential for these residues to engage in a disulfide bond.

Urea-Induced Unfolding of Calbindin D_{28k} As Monitored by CD Spectroscopy. The urea-induced unfolding of wt calbindin D_{28k} and mutants was followed by monitoring the CD signal at 222 and 223 nm (data not shown). By this method, no difference was detected between reduced and unreduced samples. Likewise, no significant differences were seen between reduced and unreduced proteins in the CD spectra scanned between 190 and 260 nm (data not shown). This indicates that the secondary structure is not affected by the redox-driven structural change.

Binding of ANS to Calbindin D_{28k} Cysteine Mutants. ANS fluorescence was used to monitor the structural differences that take place upon a change in redox potential. Initially, ANS fluorescence measurements were carried out under reducing and unreducing conditions, with and without 1 mM DTT. The data show that all mutants bind ANS. The ANS emission maximum is in all cases blueshifted from 520 nm, the value of ANS emission in water, to ca. 470 nm (data not shown). Although there is no difference in the emission wavelength between mutants, there is a significant variation in emission intensity (Figure 3). This is in accordance with earlier work on calbindin D_{28k} in which structural differences were shown to manifest themselves as a difference in ANS fluorescence intensity rather than in a change in emission wavelength (*8*). In the absence of DTT, the mutants containing C94 and C100 display a considerably stronger intensity compared to the mutants lacking these residues. The addition of DTT to mutants containing both C94 and C100 reduces the emission intensity to the same level as is observed for

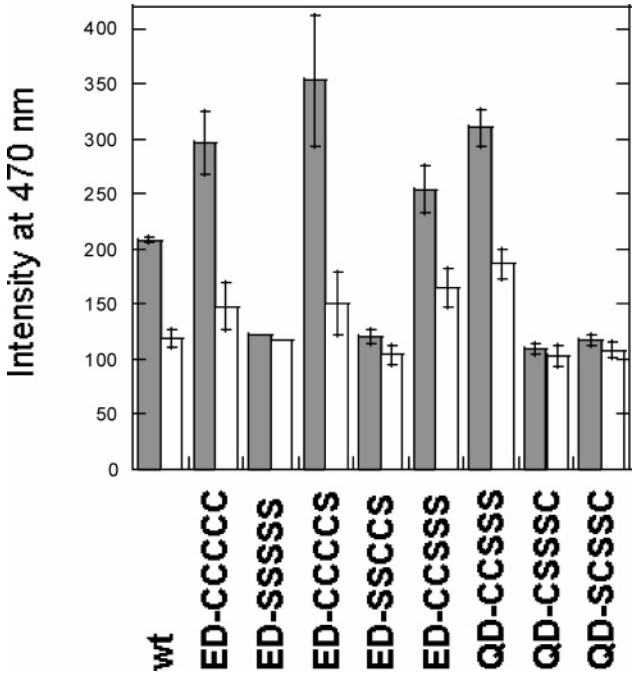

FIGURE 3: ANS binding by reduced and unreduced calbindin D_{28k} mutants. ANS fluorescence intensity at 470 nm in the presence of unreduced (closed bars) and reduced (open bars) calbindin D_{28k} mutants in 10 mM $KHPO_4$, pH 7.5, 0.15 M KCl, 0.5 mM EDTA at 25 °C. The protein concentration was 13 μM and 1 mM. DTT was included in the reduced samples. All samples were incubated overnight before ANS was added.

all other mutants (both with and without DTT). The intensity change is indicative of a structural change in the protein that is related to the oxidation state of C94 and C100. The results are in accordance with the denaturation studies described above.

