Purification and Characterization of the Recombinant Human Calcium-Binding S100 Proteins CAPL and CACY[†,‡]

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ABSTRACT: The S100 proteins CAPL and CACY are expressed in a tissue- and cell-specific manner and have been reported to be associated with the metastatic phenotype of tumor cells. In order to study the biochemical, cation-binding, and conformational properties, we produced and purified large amounts of the recombinant human proteins in *Escherichia coli*. Several characteristics of native proteins are shown to correspond to those of the bacterially expressed proteins. Both are able to form homodimers *in vitro*, probably the biologically active species, but not heterodimers. The Ca^{2+}-binding parameters were studied by flow dialysis at physiological ionic strength. Both isotherms show a maximum of two Ca^{2+} per protein and are insensitive to Mg^{2+}, indicating that the sites are of the Ca^{2+}-specific type. The isotherms show slight (CAPL, $n_H = 1.15$) or pronounced (CACY, $n_H = 1.33$) positive cooperativity with $K_{0.5}$ values of 0.32 mM (CACY) and 0.15 mM (CAPL), indicating that the sites are of the low-affinity type. Conformational changes in the Tyr microenvironment of CACY indicate that Ca^{2+} binding induces a shift of Tyr to a less polar environment. Mg^{2+} does not affect the fluorescence properties nor does it induce a difference spectrum, thus suggesting that at physiological ionic conditions it does not interact with the protein. The Ca^{2+}-induced difference spectra of CAPL are about 3 times smaller than those of CACY, suggesting that the additional Tyr84 in CACY is much more sensitive to Ca^{2+} than the two Tyr residues conserved in both proteins.

CAPL and CACY are members of the S100 family of Ca^{2+}-binding proteins. These proteins contain two distinct EF-hand motifs of 14 and 12 amino acids, respectively. The 14 amino acid loop is unique for S100 proteins. The structure of one member of this family, CaBP9k, has been studied extensively by X-ray analysis (Szebenyi, 1986) and NMR studies (Carlström & Chazin, 1993; Akke et al., 1992). The binding of calcium to S100 proteins induces a conformational change, exposing hydrophobic regions that may interact with target proteins. It has been suggested that the specificity for these distinct functions may be located in the central "hinge" region, which shows the lowest sequence homology of all S100 proteins (Kligman & Hilt, 1988; Lackmann et al., 1993).

S100 proteins were considered to have a low molecular weight (9000–12000). However, most recently large molecular weight proteins (profilaggrin and trichohyalin) have been shown to contain a domain corresponding to a complete S100 protein. These proteins are involved in the terminal differentiation of keratinocytes (Lee et al., 1993; Markova et al., 1993; Presland et al., 1992). Interestingly, their genes are located together with other cornified envelope proteins (Hohl & Roop, 1993) in the chromosomal region 1q21, the same region in which six other S100 genes form a tight gene cluster (Engelkamp et al., 1993). Since the S100 proteins are expressed in a tissue- and cell-specific manner, they must underly an individual transcriptional control. Various functions have been postulated for the members of the S100 protein family: they appear to be involved in cell cycle regulation, differentiation, growth, and metabolic control [for reviews, see Van Eldik and Zimmer (1988), Donato (1991), and Hilt and Kligman (1991)].

Members of the S100 protein family interact with annexins (Filipek et al., 1991; Kube et al., 1992; Mizutani et al., 1992; Bianchi et al., 1992), glyceraldehyde-2-phosphate dehydrogenase (Zeng et al., 1993), membrane structures (Lemarchand et al., 1992), glial fibrillary proteins (Bianchi et al., 1993), glycogen phosphorylase (Zimmer & Dubuisson, 1993), and proteins regulating contraction in smooth muscles (Mani et al., 1992). Cell transfection experiments suggest that S100 proteins can influence structural features of the cytoskeleton (Selinfreund, 1990; Masiakowski & Shooter, 1990; Lakshmi et al., 1993; Davies et al., 1993). Some S100 proteins have been shown to be secreted from cells (Lackmann et al., 1992; Watanabe et al., 1992b) and to act extracellularly on nerve growth (Van Eldik et al., 1991), secretion (Thordarson et al., 1991), and the regulation of calcium fluxes (Barger & Van Eldik, 1992).

Our interest is focused on the function of S100 proteins in tumor progression. In order to begin to understand the molecular mechanisms by which S100 proteins exert their function, we wanted to determine the biochemical and physicochemical properties of human CAPL and CACY, two S100 proteins found in metastatic cells.

CAPL was originally isolated as an mRNA preferentially expressed in metastatic rather than nonmetastatic rat mammary myoepithelial cell lines (Barraclough et al., 1987;

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[‡] As several different names have been given to S100 proteins, we used the nomenclature of the committee of the Tenth International Workshop on Human Gene Mapping (Bruns & Sherman, 1989), updated in Engelkamp et al. (1993). Synonyms for CACY are calcyclin, 2A9, PRA, 5B10, and SMCaBP-11. Synonyms for CAPL are p9ka, 42A, pEL98, mts1, metastasin, calvasculin, and 18A2. Synonyms for CAGB are CFAg, MRP14, p14, MA C387, 608Ag, L_1Ag, MIF, and NIF.

