Comparison of the Ca\textsuperscript{2+}-binding Properties of Human Recombinant Calretinin-22k and Calretinin*

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Beat Schwaller‡, Isabelle Durussel§, Doris Jermann¶, Brigitte Herrmann‡, and Jos A. Cox§

From the ‡Institute of Histology and General Embryology, University of Fribourg, Pérolles, CH-1705 Fribourg, ¶Pharma Research, Nervous System Diseases, F. Hoffmann-La Roche and Co. Ltd., CH-4002 Basle, and the §Department of Biochemistry, University of Geneva, CH-1211 Geneva 4, Switzerland

Calretinin-22k (CR-22k) is a splice product of calretinin (CR) found specifically in cancer cells, and possesses four EF-hands and a differently processed C-terminal end. The Ca\textsuperscript{2+}-binding properties of recombinant human calretinin CR-22k were investigated by flow dialysis and spectroscopic methods and compared with those of CR. CR possesses four Ca\textsuperscript{2+}-binding sites with positive cooperativity (n = 1.3) and a [Ca\textsuperscript{2+}]\textsubscript{0.5} of 1.5 μM, plus one low affinity site with an intrinsic dissociation constant (K\textsubscript{D}) of 0.5 mM. CR-22k contains three Ca\textsuperscript{2+}-binding sites with n = 1.3 and [Ca\textsuperscript{2+}]\textsubscript{0.5} of 1.2 μM, plus a low affinity site with K\textsubscript{D} of 1 μM. All the sites seem to be of the Ca\textsuperscript{2+}-specific type. Limited proteolysis and thiol reactivity suggest that the C terminus of full-length CR, but not of CR-22k, is in close proximity of site III leading to mutual shielding. Circular dichroism (CD) spectra predict that the content of α-helix in CR and CR-22k is similar and that Ca\textsuperscript{2+} binding leads to very small changes in the CD spectra of both proteins. The optical properties are very similar for CR-22k and CR, even though CR-22k possesses one additional Trp at the C-terminal end, and revealed that the Trp residues are organized into a hydrophobic core in the metal-free proteins and become even better shielded from the aqueous environment upon binding of Ca\textsuperscript{2+}. The fluorescence of the hydrophobic probe 2-p-toluidinylnaphtalene-6-sulfonate is markedly enhanced by the two proteins already in the absence of Ca\textsuperscript{2+} and is further increased by binding of Ca\textsuperscript{2+}. The tryptosinolysis patterns of CR and CR-22k are markedly dependent on the presence or absence of Ca\textsuperscript{2+}. Together, our data suggest the presence of an allosteric conformational unit encompassing sites I–III for CR-22k and I–IV for CR, with a very similar conformation and conformational changes for both proteins. In the allosteric unit of CR, site IV is fully active, whereas in CR-22k this site has a 80-fold decreased affinity, due to the decreased amphiphilic properties of the C-terminal helix of this site. Some very specific Ca\textsuperscript{2+}-dependent conformational changes suggest that both CR and CR-22k belong to the “sensor”-type family of Ca\textsuperscript{2+}-binding proteins.

Calretinin (CR),\textsuperscript{1} an intracellular Ca\textsuperscript{2+}-binding protein containing six EF-hand motifs (1), is most closely related to calbindin-D\textsubscript{28k} (2). It is abundant in neuronal tissues, especially in the olfactory bulb (3) and auditory pathways. In the rat ventral cochlear nucleus, the CR content was found to be on the order of 6.4 μg/mg of protein; however, in other regions (paraventricular nucleus of the thalamus), CR contents of approximately 4 μg/mg of protein were measured (4, 5). For calbindin-D\textsubscript{28k}, protein levels in auditory neurons were estimated to reach concentrations of up to 2 μM (6) and the concentrations of CR are expected to be of the same order of magnitude. Its physiological function is not known, but seems to be related to Ca\textsuperscript{2+} buffering and diffusion (7). In the cochlea, CR could provide a strong, fast, and mobile buffer to cope with the unusually high, localized Ca\textsuperscript{2+} spikes (8). For both CR and calbindin-D\textsubscript{28k}, a role in neuroprotection from excitotoxic insults has been postulated (9), but the results remain quite controversial (for a review, see Ref. 10). Two recent studies have shown that important amounts of CR are associated with cell membranes (11, 12), suggesting that besides the proposed role as Ca\textsuperscript{2+} buffer, CR could also have specific target ligands in the membrane.