ANS Binding to Calbindin D_{28k} at Controlled Redox Potentials. We next carried out a more detailed study to evaluate if the observed differences in ANS binding to calbindin D_{28k} due to reduction/oxidation could take place within a physiologically relevant range of redox potentials. ANS binding by ED-CCCCC was tested at redox potentials ranging from −300 (a more reducing environment) to −100 mV (a more oxidizing environment). Figure 4A shows a clear transition between −250 and −190 mV from a low to a high-intensity ANS emission at 470 nm. In contrast, ANS fluorescence in the presence of ED-SSSSS is not affected by the redox potential (Figure 4A). The concentration of oxidized glutathione is higher at −170 mV than at −250 mV. To investigate the possibility that part of the observed structural change is due to interactions (covalent or noncovalent) between glutathione and calbindin D_{28k}, ANS fluorescence was measured at different concentrations of oxidized glutathione while maintaining the redox potential at −170 mV. At increasing concentrations of oxidized glutathione, the ANS fluorescence intensity increases for ED-CCCCC while ED-SSSSS remains unaffected (Figure 4B). Because the plot of ANS emission intensity versus GSSG concentration is different for the two sets of experiments, the difference must be caused by a change in GSSG concentration in addition to a change in redox potential (Figure 4B). The disparity between the two sets of data therefore suggests that glutathione is not only affecting the redox potential, but also interacts directly with calbindin D_{28k}, thereby inducing

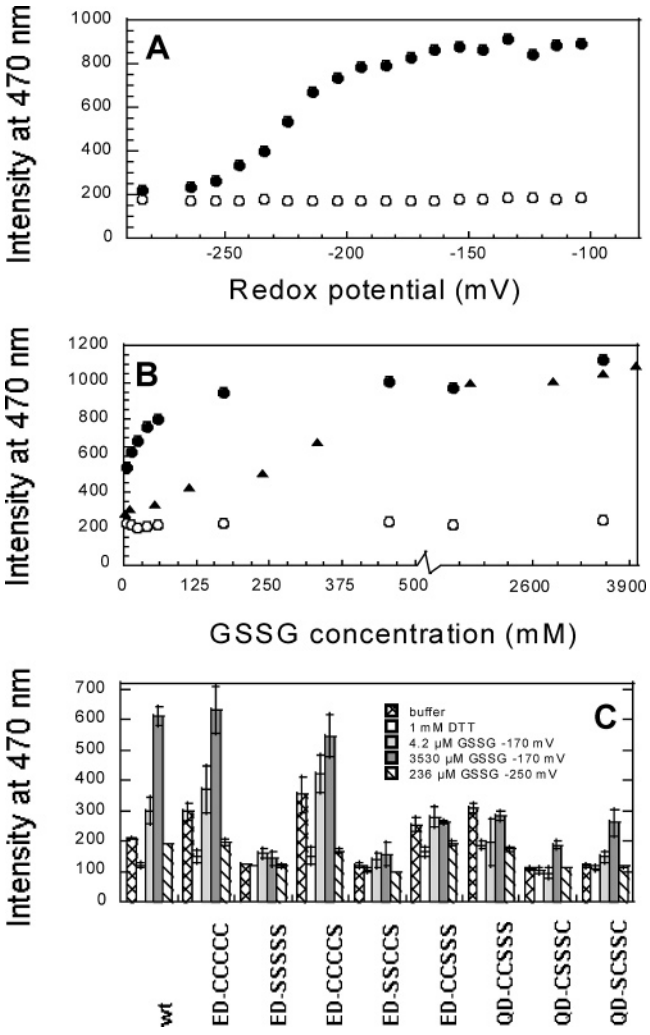

FIGURE 4: ANS binding by calbindin D_{28k} mutants at controlled redox potentials. (A) ANS fluorescence emission intensity monitored at 470 nm for ED-CCCCC (closed circles) and ED-SSSSS (open circles) at increasing redox potentials. The samples containing protein (13 μM) in 10 mM KHPO4, pH 7.5, 0.15 mM KCl, 0.5 mM EDTA, and a total of 5 mM glutathione were incubated overnight under N_2 atmosphere. The specific redox potentials were obtained by mixing reduced and oxidized glutathione as described in Experimental Procedures. (B) ANS fluorescence emission intensity monitored at 470 nm for ED-CCCCC (closed circles) and ED-SSSSS (open circles) at increasing concentrations of oxidized glutathione, with the redox potential maintained at −170 mV. For comparison, the ANS fluorescence for ED-CCCCC at different redox potentials (same data as in A) is plotted as a function of GSSG concentration. (C) ANS fluorescence intensity monitored at 470 nm for each individual calbindin D_{28k} mutant in 236 μM glutathione at −250 mV (bar #5 in each data set), in 3.5 mM glutathione at −170 mV (bar #4), or at −170 mV in 4.2 μM glutathion (bar #3). Data for the ANS fluorescence in buffer (bar #1) or in buffer containing 1 mM DTT (bar #2) are included for comparison.