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Ebralidze et al., 1989), and transfection experiments showed that CAPL can induce the metastatic phenotype in mouse mammary tumor cells (Davies et al., 1993; Lakshmi et al., 1993). The protein is also expressed in human heart, skeletal muscle, lung, and thymus (Engelkamp et al., 1992) and has been suggested to be secreted by smooth muscle cells (Watanabe et al., 1992b). Recently, we demonstrated (Pedroccchi et al., 1994) that CAPL may play a role in the acquisition of metastatic potential of human mammary epithelial cells and found a significant correlation between enhanced expression of CAPL and the presence of the invasivity marker urokinase-type plasminogen activator (uPA[1]).

CACY is specifically expressed in dividing, but not in quiescent, human fibroblasts (Calabretta et al., 1985). It has been suggested that CACY is also expressed in human metastatic cells (Weterman et al., 1992) and at sites of exocytosis (Timmons et al., 1993). The human tissues containing high levels of CACY are heart muscle, kidney, lung, thymus, and brain (Engelkamp et al., 1993), and epithelial cells, fibroblasts (Kuznicki, 1992), and neuronal cells (Filipek et al., 1993) also contain high levels of CACY. CACY has been shown to interact with annexins (Filipek et al., 1991), including CAP-50, a novel member of this protein family (Tokumitsu et al., 1992, 1993).

In this study, we describe for the first time the purification and characterization of recombinant human CAPL and CACY produced in *Escherichia coli*. We show that their physicochemical characteristics correspond to their native counterparts and that they both form homodimers *in vitro*, which might be the biologically active form. We characterized in detail the Ca^{2+}-binding properties of the recombinant proteins under physiological conditions. We also monitored, by difference spectrophotometry and fluorimetry, the Ca^{2+}-dependent changes in the environment of the Tyr residues.

MATERIALS AND METHODS

Materials

A Protein Fusion and Expression System was obtained from Promega. DTT and EGTA were from Fluka, and ammonium sulfate was from Merck. Phenylsepharose CL-4B was purchased from Pharmacia. IPTG and the restriction endonucleases were from Boehringer. Concentrated T4 DNA ligase was obtained from New England Biolabs, and BS[3] was from Pierce.

Oligonucleotides were synthesized on a Gene Assembler DNA synthesizer (Pharmacia LKB). The primers used to amplify the cDNA for cloning into pGEMEX were as follows:

CAPL-1: 5′-AATCATATGGCGTGCCCTCTGGAG-3′

CAPL-2:
5′-ACTCCTCTGATGTGGTTGGGATCCTAC-3′

CACY-1: 5′-TCACATATGGCATGCCCCCTGGAT-3′

CACY-2:
5′-GGGAAGATGGAGACACCCGGATCCTAC-3′

Underlined are the inserted restriction sites for *Nde*I and *Bam*HI. The three additional nucleotides allowed complete restriction of the PCR products.

Methods

Purification of CAPL and CACY from Human Heart. Heart tissue was minced, homogenized in extraction buffer (20 mM Tris (pH 7.5), 1 mM EDTA, 10 μg/mL trypsin inhibitor, 0.1 mM PMSF, and 5 mM βME), and centrifuged (1 h, 8000*g*). The supernatant was processed exactly as the bacterial cleared lysate (see below).

Cloning of Human CAPL and CACY into a Prokaryotic Expression System. To obtain the expression vectors pGEMEX-CACY and pGEMEX-CAPL, we amplified the coding regions of full-length CACY and CAPL clones (Engelkamp et al., 1992), using specific oligonucleotides containing either an *Nde*I site (N-terminal oligos) or a *Bam*HI site (C-terminal oligos). The N-terminal *Nde*I site was introduced using the start codon of the insert. The amplified DNA was cut with *Nde*I/*Bam*HI and introduced into an *Nde*I/*Bam*HI digested expression vector, pGEMEX (Promega), eliminating the T7 gene 10 insert. Plasmids from colonies of transformed *E. coli* TG-1 strain expressing CAPL or CACY were sequenced by the dideoxynucleotide chain termination method to check for correct open reading frames. The plasmids were then transferred to the *E. coli* BL-21 Lys S strain for bacterial expression.