Normal human colon epithelial cells do not express CR, but several colon carcinoma cell lines (e.g. WiDr) express this protein to various amounts (13, 14). In several cell lines, alternatively spliced CR mRNA leading to C-terminally truncated proteins have been detected (15) and the presence of one of the splice products, calretinin-22k, has been demonstrated. To date, the alternatively spliced mRNAs have been detected only in cancer cells but not in the cells normally expressing CR. It has been hypothesized that the splice products could, in part, be responsible for the phenotype of transformed cells. One of the splice products derived from CR mRNA lacking exons 8 and 9 (∆8,9) and coding for a protein named calretinin-22k (CR-22k), was found in several different colon carcinoma cell lines, indicative of a particular “physiological” function (14). Human CR-22k comprises the first four EF-hands of full-length calretinin, including the first 3 amino acid residues of the C-terminal helix of site IV followed by a segment of 14 residues which, due to a frameshift, are completely different from those present in bona fide calretinin. Thus, four potentially functional EF-hands are present in CR-22k, but the fourth site is different from the one in CR.

In the frame of the function of CR and CR-22k as buffers, it is important to know how sensitive and selective they are toward Ca\textsuperscript{2+}, especially since the alternative splicing caused quite dramatic effects in the C-terminal sites. If they are also activators, it is also important to monitor the type of the con-

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\textsuperscript{2}To whom correspondence should be addressed: Dept. of Biochemistry 30, Quai Ernest-Ansermet, 1211 Geneva 4, Switzerland. Fax: 22-7026483; E-mail: jos.cox@biochem.unige.ch.

\textsuperscript{3}The abbreviations used are: CR, calretinin; CR-22k, calretinin-22k; CaM, calmodulin; TNS, 2-p-toluidinylnaphtalene-6-sulfonate; ESI-MS, electrospray ionization-mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.
formational changes upon binding of Ca\(^{2+}\) and establish the differences between CR and CR-22k. In the present report, human CR-22k was expressed and its Ca\(^{2+}\)-binding properties and conformational changes are described. These properties were systematically compared with those of the full-length recombinant CR.

MATERIALS AND METHODS

Overexpression and Purification of 6xHis-CR-22k, 6xHis-CR, CR-22k, and CR—The expression vectors coding for the fusion proteins named 6xHis-CR-22k and 6xHis-CR, which contain the affinity tag MRGS(H)\(_6\)GS at the N terminus, have been described previously (14). Briefly, the cDNA of clone CR16.17 coding for CR-22k was cut with SspI (this site is located 35 nucleotides downstream of the stop codon of CR-22k), the site was filled by Klenow enzyme, and the plasmid further digested with NcoI. The NcoI site (CCATGG) contains the start codon ATG for CR-22k. The vector pDS6.1/RBSII 6xHis, NcoI-HindIII, filled) was used to ligate the isolated fragment. For the 6xHis-CR vector, the cDNA clone CR3.9 was digested with NcoI and HindIII and the isolated fragment containing the entire reading frame of CR and including the 5'-nontranslating region of the CR cDNA was cloned into the same expression vector as above (pDS6.1/RBSII 6xHis, NcoI-HindIII). To remove the affinity tag, a fragment coding for the recognition site for protease Xa was cloned in between the 6xHis tag and the CR or CR-22k cDNAs (Fig. 1). For this reason, two complementary oligodeoxyribonucleotides with NcoI half-sites were synthesized that code for the peptide Ile-Glu-Gly-Arg-Ser, and this linker was inserted into the NcoI site. After transformation and plating, positive clones from each ligase formation were chosen according to several restriction enzyme digests. The site preceding the start codon ATG remains intact and can be used as a general cloning vector containing the 6xHis affinity tag and the protease Xa cleavage site. The cleavage site for the protease Xa is marked by an arrow.

Protein samples were desalted using capillary reverse phase-HPLC (Hewlett Packard, Waldbronn, Germany). SDS-PAGE (12.5%) was used to analyze the proteins CR-22k and CR. The thiol reactivity was monitored by measuring the kinetics of the reduction of the disulphide bonds with 10 mM 2-mercaptoethanol, and then passed through a 40 \(\times\) 0.6 cm Sephadex G-25 column equilibrated in buffer A. Total Ca\(^{2+}\) and Mg\(^{2+}\) concentrations were determined with a Perkin-Elmer LS-5B spectrophotometer. The protein concentration was determined spectrophotometrically using a molar extinction coefficient at 276 nm of 26,100 M\(^{-1}\) cm\(^{-1}\) for CR-22k and CR, respectively.