additional conformational changes in the protein. The results tie in with the denaturation studies described above in which residues C187 and C219 appear to modulate the stability of the protein when residues C94 and C100 are present. Thus, in the presence of GSSG, additional structural changes, beyond the formation of a disulfide bridge between residues C94 and C100, may take place during oxidation of the protein. The ANS fluorescence data obtained at high versus low concentrations of GSSG further support this conclusion. Figure 4C shows ANS fluorescence for all mutants at −250 and at −170 mV at either high or low GSSG concentration. The data from the experiments in neat buffer or in buffer containing DTT are included for comparison. At −250 mV, or after reduction with DTT, the emission intensity at 470 nm is low for all mutants. In a more oxidizing environment, at a redox potential of −170 mV and low GSSG concentration, a more intense ANS fluorescence is observed for mutants containing residues C94 and C100. A similar, high ANS intensity is obtained for samples incubated in plain buffer, indicating that these conditions are as oxidizing as maintaining the redox potential at −170 mV. Under these conditions, the intensity is similar for each individual mutant. At −170 mV and high GSSG concentration, ANS fluorescence is stronger for wt calbindin D_{28k}, ED-CCCCC and ED-CCCCS compared to at −170 mV and low GSSG (or in plain buffer). No increase in ANS binding is seen for ED-CCSSS, QD-CCSSS, or ED-SSCCS, suggesting that the additional conformational changes induced by the presence of high GSSG concentration requires C94 and C100 (to form a disulfide bridge) as well as additional cysteine residues. The cysteine mutants, QD-CSSSC and QD-SCSSC, also display a somewhat stronger ANS fluorescence at high, compared to low, concentration of GSSG, which is consistent with structural modifications due to glutathione binding.

Urea-Induced Unfolding of Calbindin D28k in 5 mM GSSG. The ANS binding experiments indicate that, in addition to promoting the formation of a disulfide bond between residues C94 and C100, glutathione promotes additional structural changes in calbindin D_{28k} by binding to the protein. Therefore, we went back to study the urea-induced unfolding in the presence of 5 mM GSSG. The unfolding of ED-CCCCC and ED-SSSSS in 5 mM GSSG is shown in Figure 5, together with data obtained in plain buffer and in buffer containing 0.2 mM DTT. In the presence of oxidized glutathione, the unfolding of ED-CCCCC occurs at very low urea concentrations. In contrast, GSSG has no or little effect on the unfolding of ED-SSSSS.

Mass Spectrometry Analysis of Glutathione-Treated Calbindin D_{28k}. As described above, the structure of calbindin D_{28k} is redox sensitive. In addition, glutathione appears to influence the structure of calbindin D_{28k} in a manner that is dependent on the concentration of oxidized glutathione. One possible explanation for this behavior is that glutathione forms mixed disulfides with cysteine residues. To test this hypothesis, ED-CCCCC was incubated with 5 mM glutathione at −170 mV and subjected to mass spectrometry. Four different species were detected for ED-CCCCC (Table 2). The molecular weight of 29 928 is 32 units above the expected theoretical molecular weight for the protein (reduced form) and corresponds to two oxygen atoms. The molecular weights of 30 202 and 30 814 are higher by 306 and 918 mass units, corresponding exactly to the addition of one and three glutathione residues, respectively. The fourth species has a molecular weight of 30 540, which is 644 higher than the expected value, corresponding to the addition of two glutathiones (+612) and two oxygen atoms (+32). The glutathione modifications are cysteine specific, as ED-SSSSS only produces one peak in the mass spectrum corresponding to the expected parent molecular weight. Similarly, ED-CCSSS only furnishes the expected peak of the unmodified protein. ED-CCCCS and ED-SSCCS are both modified with one or two glutathione residues. This implies