Expression and Purification of Recombinant Human CAPL and CACY. LB medium (250 mL) containing 0.2% glucose and 100 mg/L ampicillin was inoculated with a 5-mL overnight culture of an *E. coli* BL-21 Lys S clone expressing the human CACY or CAPL protein. At an OD_{600} of 0.5, the expression of the recombinant proteins was induced with 0.5 mM IPTG. The cultures were further incubated for 3.5 h. Cells were then harvested by centrifugation at 4000*g* and resuspended in 6 mL of lysis buffer (20 mM Tris-HCl (pH 7.4), 300 mM KCl, 10% glycerin, 1 mM EDTA, 1 mM PMSF, 5 μg/mL leupeptin, and 2 mM DTT). Cell lysis was performed by freezing and thawing twice and sonicating for 1 min on ice. All subsequent procedures were performed at 4 °C. The suspension was ultracentrifuged at 150000*g* for 50 min. The cleared lysate was precipitated by slowly adding ammonium sulfate to 30% and centrifuging at 3000*g* for 15 min. The supernatant was brought to 2 mM $CaCl_2$, applied to a phenylsepharose affinity column (0.8 × 4 cm; flow, 400 μL/min), chromatographed, washed, and eluted with 20 mM Tris-HCl (pH 7.5) and 5 mM EGTA. The fractions containing CAPL or CACY were adjusted to 5 mM $CaCl_2$ and stored at −20 °C.

Mass Spectrometry. For analysis by electrospray ionization mass spectrometry, 10 μg CAPL or CACY was desalted by reverse-phase HPLC using a C2-RF precolumn, dialyzed, evaporated to a small volume, and diluted with 50% acetonitrile and 1% formic acid. Electrospray ionization mass spectrometry spectra were obtained with a Sciex Api III equipped with an ion spray source, as described.

Calcium Blots. Either recombinant human CACY or CAPL or purified rat parvalbumin (3 μg) as a positive control was run on 15% SDS–PAGE using the Tricine system (Schägger & Jagow, 1987; Föhr et al., 1993), blotted onto nitrocellulose, and incubated with $^{45}Ca^{2+}$ in 60 mM KCl, 5 mM $MgCl_2$, and 10 mM imidazole (pH 6.8) for 10 min at room temperature. The overlay was performed using 0.1 mCi of $^{45}Ca^{2+}$, corresponding to a Ca^{2+} concentration of 88 μM. Unbound $^{45}Ca^{2+}$ was washed away, and the bound radioactivity was visualized by exposing an X-ray film (48 h at −80 °C).

[1] Abbreviations: βME, β-mercaptoethanol; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; BS, bis(sulfosuccinimidyl) suberate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; uPA, urokinase-type plasminogen activator.

Isoelectric Focusing. This was performed under nondenaturing conditions using the Rotofor cell system by Bio-Rad, according to the manufacturer's manual.

Protein Dimerization Studies. (a) In the absence of a cross-linker: 2.5 μg of the mixture of both proteins was incubated either in 0.1% Triton X-100, 10 mM EGTA, and 5 mM calcium or in 100 mM DTT, followed by analysis of the protein monomers and dimers on a nonreducing SDS–polyacrylamide (15%) gel. (b) In the presence of a cross-linker: 5 μg of either CAPL or CACY or 2.5 μg of each protein was preincubated with either 5 mM calcium or 100 mM DTT for 6 h at 4 °C. The solutions were subsequently incubated with 0.1 mM bifunctional cross-linker BS^3, a water-soluble disuccinimidyl suberate derivate. After 1 h of incubation at room temperature, the reaction was stopped by adding 10 mM ethanolamine, 3 mM *N*-ethylmaleimide, and 10 mM sodium phosphate. The proteins were precipitated as described (Wessel, 1984), and the pellet was resuspended directly in gel loading buffer. The products were separated by 15% SDS–PAGE using the Tricine system described (Föhr et al., 1993). After blotting, the nitrocellulose blot was incubated with a polyclonal antiserum against either human recombinant CACY or CAPL. The specificity of these antisera will be described elsewhere (Pedrocchi et al., manuscript in preparation). The sera were diluted 1:500 and visualized by a secondary goat anti-rabbit antibody coupled to horseradish peroxidase and diluted 1:2000.

Direct Ca^{2+}- and Mg^{2+}-Binding Studies. For the removal of contaminating metal ions and for complete equilibration of the proteins in the assay buffer, CACY and CAPL were precipitated with 3% trichloroacetic acid (TCA) and then passed through a 1 × 40 cm Sephadex G-25 column equilibrated in the assay buffer. The TCA-treated proteins showed the same electrophoretic mobility and Ca^{2+}-binding properties as the EDTA-treated protein (I. Durussel and J. A. Cox, unpublished results). The buffers were freed of contaminating metals by passage over a column of EDTA agarose (Haner et al., 1984). Ca^{2+} contamination was always less than 0.1 mol/mol of protein. Total Ca^{2+} concentrations were determined with a Perkin-Elmer Cetus Instruments 2380 atomic absorption spectrophotometer. For the atomic absorption measurements, up to 1 mM EDTA was added to all solutions, including the standards, in order to normalize the quenching effects. The protein concentration was determined from the UV absorption spectrum using molar extinction coefficients at 278 nm of 4600 and 7200 M^{-1} cm^{-1} for metal-free CACY and CAPL, respectively. These values were measured on protein stock solutions in bidistilled water, whose concentrations were determined by dry weight. Protein concentration was also determined by quantitative amino acid analyis. This was carried out by gas-phase HCl hydrolysis, conversion with dansyl chloride, and quantitation of the derivatized products with a Beckman System Gold HPLC instrument.