Conformational Changes Monitored by Near Ultraviolet Absorption—UV-absorption spectra and difference spectra were measured with a Perkin-Elmer Lambda 5 spectrophotometer at room temperature. The Ca\(^{2+}\)-sensitive metal-protein solution (40 \(\mu\)M) was equilibrated in buffer A and 20 \(\mu\)M Ca\(^{2+}\) was added. Spectra were obtained at a flow rate of 0.1 \(\mu\)l/min in a cell of 1 mm optical path length (in mm), c the molar concentration of the protein, and N the total number of residues in the protein.

Spectral analysis—The Ca\(^{2+}\)- and Mg\(^{2+}\)-dependent changes in hydrophobicity of CR-22k and CR were followed by monitoring the fluorescence properties of TNS as described (20). Solutions of 30 \(\mu\)M TNS and 2 \(\mu\)M metal-free protein were excited at 328 nm and the emission spectra recorded with slits of 5 nm. EGTA (20 \(\mu\)M), MgCl\(_2\) (5 mM), or CaCl\(_2\) (1 mM) were added subsequently to obtain the metal-free, Mg\(^{2+}\), Ca\(^{2+}\), and denatured forms, respectively. Difference spectra were taken on solutions with an optical density at 278 nm of 1 and normalized to an optical density of 1.0 at 278 nm.

Analysis of Tryptic Fragments—Protein samples were desalted using capillary reverse phase-HPLC (Hewlett Packard, Waldbronn, Germany). Many, model 1090, LC packings POROS R2/H, cartridge 5 mm \(\times\) 0.8 mm) and an acetonitrile, 0.1% trifluoroacetic acid gradient for elution. The desalted fractions were lyophilized, taken up in 10 \(\mu\)l of acetonitrile, 1 \(\mu\)l acetic acid (1:1), and flow-injected with the HPLC system into a Perkin Elmer/Spectra (Concord, Canada) model A-132 linear spectrophotometer.
RESULTS

Expression and Purification of Recombinant Proteins—The protein 6xHis-CR-22k was overexpressed and purified to homogeneity on a nickel chelate column as described previously (14) and additionally characterized by MALDI-TOF and ESI-MS. The molecular mass estimated by MALDI-TOF was 23,572, which was slightly too high due to a small amount of dimer \( M_r = 47,144 \) not separated from the peak of \( M^+ \). The \( M_r \) of 6xHis-CR-22k was additionally measured by ESI-MS, and the calculated mass of 23,555 ± 1.5 Da was as expected from the calculated molecular weight. Since an interference by the 6xHis affinity tag on the cation binding properties cannot be ruled out, an expression system was developed, with a recognition sequence for protease Xa between the 6xHis affinity tag and the CR or CR-22k protein. After cleavage with protease Xa, CR-22k and CR were identical to the human recombinant proteins except for an additional serine residue at the N terminus. The two proteins were produced in \( E. \) coli and purified as described under “Materials and Methods,” and SDS gels of the purified proteins CR and CR-22k are shown in Fig. 2. The size difference between CR-22k and CR before and after cleavage at the protease Xa cleavage site is clearly visible and is a result of the removal of the peptide MRGS(H)_5GSIEGRS from the N termini of both proteins.

Kinetics of Removal of the Affinity Tag by the Protease Xa and Trypsin Digestion Experiments—In preliminary proteolytic experiments during the purification of CR-22k, a protein band appeared of approximately 17–18 kDa, significantly smaller than the expected size of CR-22k (Fig. 2). Amino acid sequence comparison revealed that segment 36–39\(^2\) (Ile-Glu-Gly-Lys) in the loop of EF-hand site I is almost identical to the sequence recognized by protease Xa (Ile-Glu-Gly-Arg). Since this cleavage was not detected during the purification of CR, we determined the time course of the digestion of both proteins.

Whereas for CR-22k the cleavage was complete after 20 min, in the case of CR undigested precursor protein was still present after 40 min (Fig. 2). Prolonged incubation of CR with protease Xa did not lead to the internal cleavage presumably after amino acid Lys-39, whereas the cleavage of CR-22k at this site was complete under identical conditions (Fig. 2). The different kinetics of cleavage at the introduced specific site and the different sensitivity toward the internal cleavage site demonstrated that the accessibility for the protease Xa was different for the two proteins in the region of \( Ca^{2+} \)-binding site I.