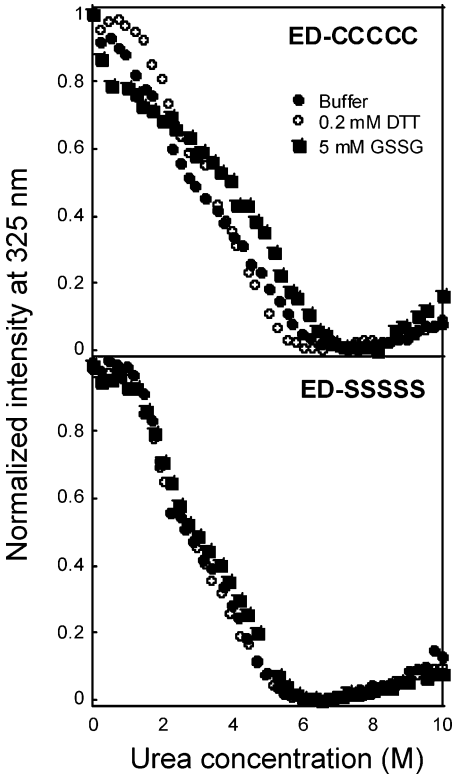

FIGURE 5: Urea-induced unfolding of calbindin D_{28k} mutants in 5 mM glutathione. The unfolding of ED-CCCCC and ED-SSSSS in 5 mM oxidized glutathione was followed by monitoring the intrinsic tryptophane fluorescence at 325 nm (with the remaining conditions as in Figure 4). Data for ED-CCCCC and ED-SSSSS in unreduced buffer or in 0.2 mM DTT are included for comparison.

Table 2: Molecular Mass Species of Calbindin D_{28k} after Incubation in 5 mM Glutathione, −170 mV

mutants	mass	mass difference[a]	number of glutathione
ED-CCCCC	29894	−2	−
(not modified)	29926	30	−
	29957	61	−
ED-CCCCC	29928	32	−
	30202	306	1
	30540	644	2 + 32
	30814	918	3
ED-SSSSS	29813	−3	−
ED-CCCCS	29875	−5	−
	30182	302	1
	30488	608	2
ED-SSCCS	29850	2	−
	30158	310	1
	30463	615	2
ED-CCSSS[b]	29845	−3	−
QD-CSSSC[c]	29844	−3	−
	30150	303	1
QD-SCSSC	29845	−2	−
	30150	303	1

[a] The difference in mass from the calculated mass without the first methionine residue. [b] A small proportion of ED-CCSSS has a mass corresponding to one added glutathione molecule. [c] A small proportion of QD-CSSSC has a mass corresponding to two added glutathione molecules.

that C187, C219, and C257 are the residues targeted for modification by glutathione. Interestingly, the additional mass of 32 noted for ED-CCCCC is not seen in ED-CCCCS, suggesting that additional mass of 32 can only occur when C257 is present.