Ca^{2+} binding was measured at 25 °C by the flow dialysis method of Colowick and Womack (1968) in 50 mM Tris buffer (pH 7.5), 150 mM KCl, and 7.5 mM mercaptoethanol (buffer A). The protein concentrations were 100–120 μM. Treatment of the raw data was as described by Cox et al. (1990). The binding data were analyzed by means of the Adair (1925) equation for two binding sites, and the stoichiometric binding constants (K) were calculated with the curve-fitting procedure of SigmaPlot. The intrinsic association constants (K') were obtained from the stoichiometric constants by applying the statistical factors of $^1/_2$ and 2 for the respective binding steps (Cornish-Bowden & Koshland, 1975).

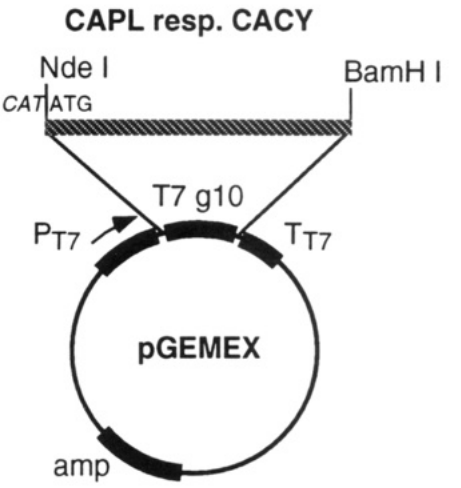

FIGURE 1: Bacterial expression plasmid for CACY and CAPL. Human cDNAs for CACY and CAPL were obtained by PCR techniques and cloned *in frame* into the *Nde*I site after the promoter region, eliminating the T7 gene 10: hatched region, coding region of the human CAPL or CACY cDNA; ATG, translational start site; amp, resistance gene to ampicillin; T7 g10, gene for the production of fusion proteins, excised for this work; and P_{T7} and T_{T7} stand for the T7 promoter and terminator, respectively.

Optical Methods To Probe the Tyr Environment. Emission fluorescence spectra were taken with a Perkin-Elmer LS-5B spectrofluorimeter interfaced with a computer. The measurements were carried out on 30 μM TCA-treated, metal-free CACY and CAPL at room temperature, with excitation and emission slits of 5 nm. EGTA (50 μM), 5 mM $MgCl_2$, 2 mM $CaCl_2$, or 10 mM Zn^{2+} was added subsequently to obtain the metal-free Mg^{2+}, Ca^{2+}, or Zn^{2+} forms, respectively.

UV absorption spectra and difference spectra were measured with a Perkin-Elmer Cetus Instruments lambda 5 UV/vis spectrophotometer interfaced with a computer. The metal-free protein was dissolved in buffer A. Difference spectra were taken at room temperature on solutions with an optical density at 280 nm of 1. Ca^{2+} titrations were performed with 333 μM CACY and 380 μM CAPL in buffer A. In order to monitor changes in both the Tyr and Phe environments, spectra were taken after each Ca^{2+} increment.

RESULTS AND DISCUSSION

Purification of Recombinant Human S100 Proteins CACY and CAPL. We introduced the human cDNA's of CACY and CAPL into the prokaryotic expression vector pGEMEX (Figure 1). The cDNA was inserted in-frame without a fusion peptide to obtain the correctly folded and active products. Correct cloning and the full-length sequence were tested by sequencing the inserted cDNA, including the insertion sites. The vector was transformed into BL-21 Lys S cells, an *E. coli* stem protected from the potential toxicity of leaking recombinant protein by low levels of lysozyme production.

Extracts of bacterial cultures transformed with pGEMEX-CACY, pGEMEX-CAPL, or pGEMEX without any insert and induced with IPTG were subjected to SDS–PAGE (Figure 2). Bands can be seen at 28 kDa for the control vector (T7 gene 10 leader peptide, lane 2), 6.2 kDa for CACY (lane 4), and 8.2 kDa for CAPL (lane 7). These values are lower than the molecular masses calculated from sequence data and represent only apparent masses.

S100 proteins are known to be soluble even at 100% ammonium sulfate. However, we observed that at 50% ammonium sulfate a large portion of the recombinant proteins had already precipitated, probably due to their high concen-