Trypsin digestion experiments in the presence or absence of \( Ca^{2+} \) with CR-22k containing the affinity tag are shown in Fig. 3 (A and B). In the presence of 1 \( mM \) \( Ca^{2+} \), the protein was cleaved at Lys-60, giving rise to fragments of 15 and 7 kDa. The 15-kDa fragment was then further cleaved yielding a fragment of 11 kDa. In the absence of \( Ca^{2+} \), several fragments were already visible after 0.5 min and also the 15-kDa fragment was rapidly cleaved into very short fragments. When the digestion experiment was repeated on CR-22k from which the affinity tag had been previously removed, the pattern (not shown) was identical to that shown in Fig. 3 (A and B). Since in the latter experiment time points were chosen so that many different fragments accumulated, these fragments could be analyzed by ESI-MS; the results are listed in Table I and Fig. 3C. In the presence of \( Ca^{2+} \), CR-22k was preferentially cleaved at Lys-60, while in the apo form the protein was additionally cleaved at residues Lys-141 and Lys-170, as well as at other sites, which could not be unambiguously identified.

Digestion of human CR with trypsin in the presence or absence of \( Ca^{2+} \) resulted also in characteristic fragmentation patterns (Fig. 4A). The major cleavage site was at Lys-60, which was accessible in the presence as well as in the absence of \( Ca^{2+} \), as has been shown previously for rat CR (27). In the presence of either 0.1 \( mM \) or 1 \( mM \) \( Ca^{2+} \), this remained the major cleavage site, whereas in the apo form CR was cleaved into many more fragments. These were characterized by ESI-MS and MALDI-TOF, and the results are shown in Tables.
I and II and Fig. 4B. The digestion experiments with either CR-22k or CR demonstrated that the accessibility for trypsin is significantly changed depending on the metal-binding status of both proteins, indicative of significant Ca\(^{2+}\)-dependent conformational changes.

**Direct Binding Studies**—Ca\(^{2+}\)-binding studies by flow dialysis revealed that CR-22k binds 4 mol of Ca\(^{2+}\)/mol of protein and contains three high affinity sites and one low affinity site (Fig. 5A). For comparison, CR contains four high affinity sites and one low affinity site (Fig. 5B). The stoichiometries of the high and low affinity sites in both proteins were confirmed by selected Hummel-Dryer experiments (data not shown). Binding to the high affinity sites occurs with moderate positive cooperativity (\(n_H = 1.3\)) and [Ca\(^{2+}\)]\(_{b,5}\) values of 1.2 and 1.5 \(\mu\)M for CR-22k and CR, respectively. The low affinity site displays a dissociation constant of 1 \(\mu\)M (CR-22k) and 0.5 \(\mu\)M (CR).

Analysis according to the Adair equation yielded sets of intrinsic binding constants listed in the legend of Fig. 5. This analysis confirms the general conclusion that the alternative splicing selectively affects the set of high affinity sites by removing one site from the cooperative pool to yield a low affinity site. Mg\(^{2+}\) at the physiological concentration of 2 mm has no significant effect on the Ca\(^{2+}\)-binding profile of CR (Fig. 5B, *), suggesting that all the sites of this protein are so-called Ca\(^{2+}\)-specific sites.

**Circular Dichroism**—The far UV circular dichroic spectra of the Ca\(^{2+}\), metal-free, and denatured forms of CR-22k and CR are presented in Fig. 6 (A and B). The mean residue ellipticities are very similar for the two native proteins. For both proteins, very small differences (less than 10% at 222 nm) were observed between the Ca\(^{2+}\) and metal-free state, indicating that the main peptide backbone folding is not importantly altered by binding or dissociation of Ca\(^{2+}\). Small changes are quite common in Ca\(^{2+}\)-binding proteins of the EF-hand family (25). An estimation of the contents of secondary structure according to Chang et al. (34) by comparison of the 195–240 nm zone of the spectra to reference spectra yielded the following approximate values: for CR-22k, 37% \(\alpha\)-helix, 23\% \(\beta\)-sheet, and 8\% \(\beta\)-turn; and for CR, 41\% \(\alpha\)-helix, 20\% \(\beta\)-sheet, and 12\% \(\beta\)-turn. All this structure is converted to random coil in the presence of 3 \(M\) guanidine HCl.