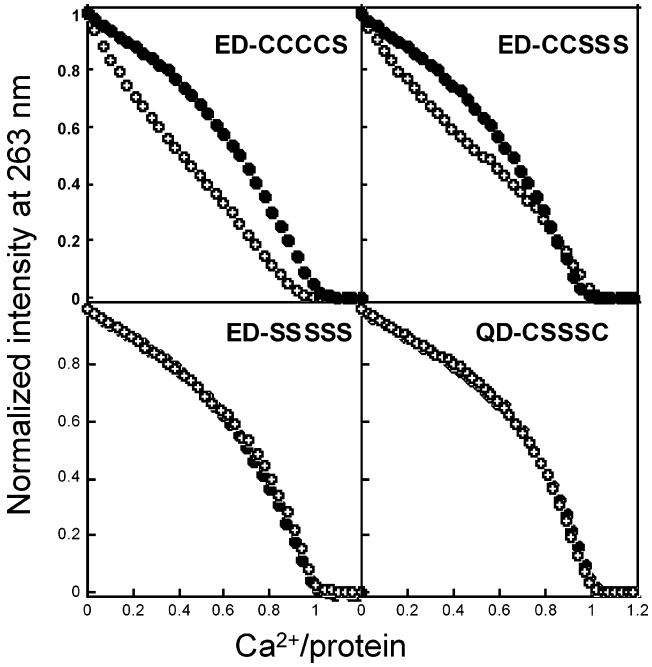

FIGURE 6: Ca^{2+} binding by reduced and unreduced calbindin D_{28k} mutants. Normalized absorbance at 263 nm as a function of total Ca^{2+} concentration for quin 2 in the presence of calbindin D_{28k}, wt and mutants. Approximately 20 μM of each calbindin D_{28k} mutant in Ca^{2+}-free 2 mM Tris-HCl buffer, pH 7.5, was titrated with $CaCl_2$ in the presence of the Ca^{2+} chelator Quin-2. The Ca^{2+} titrations were performed after overnight incubation in buffer (open circles) or in buffer with 1 mM DTT (closed circles). The absorbance change at 263 nm reports on Ca^{2+} binding to quin 2. The total Ca^{2+} concentration is normalized such that 1.0 corresponds to four Ca^{2+} binding sites in calbindin D_{28k} plus one in quin 2.

Mass spectrometry data of unmodified ED-CCCCC reveal the presence of three species. Most prevalent is the peak observed at 29 894, which corresponds to unmodified protein. The other two peaks have additional masses of 30 and 61, showing that the extra mass of 32 is not specific for calbindin D_{28k} modified with glutathione. The extra masses correspond to two (+32) and four (+64) oxygen atoms, suggesting that cysteine thiols have been converted to a higher oxidation state (see Discussion).

Calcium Binding by Reduced and Unreduced Calbindin D_{28k} Cysteine Mutants. Ca^{2+} binding was assessed through a series of Ca^{2+} titrations of calbindin D_{28k} cysteine mutants in the presence of the chromophoric Ca^{2+} chelator Quin-2. The data show that ED-CCCCS, ED-CCSSS, and QD-CCSSS bind Ca^{2+} in a redox-dependent manner (Figure 6, and data not shown). The titration data indicate a lower Ca^{2+} affinity for the unreduced proteins (in the absence of DTT), which is in agreement with the results obtained for wt calbindin D_{28k} and ED-CCCCC.[2] No redox dependence of Ca^{2+} binding is observed for ED-SSSSS, ED-SSCCS, QD-CSSSC, and QD-SCSSC (Figure 6, and data not shown), implying that C94 and C100 are necessary for the redox-dependent behavior. A more thorough study would include Ca^{2+} titration in buffers containing glutathione instead of DTT. Although several attempts were made, no complete set of data of the Ca^{2+} binding properties for wt and mutant proteins in the presence of glutathione could be obtained due to precipitation problems. However, preliminary data obtained for ED-CCCCS and ED-CCSSS suggest that the presence of GSSG accentuates the redox dependency.

Previous Ca^{2+} binding studies carried out on wt calbindin D_{28k} using the Quin-2 method show that the protein binds four Ca^{2+} with high affinity (*7*). Likewise, the Ca^{2+} titration data obtained for the cysteine mutants also fit to a model that assumes four high-affinity binding sites. Comparing the data for oxidized and reduced ED-CCCCS, ED-CCSSS and QD-CCSSS indicate that for each mutant the oxidized form binds Ca^{2+} with an overall lower affinity than the reduced form of the proteins.

Effect of D_{28k} Cysteine Mutants on IMPase at Different Redox Potentials. Calbindin D_{28k} interacts with myo-inositol monophosphatase (IMPase) and enhances its enzymatic activity (*9*). In this study, we investigated if this activation is redox dependent. The activity of IMPase in the presence of several calbindin D_{28k} mutants was studied using a colorimetric assay that quantifies the amount of released phosphate (*31*). The degree of activation exerted by calbindin D_{28k} is strongly dependent on substrate concentration, pH, and protein concentration (*9*). Under oxidizing conditions, at −170 mV, wt calbindin D_{28k}, ED-CCCCC and ED-CCCCS show a strong effect on the IMPase activity (Figure 7A), while only a modest activation is seen with ED-SSSSS and ED-SSCCS. This indicates that residues C94 and C100 are important for the ability of calbindin D_{28k} to affect the activity of IMPase. However, ED-CCSSS, QD-CCSSS, QD-CSSSC, and QD-SCSSC enhance the enzymatic activity less than wt calbindin D_{28k} and ED-CCCCC (Figure 7C), suggesting that optimal effects can only be achieved when the four N-terminal cysteine residues are present.