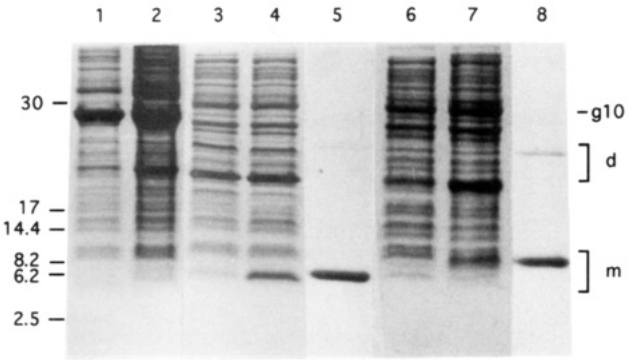

FIGURE 2: SDS–PAGE (15%) showing induction and purification of recombinant CACY and CAPL. *E. coli* BL-21 Lys S were transformed with the prokaryotic expression vectors carrying the cDNA inserts for human CACY and CAPL and were induced to produce the recombinant proteins by IPTG. Bacterial pellets corresponding to 100 μl of culture (ca. 100 μg of protein) were suspended in loading buffer and subjected to SDS–PAGE. Lanes 1 and 2, pGEMEX expressing the T7 gene 10; lanes 3 and 4, pGEMEX-CACY; and lanes 6 and 7, pGEMEX-CAPL. Lanes 1, 3, and 6 are noninduced cultures, 2, 4, and 7 are cultures induced with 0.5 mM IPTG for 3.5 h. Lane 5 contained 5 μg of recombinant CACY, and lane 8 5 μg of recombinant CAPL after purification. m, monomeric; d, dimeric; g10, induced T7 gene 10.

trations in the solute. We therefore precipitated the soluble fraction of the bacterial extracts with only 30% ammonium sulfate. The supernatant was loaded directly onto a phenylsepharose column in the presence of 2 mM $CaCl_2$, and the recombinant proteins were eluted with 5 mM EGTA. Lanes 5 and 8 in Figure 2 show that the recombinant proteins represent more than 95% of the eluting proteins. In addition to the monomeric bands, faint bands are visible that migrate at about 20 kDa, corresponding to the respective dimers of CACY and CAPL (see below). We obtained several milligrams of purified protein per liter of bacterial culture. Interestingly, for all of the subclones tested, the yields of CAPL (10–15 mg/L) were consistently lower than those of CACY (20–30 mg/L), although both the vector and the insertion site were identical. The production of large amounts of CAPL seems to affect either the growth properties or the translation of *E. coli* differently from the production of large amounts of CACY. During purification, the recombinant proteins behaved identically to the native proteins, which were purified by the same method from human heart.

Biochemical Properties of Human CAPL and CACY. As mentioned above, the apparent molecular masses of both recombinants on SDS–PAGE were notably lower than the real masses deduced from their cDNAs. The same result was also obtained with the native proteins purified from human heart (not shown). To ascertain that this divergence derives from the behavior of the recombinant and native proteins in our gel system, and not from any errors in cloning or protein synthesis, we determined their exact molecular masses by electrospray ionization mass spectrometry (ESI-MS). For comparison of our data, it must be taken into account that the N-terminal methionine is usually processed in bacterial systems (−131 mass units), and no acetylation of the terminal amino acid takes place. The measured value for recombinant CACY was found to be 10 053 Da (calculated and corrected value from the sequence: 10 050 Da), and that for recombinant CAPL, 11 594 Da (calculated value: 11 599 Da). The masses we obtained for the native human heart proteins were 10 089 Da for CACY and 11 646 Da for CAPL. These results suggest that the only difference between the recombinant and native proteins is the lack of N-terminal acetylation, and thus the

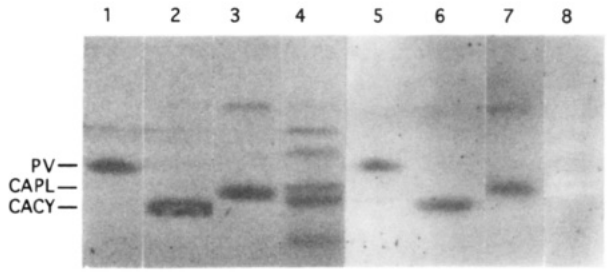

FIGURE 3: $^{45}Ca^{2+}$ overlay blot of recombinant proteins. CACY, CAPL, and rat parvalbumin (control) were separated on 15% SDS–PAGE, blotted onto nitrocellulose, and incubated with $^{45}Ca^{2+}$. Lanes 1–4, ponceau red staining; lanes 5–8, $^{45}Ca^{2+}$ overlay. The lanes were loaded with rat parvalbumin (1 and 5), recombinant human CACY (2 and 6), recombinant human CAPL (3 and 7), and protein molecular weight markers (Bio-Rad SDS-17) (4 and 8). PV, rat parvalbumin. 3 μg of protein was applied on each lane.

recombinant proteins are likely to behave identically to the native ones. Interestingly, no other posttranslational modifications were observed for native CAPL and CACY, showing that they are processed differently from the S100 protein CAGB (also named p14), which has been shown to become phosphorylated depending on intracellular calcium concentrations (Edgeworth et al., 1991). We conclude that our recombinant CACY and CAPL represent the full-length proteins and are identical to the native proteins, since their mass values correspond to the calculated molecular masses.

We assessed the isoelectric point by native isoelectric focusing in a 4–10% gradient gel. For recombinant CACY the value was 5.25 and for CAPL 6.15, in agreement with the calculated values of 5.25 and 5.97, respectively. Interestingly, in our gel system we had to use 10 times more of the recombinant CAPL than of CACY for detection by Coomassie blue staining.