**TABLE I**

| Protein fragments | Amino acid | Molecular mass determined by ESI-MS | Calculated molecular mass deduced from amino acid sequence |
|-------------------|------------|----------------------------------|-------------------------------------------------------------|
| CR-22k (+ Ca\(^{2+}\)) | 1–60 | 6931.8 | 6930.8 |
| | 61–192 | ND\(^a\) | 15329.4 |
| CR-22k (− Ca\(^{2+}\)) | (75–170) | 11167.3 | 11187.6 |
| | (−1–60) | 6932.0 | 6930.8 |
| | 142–170 | 3441.8 | 3441.8 |
| CR (+ Ca\(^{2+}\)) | 61–271 | ND | 24714.2 |
| CR (− Ca\(^{2+}\)) | 246–255 | 991.7 | 991.5 |
| | 246–258 | 1424.8 | 1424.7 |
| | 233–244 | 1537.2 | 1537.7 |
| | 162–178 | 1889.3 | 1870.1 |
| | 145–161 | 2335.2 | 2335.6 |
| | (−1–52) | 6124.5 | 6123.8 |
| | 51–103 | 6124.5 | 6124.1 |

\(^a\) positions corresponding to the published sequence; the first Met (ATG) corresponds to residue 1; −1 denotes the additional Ser residue at the amino terminus of the purified protein. Calculated sizes \(>1000 Da\) are average molecular weights, while for the fragment \(<1000 Da\) the monoisotopic molecular weight was calculated. Amino acids in parentheses denote possible fragments where the observed and the calculated sizes are not perfectly correlated. Underlined fragments were identified by amino acid sequencing of the N-terminal amino acids and by size on the SDS gels.

**TABLE II**

| Protein fragments | Amino acid | Molecular mass determined by MALDI-TOF | Calculated molecular mass deduced from amino acid sequence |
|-------------------|------------|----------------------------------|-------------------------------------------------------------|
| | 27–39\(^a\) | 1422.1 | 1421.5 |
| | 40–52 | 1652.8 | 1651.8 |
| | 104–117 | 1635.3 | 1633.7 |
| | 118–133 | 1901.1 | 1900.9 |
| | 119–133 | 1773.1 | 1772.8 |
| | 142–161 | 2463.5 | 2462.3 |
| | 143–161 | 2335.4 | 2334.2 |
| | 152–161 | 1264.3 | 1263.7 |
| | 195–211 | 2095.6 | 2094.9 |
| | 233–244 | 1537.3 | 1536.7 |
| | 234–244 | 1409.3 | 1408.7 |
| | 245–258 | 1552.2 | 1552.8 |

\(^a\) positions corresponding to the published sequence; the first Met (ATG) corresponds to residue 1.
Fluorescence Characteristics—After excitation at 278 nm, Ca\(^{2+}\)-saturated CR-22k (1 \(\mu\)M) shows an emission spectrum with a maximum at 332 nm, which is decreased by a factor of 1.5 upon addition of EGTA (Fig. 7A). The EGTA spectrum is not influenced by the addition of up to 5 \(\text{mM} \) Mg\(^{2+}\). Upon denaturation by 4 \(\text{M} \) guanidine HCl, the maximum of the fluorescence is shifted to 350 nm without any decrease in intensity. The spectra of the denatured and metal-free protein show an isosbestic point at 341 nm, and those of the denatured and Ca\(^{2+}\) form an isosbestic point at 360 nm. The corresponding spectra of a 1 \(\mu\)M CR solution (Fig. 7B) are qualitatively similar to those of CR-22k, taken into account that CR contains two Trp and CR-22k three. The fluorescence of the Ca\(^{2+}\) form of CR is comparatively higher with a red shift of the isosbestic point to 366 nm. To compare the stabilities of the two proteins in their metal-free forms, the fluorescence changes were followed during titration with guanidine HCl (data not shown). For both proteins [Gu-HCl]\(_n\) \(_{50}\) was 1.74 M, indicating that hydrophobic cores of the metal-free proteins are similar.