The small effects on IMPase activity exerted by ED-SSSSS and ED-SSCCS are independent of the redox potential. In contrast, the effect of wt calbindin D_{28k}, ED-CCCCC, and ED-CCCCS is significantly attenuated at −250 mV compared to −170 mV, suggesting that the redox state of the cysteine residues is important for calbindin D_{28k}'s ability to increase the enzymatic activity of IMPase. Upon reduction with 1 mM DTT, wt calbindin D_{28k} and ED-CCCCC display a weak capacity to activate IMPase, and are as ineffective as ED-SSSSS (Figure 7A). This strongly supports the idea that the redox state of the cysteine residues is important for IMPase activation by calbindin D_{28k}.

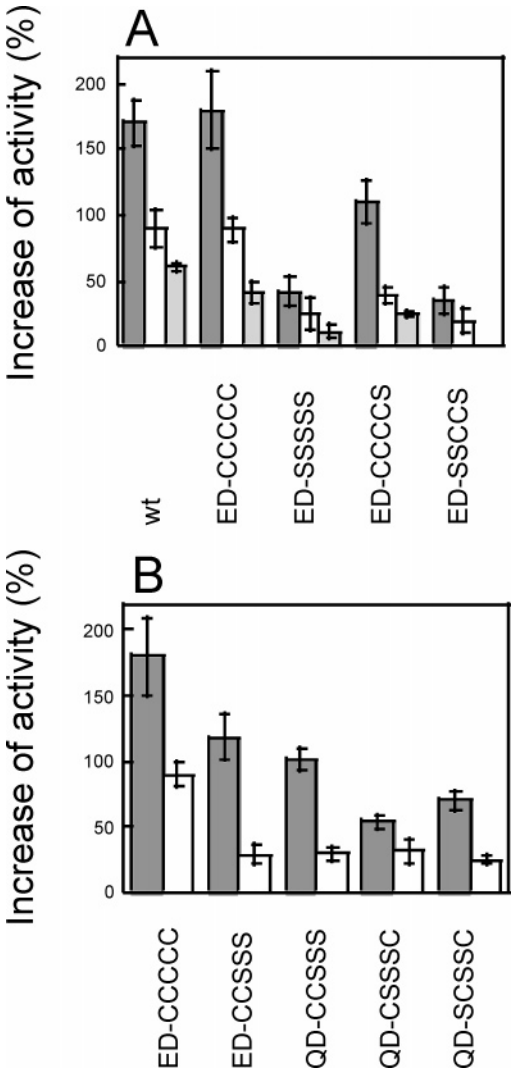

FIGURE 7: The effect of calbindin D_{28k} mutants on IMPase activity. Increase in IMPase enzyme activity due to the presence of 15 μM protein in 50 mM Hepes-KOH, pH 7.5, 150 mM KCl, 2 mM $MgCl_2$, and 0.2 mM EGTA with 1 mM DTT, or with 5 mM total glutathione at −170 or −250 mV. The effect of calbindin D_{28k} is reported as the percent increase of the IMPase activity compared to samples containing no calbindin. (A, B) Data at 1 mM DTT (light gray bars), −250 (open bars), or −170 mV (dark gray bars).

DISCUSSION

In this study, we have shown that human calbindin D_{28k} is structurally and functionally regulated by the redox state of its five cysteine residues. Within the biologically relevant range of redox potentials (−170 to −250 mV), calbindin D_{28k} undergoes conformational changes and chemical modifications that alter its Ca^{2+} binding properties and regulatory interactions with IMPase.