The fact that both recombinant proteins could be purified by calcium-dependent affinity chromatography suggests that they have retained their Ca^{2+}-binding properties. This was then verified by subjecting both proteins to SDS–PAGE, followed by $^{45}Ca^{2+}$ overlay. The autoradiography in Figure 3 shows that both recombinant proteins (lanes 6 and 7) can bind radioactive Ca^{2+} in the same way as purified rat parvalbumin used as a control (lane 5).

Dimer Formation of CAPL and CACY in Vitro. S100 proteins are known to form dimers (Selinfreund et al., 1991; Linse et al., 1993), and the dimeric form is assumed to represent the active form of these proteins (Edgeworth et al., 1991; Barger et al., 1992). We therefore studied the ability of our recombinant proteins to form dimers *in vitro* under various conditions and visualized the complexes on a nonreducing polyacrylamide gel (Figure 4).

Under the conditions applied, CAPL and CACY form dimers but no higher oligomers (lanes 3–8; a double band is often seen for CAPL). The dimer is probably stabilized by cysteine bridges, since high concentrations of the reducing agent dissociate these dimers (lanes 1 and 2). Interestingly, the presence of neither Triton X-100 (lanes 3 and 6) nor EGTA (lanes 4 and 7) influences dimer formation and stability. CAPL dimers are more stable than CACY dimers, resisting harsh treatment with DTT much longer (not shown). The ability to form homodimers confirms the observations made for native mouse CACY (Kuznicki, 1989) and bovine CAPL (Watanabe et al., 1992).

Some members of the S100 protein family, namely, S100α and S100β (Isobe et al., 1977), or CAGA and CAGB (Teigelkamp et al., 1991) form *hetero*dimers. This suggests

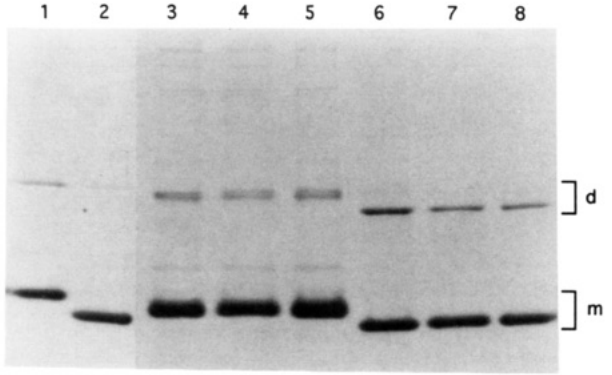

FIGURE 4: SDS–PAGE (15%) of recombinant CACY and CAPL under nonreducing conditions. Recombinant CACY (2 and 6–8) or CAPL (1 and 3–5) was incubated with 100 mM DTT (1 and 2), 10 mM Triton X-100 (3 and 6), 10 mM EGTA (4 and 7), or 5 mM calcium (5 and 8) and applied to a 15% nonreducing polyacrylamide gel. m, monomeric; d, dimeric. 5 μg of protein was applied on each lane.

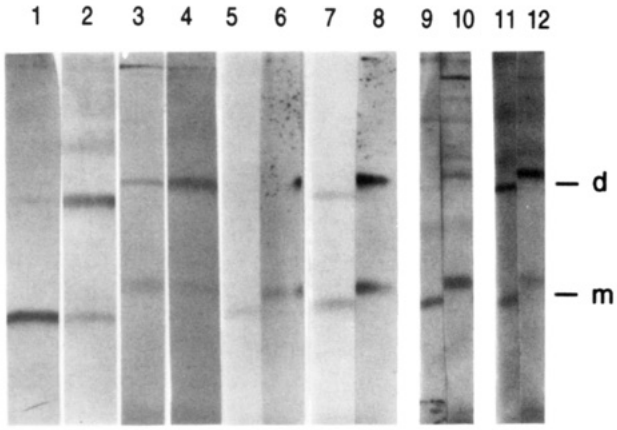

FIGURE 5: Cross-linking of human CACY and CAPL. 3 μg of recombinant CACY (1 and 2) or recombinant CAPL (3 and 4), 1.5 μg of both proteins (5–8), or 7 μg of native S100 proteins from human heart (9–12) was incubated with 0.1 mM cross-linker BS^3 in phosphate-buffered saline (pH 7.4) in the presence of either 100 mM DTT (1, 3, 5, 6, 9, and 10) or 5 mM calcium (2, 4, 7, 8, 11, and 12) and separated on 15% SDS–PAGE under reducing conditions. The proteins were visualized with polyclonal antisera against human recombinant CACY (1, 2, 5, 7, 9, and 11) or CAPL (3, 4, 6, 8, 10, and 12). m, monomeric; d, dimeric.