Near UV Difference Spectrophotometry—The near UV spectrum (Fig. 8A, inset) of CR-22k is characteristic for a protein rich in Trp (3 residues) and Tyr (7 residues). The difference spectrum induced by Ca\(^{2+}\)-binding shows positive and negative peaks at 293 and 290 nm (Fig. 7), merely due to rearrangement in the Trp environment. The Trp perturbation is confirmed by an important positive peak (with \(\Delta\text{OD} > 0.2\)) at 236 nm (not shown). The Phe environment does not seem to be sensitive to Ca\(^{2+}\) binding, although the folding of the protein (dashed and dotted lines in Fig. 8A) to the metal-free state clearly shows the rearrangements in both the Phe and Trp environments. The spectrum is not influenced by the addition of Mg\(^{2+}\). Despite the fact that CR contains less Trp (2 instead of 3) but more Tyr (12 instead of 7) than CR-22k, the spectra and difference spectra of the two proteins (Fig. 8, A and B) are very similar, including the Ca\(^{2+}\)-induced perturbation of the Trp environment and the absence of a Mg\(^{2+}\) effect. Only the intensities are distinctly higher in CR, suggesting a more compact hydrophobic core.

Interaction with the Hydrophobic Probe TNS; Comparison with Calmodulin (CaM)—Upon binding of Ca\(^{2+}\), CaBPs of the activator type, such as CaM (22) and neuron-specific CaBPs (23), usually display hydrophobic patches on their surface, which can be monitored with particular fluorescent probes such as TNS. Fig. 9A shows that the Ca\(^{2+}\) form of CR-22k induces a 12-fold increase in fluorescence enhancement with maximal fluorescence at 436 nm, whereas the metal-free protein shows a 10-fold enhancement. Contrary to fluorescence and difference

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**Fig. 5.** Ca\(^{2+}\) binding to recombinant human CR-22k (A) and CR (B). Ca\(^{2+}\)-binding was measured by the flow dialysis method on 25–35 \(\mu\)M protein solutions at 25 °C. The different symbols correspond to separate experiments; three experiments were done on CR-22k and two on CR in the absence of Mg\(^{2+}\); one on CR was done in the presence of 2 \(\text{mM} \) Mg\(^{2+}\) (*). The theoretical isotherms (lines) were generated using Eq. 1 with the following intrinsic constants (\(K_i\) to \(K_n\)): 2.7 \(\times\) 10\(^5\), 4.0 \(\times\) 10\(^5\), 6.0 \(\times\) 10\(^5\), and 1.0 \(\times\) 10\(^4\) M\(^{-1}\) for CR-22k and 2.2 \(\times\) 10\(^5\), 3.2 \(\times\) 10\(^5\), 4.7 \(\times\) 10\(^5\), 8.0 \(\times\) 10\(^5\) and 2.0 \(\times\) 10\(^4\) M\(^{-1}\) for CR.

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**Fig. 6.** Circular dichroic spectra of CR-22k (A) and CR (B) at 25 °C. Protein solutions (0.25 mg/ml) in 5 \(\text{mM}\) phosphate buffer, pH 7.5 were supplemented with 100 \(\text{mM} \) Ca\(^{2+}\) (solid lines), 100 \(\text{mM}\) EGTA (dotted lines), or 3 \(\text{M}\) guanidine HCl (dashed and dotted lines). Due to strong light absorbance, the spectra in the presence of guanidine HCl are meaningful from 250 to 210 nm.
spectrophotometry where the metal-free and the Mg$^{2+}$ spectra are indistinguishable (Figs. 7 and 8), this optical method shows that the fluorescence spectra of both proteins are slightly altered in the presence of 5 mM Mg$^{2+}$. At the same protein concentration, CR shows an enhancement of the TNS fluorescence, which is reminiscent of that of CR-22k (Fig. 9C), but the maximal fluorescence is blue-shifted by 4–5 nm and the intensities are somewhat lower. Under identical experimental conditions, Ca$^{2+}$-saturated CaM enhances the fluorescence of TNS 11-fold, but it differs in two aspects from CR-22k and CR. 1) The fluorescence maximum is red-shifted to 456 nm; 2) the enhancement by apo-CaM is negligible (Fig. 9B).

**Fig. 7. Fluorescence spectra of CR-22k (A) and CR (B).** The protein concentration was 1 μM. Ca$^{2+}$, Mg$^{2+}$, or guanidine HCl were added up to 1 mM, 5 mM, and 4 M, respectively. Solid line, Ca$^{2+}$ form; dashed line, Mg$^{2+}$ form; dotted line, metal-free form; dashed and dotted line, guanidine HCl-denatured form.