The urea-induced denaturation of calbindin D_{28k}, as followed by intrinsic fluorescence emission spectroscopy, shows that calbindin D_{28k} is more stable with than without DTT. In contrast, when denaturation is followed by CD spectroscopy, no change is observed between the two redox states, indicating that the structural differences occur on the tertiary rather than the secondary level.

Calbindin D_{28k} has recently been shown to be structurally sensitive to changes in H^+ and Ca^{2+} concentrations (*7*, *8*). For example, both the apo and the Ca^{2+} loaded forms of calbindin D_{28k} have exposed hydrophobic surfaces, but they bind ANS to a different degree (*8*). In these studies, the effects on ANS binding predominantly manifest themselves in a difference in fluorescence emission intensity rather than as a shift in emission wavelength. Other techniques, including chromatography on a hydrophobic resin and near-UV CD spectroscopy, verified that the difference in ANS fluorescence intensity was based on structural differences between the apo and holo forms of calbindin D_{28k}. Here we show that unreduced calbindin D_{28k} elicits stronger ANS fluorescence than the reduced protein, consistent with structural differences. The structural changes that take place upon oxidation involve the exposure of a more hydrophobic surface, and could be linked to a regulatory function for calbindin D_{28k}. Here we present data that provide a possible mechanism for regulation of calbindin D_{28k}, which is based on the redox potential in the cell.

An intramolecular disulfide bond between C94 and C100 has earlier been reported for rat calbindin D_{28k} (*27*) and recently for human calbindin.[2] Through ANS binding and

urea-induced unfolding experiments, this study shows that only mutants containing both C94 and C100 undergo the redox-driven structural change. The requirement of both C94 and C100 for the structural change to occur strongly suggests a mechanism in which these residues form a disulfide bond upon oxidation. The mutants that only contain C94 and C100 display a less significant difference in ANS binding at the two extreme redox potentials than mutants containing C94 and C100 together with additional cysteine residues. This indicates that the other cysteine modifications discussed above are responsible for the additional structural changes. These data are further supported by the urea-induced denaturation experiments. Part of the unfolding of unreduced ED-CCSSS and QD-CCSSS occurs at lower urea concentrations compared to wt calbindin D_{28k}, ED-CCCCC, and ED-CCCCS, indicating that the redox state of C187 and C219 affects the stability of the protein. Likewise, the presence of C187 and C219 in the unreduced protein increases ANS fluorescence, but only when C94 and C100 are present. This suggests an intricate interplay between the cysteine residues in calbindin D_{28k} in which the redox state of one cysteine residue affects the relative population of the oxidized and reduced states of another cysteine.

The four N-terminal cysteine residues in human calbindin D_{28k} are conserved among mammalian species with the fifth cysteine residue, C257, at the C-terminal end, being unique for human calbindin D_{28k}. Consequently, a comparison between ED-CCCCS, wt calbindin D_{28k}, and ED-CCCCC would provide information concerning the structural and functional differences between calbindin D_{28k} from different species. The urea-induced unfolding and the ANS fluorescence experiments suggest redox-driven structural changes in ED-CCCCS, which are similar to those occurring for wt calbindin D_{28k} and ED-CCCCC. However, the apparent ANS fluorescence generated by ED-CCCCS in the absence of DTT (unreduced conditions) is more intense compared to wt calbindin D_{28k} and ED-CCCCC, suggesting that its surface is more hydrophobic. This is not due to artifacts resulting from concentration differences, as ANS fluorescence to the three reduced proteins is the same. It is, however, noteworthy that there are no differences in ANS binding between ED-CCCCS, and wt calbindin D_{28k} and ED-CCCCC in the presence of glutathione. The mass spectrometry data reveal further differences between ED-CCCCC and ED-CCCCS. In the presence of oxidized glutathione, ED-CCCCC is modified with up to three glutathione residues. Not surprisingly, ED-CCCCS is modified with one less glutathione. However, in addition to the extra mass of glutathione, ED-CCCCC also has an additional mass of 32 Da, which is not seen with ED-CCCCS. These structural differences between ED-CCCCS, and wt calbindin D_{28k} and ED-CCCCC may have functional implications.