that CAPL and CACY also might form heterodimers. We tested this possibility by *in vitro* cross-linking experiments and incubated equivalent amounts of CACY and CAPL, separately or combined, in the presence of either DTT or Ca^{2+}. The suspensions were treated with the bifunctional cross-linker BS^3, a disuccinimidyl suberate derivative, and the proteins were separated by SDS–PAGE and analyzed for dimer formation by Western blotting. Specific polyclonal antisera raised against each of the recombinant proteins were applied. Figure 5 shows that both CAPL and CACY predominantly form homodimers, but no heterodimers. In the presence of Ca^{2+}, the single proteins cross-linked to homodimers (lanes 2 and 4). Even in the presence of DTT, faint dimeric protein bands are visible (lanes 1 and 3) when visualized with antibodies. When CAPL and CACY were mixed before incubation with the cross-linker, monomeric and dimeric bands were again detected (lanes 5–8). However, the homodimers for CAPL and CACY migrate differently, and the two antisera do not recognize any common band in addition to the respective homodimer. The same results were obtained when the native proteins purified from human heart (lanes 9–12) were used for the cross-linking. We conclude

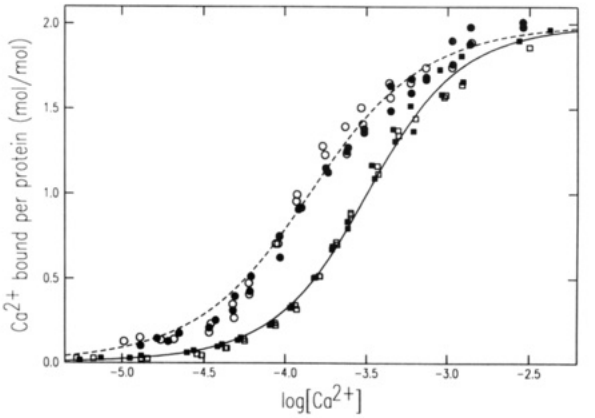

FIGURE 6: Ca^{2+} binding to CACY (■, □) and CAPL (●, ○) in the absence (□, ○) and presence of 1 mM Mg^{2+} (■, ●). Ca^{2+} binding was monitored by the flow dialysis method at 25 °C. The lines represent the isotherms calculated with the Adair equation using the following stoichiometric constants: for CACY, $K_1 = 2.2 \times 10^3$ M^{-1}, $K_2 = 4.4 \times 10^3$ M^{-1}; for CAPL, $K_1 = 1.0 \times 10^4$ M^{-1}, $K_2 = 4.5 \times 10^3$ M^{-1}.

that, in contrast to some other S100 proteins, only homodimers are formed *in vitro* in spite of the high structural similarity of CAPL and CACY.

Direct Ca^{2+}-Binding Studies. The Ca^{2+}-binding isotherms of both CACY and CAPL at 25 °C and in physiological KCl concentrations are presented in Figure 6. Both proteins show the maximal binding of 2 Ca^{2+} per mole of protein and display half-maximal saturation at 0.32 and 0.15 mM free Ca^{2+}, respectively. The isotherms are not noticeably influenced by 1 mM Mg^{2+}, indicating that the two proteins contain exclusively Ca^{2+}-specific sites and are of the low-affinity type. Since the steepness of both isotherms indicates positive cooperativity, they were analyzed according to the Adair equation for two binding sites. The intrinsic constants, K'_{Ca}, calculated from the stoichiometric constants, as described by Cornish-Bowden and Koshland (1975), amount to 1.1×10^3 and 8.8×10^3 M^{-1} for CACY and 5.0×10^3 and 9.0×10^3 M^{-1} for CAPL, corresponding to Hill coefficients of 1.47 and 1.16, respectively. The positive cooperativity observed may be partly due to intermolecular associations since the turbidity of the protein solution increases upon titration with Ca^{2+}, especially for CAPL.

For SMCaBP-11 (probably chicken CACY), Mani and Kay (1990) reported that this 10-kDa protein forms homodimers, binds 2 mol of Ca^{2+} per mole of monomer with pronounced cooperativity, and shows characteristic Ca^{2+}-dependent changes in its Tyr environment. The reported higher affinity of SMCaBP-11 for Ca^{2+} likely is due to the low ionic strength buffer. Similar findings were reported for bovine calvasculin (probably the homologue of human CAPL) (Watanabe et al., 1992a).

Conformational Changes in the Tyr Microenvironment. Human CACY and CAPL contain two well-conserved Tyr residues in the A and D helices [for code, see Strynadka and James (1989)], whereas CACY contains an additional Tyr in the C-terminal segment at position 84. Figure 7 shows the fluorescence spectra of the metal-free form and of the Ca^{2+}-, Mg^{2+}-, and Zn^{2+}-loaded CACY (a) and CAPL (b) after excitation at 280 nm. For CACY, maximal emission occurs at 308 nm and the fluorescence is sensitive to Ca^{2+} and Zn^{2+}, but not to Mg^{2+}, in line with the absence of a Mg^{2+} effect on Ca^{2+} binding. The Zn^{2+}-dependent fluorescence increase of mouse CACY, also observed by Filipek et al. (1990), is half-maximal at 2 mM free Zn^{2+}, indicating a low affinity. The