**Fig. 8. Ca$^{2+}$ or Mg$^{2+}$-induced difference spectra of CR-22k (A) and CR (B) at room temperature.** The protein concentrations were 40 μM in buffer A. Ca$^{2+}$ and Mg$^{2+}$ were present at 100 μM and 5 mM, respectively. For comparison spectra were also taken after denaturation of the protein by 4 M guanidine HCl. Ca$^{2+}$ form versus apo form (solid line); Mg$^{2+}$ form versus apo form (dashed line); metal-free form versus denatured form (dashed and dotted line). Insets, the near UV spectra of the two proteins.
tains one more Cys in position 266 (Fig. 4B), and the total number of thiols titratable with 5,5'-dithiobis(2-nitrobenzoic acid) was 1.78. In both the Ca\(^{2+}\)- and metal-free form of CR, one thiol reacted very rapidly and one rather slowly (t\(_{1/2}\) approximately 1 min). Since Cys-101 of CR-22k also reacted rapidly, Cys-266 probably is the slowly reacting thiol in the full-length protein.

**DISCUSSION**

Recent structural data of EF-hand proteins revealed significant differences in Ca\(^{2+}\)-induced conformational changes. While proteins such as calmodulin or troponin C undergo substantial conformational changes, the Ca\(^{2+}\)- and the apo form of calbindin-D9k are essentially the same (25). On the basis of these results and the known functions for several EF-hand proteins, proteins with regulatory functions (e.g. troponin C in muscle) have been termed “Ca\(^{2+}\) sensor proteins”, while those involved in Ca\(^{2+}\) transport and buffering (e.g. calbindin-D9k in intestine) have been named “Ca\(^{2+}\) buffers” (for a review, see Ref. 26). It is proposed that proteins of the “sensor” type show characteristic Ca\(^{2+}\)-dependent conformational changes exposing more hydrophobic surface, which is necessary for their biological activities. On the other hand, “buffers” only need to bind Ca\(^{2+}\) fast and efficiently. Calretinin has been classified as a Ca\(^{2+}\) buffer protein due to its close homology to calbindin-D9k, but recent reports have demonstrated CR can undergo Ca\(^{2+}\)-induced conformational changes and bind to octyl-agarose in a partially Ca\(^{2+}\)-dependent manner (24, 27). In this report, we aimed to investigate the Ca\(^{2+}\)-binding characteristics of calretinin-22k and properties that are related to the Ca\(^{2+}\)-binding status.

The Ca\(^{2+}\)-affinity profiles of CR-22k and CR are very similar, except that one high affinity site is lost in the alternatively spliced CR-22k. This is predicted since CR contains five and CR-22k four canonical EF-hand motifs. The affinity of human recombinant CR for Ca\(^{2+}\) is approximately 2–4 times lower than that of chicken (28, 29) and rat CR (24). Positive cooperativity (n\(_H\) = 1.9) was also reported for chicken CR (29). The reason for the discrepancy between the values obtained for chick or human CR could be related either to species differences or differences in the buffer compositions that were used in the different studies. The affinities for rat CR have been obtained from fluorescence spectrophotometry measurements (24) and not from direct metal-binding measurements. Two questions can be raised. 1) Which Ca\(^{2+}\)-binding site is present in CR and absent in CR-22k? 2) Which is the low affinity site in both proteins? Since site VI certainly is inactive due to an amino acid sequence not conforming to the EF-hand consensus sequence (1) and functional domains occur as pairs of EF-hands with short anti-parallel \(\beta\)-sheet interactions between the two loops (30), we are tempted to consider that in CR site V is the low affinity site. For rat CR, the fusion protein consisting of sites III–VI had the lowest affinity (K\(_D\) = 1 \(\mu\)M); moreover, the protein fragment consisting of sites IV to VI did not change its intrinsic fluorescence significantly in the presence or absence of Ca\(^{2+}\) (24), further supporting the hypothesis that the low affinity site likely is site V. Sites I–IV in CR are then the high affinity sites and display positive cooperativity. The Hill coefficient is identical for CR-22k and CR, and the optical data suggest that the allosteric conformations, R and T, and transitions are quite similar in both proteins. This implies that the alternative splicing leads to the elimination of one site, but otherwise not to dramatic alterations in the overall architecture of the allosteric domain. However, in CR-22k, the allosteric superdomain contains only three, instead of four active sites. We hypothesize that site IV is the site with the lowered affinity, because the helices of the EF-hands are usually amphiphilic, whereas the C-terminal helix of site IV in CR-22k is not (Fig. 10).