The cysteine residues in calbindin D_{28k} can be chemically modified with oxidizing agents. In an earlier study, up to four cysteine residues were found to be S-nitrosylated, and one cysteine residue, C187, was labeled hyperreactive because it is highly susceptible to S-thiolation (*32*). In the present study, mass spectrometry data of glutathione-treated calbindin D_{28k} show molecular weights corresponding to the addition of one to three glutathione residues. ED-CCCCS may be modified with up to two glutathiones. Since ED-CCSSS is not modified, the affected residues in ED-CCCCS are believed to be C187 and C219. In the case of ED-CCCCC, three residues are modified by glutathione (M+3GSH), most likely C187, C219, and C257. In addition, other species with an added molecular weight of 32 (M+32) and 644 (M+2GSH+32) were observed for ED-CCCCC. The +32 modifications are not seen with any of the other mutants, including ED-CCCCS, suggesting that C257 is required for this particular modification to occur. The most likely types of modifications would correspond to the oxidation of a cysteine thiol to a sulfenic acid or a disulfide to a disulfide-*S*-monoxide (*33*).

One of the functional roles of calbindin D_{28k} is to bind Ca^{2+}. It has been shown that deamidated calbindin D_{28k} binds Ca^{2+} with a lower affinity than nondeamidated wt protein.[2] The same study found that the Ca^{2+} affinity of wt calbindin D_{28k} is lowered upon formation of a disulfide bond. In the present study, we used a set of cysteine mutants to investigate the influence of particular cysteine residues and their redox state on the Ca^{2+} binding properties of calbindin D_{28k}. Our data show that the redox effect involves cysteines 94 and 100, and that the Ca^{2+} affinity is reduced when these two residues are oxidized. These are the same two cysteines that are involved in the redox-dependent conformational change discussed above.

The ability of calbindin D_{28k} to affect the activity of IMPase appears to be regulated by the redox potential. As discussed above, calbindin D_{28k} undergoes a redox-driven conformational change, which enhances or modifies the manner in which calbindin D_{28k} binds to IMPase, and hence, its activity. The difference in activation of IMPase between −250 and −170 mV is considerably more pronounced for ED-CCCCS than for wt calbindin D_{28k} or ED-CCCCC, suggesting differences in the structural response to a change in redox potential. The current findings parallel those obtained in earlier work, which show that the activation of IMPase is enhaced at acidic pH (*9*).

As described in the introduction, calbindin D_{28k} is proposed to protect cells from apoptosis. One of the features of apoptotic cells is a change of the cytosol environment to a more oxidizing redox potential. In this study, we show that the structure and function of calbindin D_{28k} is redox regulated, which is consistent with a role in which this protein protects the cell from death. Although it is premature to speculate about a specific role for a redox-dependent regulation of the IMPase activity during apoptosis, it is interesting to note that inositol, the product of the IMPase activity, has been reported to protect cells from apoptosis (*36*). Calbindin D_{28k} has also been shown to bind caspase 3 (*4*), which is a central protein in one of the apoptotic pathways. Recent studies show that the binding of calbindin D_{28k} to caspase 3 has a direct effect on inhibiting apoptosis in bone cells (*35*). It would be very interesting to learn more about the effects of the redox potential on this interaction. Philips et al. (*36*) introduced calbindin D_{28k} into the hippocampus of living rats via gene transfer. The redox regulation of the cells was disrupted by administering a drug that interferes with the generation of NADPH. While this treatment increased the incidence of apoptosis in neurones, cells that overexpressed calbindin D_{28k} were protected. These recent investigations suggest that the protective role of calbindin D_{28k} may include other mechanisms besides the removal of toxic levels of Ca^{2+}. This nicely ties in with the

results of the present work and should stimulate further in vivo studies relating the effects of calbindin D_{28k} to the redox state of the cell. Assigning a regulatory role to calbindin D_{28k} does not exclude it from protecting the cell from toxic levels of Ca^{2+}. The growing understanding of the multifaceted and complex functional roles displayed by calbindin D_{28k} can only increase the interest for this protein.

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