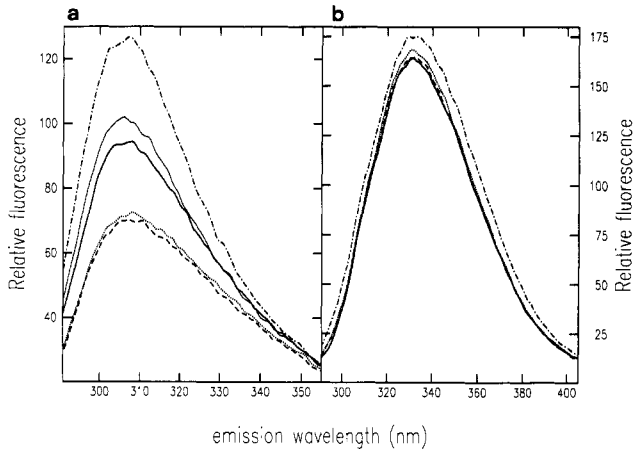

FIGURE 7: Tyrosine fluorescence spectra of CACY (a) and CAPL (b) after excitation at 280 nm. Emission spectra in the absence of metals (···) and in the presence of 5 mM $MgCl_2$ (- - -), 2 mM $CaCl_2$ (—), 10 mM $ZnCl_2$ (– · –), or both 2 mM $CaCl_2$ and 10 mM $ZnCl_2$ (—). The protein concentration was 30 μM. The spectra were corrected for the buffer contribution.

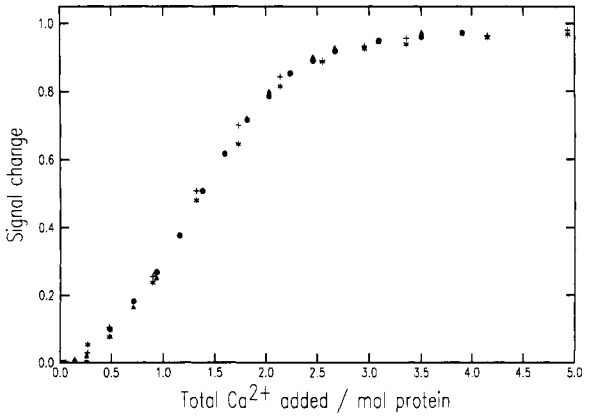

FIGURE 8: Conformational titrations of 333 μM CACY (●, ▲) and 190 μM CAPL (+, *) with Ca^{2+}. The Tyr conformational change was followed by monitoring the different spectrophotometric signals, D_{signal} 288 minus 285 nm (*, ▲) and 281 minus 285 nm (●, +). All of the signal changes, which are 3 times higher for CACY than for CAPL (for protein solutions with an optical density of 1.0 at 278 nm), were normalized to a maximal change of 1.

spectrum of the Ca^{2+}+Zn^{2+}-loaded human CACY is slightly, but significantly, different from that of the Ca^{2+} or Zn^{2+} form, suggesting that these cations bind to different sites. CAPL displays an unusual emission spectrum with a maximum of 332 nm; this spectrum is insensitive to Ca^{2+} and Mg^{2+}, but is slightly influenced by Zn^{2+}. Preliminary spectrophotometric and fluorimetric data (not shown) suggest that some of the Tyr residues in CAPL could be ionized upon excitation at neutral pH (Lakowicz, 1983), which would explain the atypical spectrum in Figure 7b.

We measured the UV difference spectra of CACY and CAPL induced by increasing concentrations of Ca^{2+} (not shown). In CACY, important perturbations can be observed in the microenvironment of Tyr that are similar to those observed when a tyrosine methyl ester is transferred from an aqueous to a nonpolar solution (Bailey et al., 1968). The absence of discrete peaks in the 250–270-nm region indicates that the environment of the Phe residues is nearly not affected by the binding of Ca^{2+}. Unusual behavior in the difference spectra is the continuous rise in the base line upon the addition of Ca^{2+}; this can be explained by Ca^{2+}-dependent aggregation of the protein, so that the solution becomes turbid. The Ca^{2+} dependence of two difference spectral changes, i.e., the D_{signal} 288–285 and 281–285 nm, are shown in Figure 8. The discrete signal changes of the Tyr peaks level off at a molar ratio of 2 Ca^{2+}/CACY but they show a sigmoidal Ca^{2+} dependence, which confirms that allosteric conformational changes occur in the two proteins. Given the phenomenon of protein aggregation, a more detailed interpretation was not attempted. In the case of CAPL, the difference spectra induced upon the binding of Ca^{2+} showed that the Tyr-related peaks are 3 times weaker than those in the case of CACY (not shown); moreover, the turbidity increase is about 10-fold higher than that for CACY. The Ca^{2+} dependence of the signal changes, normalized to a maximal change of 1, is very similar to those of CACY, thus confirming that these proteins belong to the same family, even if their functions may be different.

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