Our conformational data suggest that all the sites are of the Ca\(^{2+}\)-specific type, i.e. Mg\(^{2+}\) does not compete at these sites, which are thus empty in a cell under resting conditions. This may be particularly relevant for the Ca\(^{2+}\)-buffering function of the protein; a buffer that displays only Ca\(^{2+}\)-specific sites is very fast and dampens the Ca\(^{2+}\) signal within a time laps of 100 \(\mu\)s. In hair cells, this type of buffer is needed to handle Ca\(^{2+}\) signals that exceed 100 \(\mu\)M, oscillate at approximately 100 Hz, and spread only in the order of 100 nm from each active zone (31). Results on chick CR (29) suggest that Mg\(^{2+}\) competes with Ca\(^{2+}\) at the same sites, albeit with a low affinity (K\(_D\) = 4.5 mM).

Although circular dichroism experiments revealed little changes in secondary structure upon binding of Ca\(^{2+}\), evidence for Ca\(^{2+}\)-induced conformational changes of CR-22k and CR comes from controlled proteolysis, fluorescence end spectrophotometric experiments. In the Ca\(^{2+}\)-bound form, CR-22k is rapidly cleaved at Lys-60, but the remaining fragment 61–192 is fairly resistant to further degradation. Under the same conditions, the apo form of CR-22k is rapidly digested into smaller...
Ca2+

the former Trp residues are strongly changing by the binding of likely that this C-terminal Trp is silent. The environments of whereas the third is not present in full-length CR. It is thus all the conformational methods explored in this study. CR-22k due to a rearrangement of neighboring amino acids.

VI are almost selectively cleaved in the apo- but not in the Indeed, in CR, the Lys and Arg residues in EF-hand sites I and 5m M Tris-HCl buffer, 0.5 m M Ca2+

Programs.

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fragments and identified cleavage sites include Lys-141 (between EF-hand sites III and IV) and Lys-170 (in the loop of EF-hand IV). These results are in good agreement with the data of the metal-binding properties and spectroscopic data, which support the model that, in the presence of Ca2+, the EF-hand sites I–III of CR-22k form a compact allosteric unit and that the protein exposes more hydrophobic surface, thus burying potential trypsin cleavage sites.

For CR, a similar cleavage pattern is observed with a first cut at Lys-60, which is Ca2+-independent. However, although in the presence of Ca2+, this is the major proteolytic site, the apo form is rapidly cleaved at approximately 20 sites. The cleavage sites, identified by ESI-MS or MALDI-TOF, are between (12 sites) as well as within EF-hand sites (8 sites). Our tryptic digestion results are partially contradictory to the ones obtained with rat CR. Kuznicki et al. (27) showed that in the apo form CR is preferentially and almost selectively cleaved in the region between EF-hand sites I and II and in the E-helix of site II, whereas in the presence of 0.1 mM Ca2+ rat CR was additionally cleaved in the C-terminal half of the protein. These differences of digestion patterns are quite surprising considering the high sequence identity (98.9%) between rat and human CR with 26 Lys and 9 Arg residues at identical positions; the only difference is one additional Arg at position 211 in human CR, which is Gly in rat.

Our data also shed light on an intramolecular contact in CR. Indeed, in CR, the Lys and Arg residues in EF-hand sites I and VI are almost selectively cleaved in the apo- but not in the Ca2+-bound form, suggesting that the C-terminal part of CR comprising sites V and VI are in close proximity of EF-hand site I in the metal-bound form, leading to mutual shielding of putative cleavage sites. This is confirmed by the relatively slow thiol reactivity of Cys-266 of CR as well as by our findings that the putative protease Xa recognition site 36–39 is accessible in CR-22k, but not in CR. The slow thiol reactivity of Cys-266 could be due to a direct shielding effect of EF-hand site I; an alternative explanation is a change of the pKa of the -SH group due to a rearrangement of neighboring amino acids.

The optical properties of CR-22k and CR are very similar in most colon adenocarcinoma cells at rest, CR-22k is distributed rather homogeneously within the cells, including the nucleus (14). In the presence of sodium butyrate as an inducer of differentiation in WiDr cells, CR-22k is transiently translocated into the nucleus and the immunoreactivity remaining in the cytoplasm has a net-like appearance (33). This suggests that CR-22k could interact with specific targets in WiDr cells under specific conditions. The identification of these targets in the cytoplasm as well as in the nucleus will be the next step in elucidating the role of CR-22k in these cells.

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