Expression of Bovine Intestinal Calcium Binding Protein from a Synthetic Gene in *Escherichia coli* and Characterization of the Product†

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ABSTRACT: Intestinal calcium binding proteins (ICaBP's) constitute a group of small vitamin D inducible proteins considered to play an important role in the absorption of dietary calcium. The mammalian ICaBP's are representatives of the "EF-hand" family of calcium binding proteins. As a first step in the application of protein engineering techniques to the study of structure–function relationships in mammalian ICaBP's, we have synthesized a gene encoding the minor A form (the native form lacking the two N-terminal amino acids) of bovine ICaBP employing a rapid, microscale gene synthesis technique based on "shotgun ligation" of sets of oligonucleotides. Expression of the synthetic gene from a plasmid containing the tac promoter in a *lon* protease deficient strain of *Escherichia coli* yielded the desired product at a level of about 1–2 wt % of total protein. During the purification of the ICaBP expressed in *E. coli*, a contaminant was strongly adhering to it but was efficiently removed by gel filtration after denaturation with urea. The minor A form of ICaBP produced in *E. coli* was characterized by its mobility during sodium dodecyl sulfate–polyacrylamide gel electrophoresis and by its total amino acid composition, partial amino acid sequence, UV absorption spectrum, and 360-MHz ^{1}H NMR spectrum, showing beyond reasonable doubt its identity with the minor A form of ICaBP obtained from bovine intestines.

The molecular events behind the absorption of dietary calcium in mammals and other animals are largely unknown. The intestinal calcium binding proteins (ICaBP's)[1] are a group of proteins considered to play an important role in this process [for reviews, see Wasserman et al. (1978) and Wasserman & Fullmer (1982)]. The synthesis of ICaBP's is inducible with the hormonal form of vitamin D: 1,25-dihydroxyvitamin D_3 (Wasserman et al., 1978; Lawson, 1978). ICaBP's with molecular weights around 9000 have been found in mammalian intestines while the ICaBP's in birds have a higher molecular weight (around 28 000). The ICaBP's belong to a larger family of calcium binding proteins, including among others parvalbumin, troponin C, and calmodulin all having the same helix–loop–helix structure of their calcium binding sites, the so-called "EF-hand" structure (Kretsinger, 1972; Kretsinger & Nockolds, 1973; Herzberg & James, 1985; Babu et al., 1985). Whereas skeletal muscle troponin C and calmodulin have four such calcium binding sites, parvalbumin and mammalian ICaBP's have only two calcium binding sites. In mammalian ICaBP's, one of the sites has a structure that deviates from that of the archetypical EF hand and has been termed the "pseudo-EF-hand" site (Szebenyi et al., 1981; Szebenyi & Moffat, 1983). The mammalian ICaBP's bind calcium with different affinity to their two types of sites (Bryant & Andrews, 1984).

The amino acid sequence has been determined for the porcine and bovine forms of the ICaBP (Hofmann et al., 1979; Fullmer & Wasserman, 1981; Desplan et al., 1983), and cDNA clones encoding a part of the rat ICaBP and the complete chicken ICaBP have been isolated and sequenced (Desplan et al., 1983; Wilson et al., 1985). The three-dimensional crystal structure has been established to high resolution for the minor A form of the bovine ICaBP, i.e., the form lacking the two N-terminal amino acids of the native protein (Szebenyi et al., 1981; Szebenyi & Moffat, 1983). Also, ^{1}H NMR studies have been performed on the minor A form of the bovine protein (Dalgarno et al., 1983) as well as on the native porcine protein (Shelling et al., 1983).

The bovine ICaBP constitutes a very suitable system for biophysical studies of structure–function relationships in the EF-hand family of calcium binding proteins because it is the smallest EF-hand protein with an established three-dimensional structure. The small size is particularly attractive in connection with two-dimensional high-resolution ^{1}H NMR studies (Wüthrich et al., 1982). In addition, no posttranslational modifications of the minor A form of bovine ICaBP take place. The gene for bovine ICaBP has, however, not been cloned. We have therefore, as a basis for future protein engineering applications, utilized a microscale gene synthesis technique employing "shotgun ligation" of sets of oligonucleotides (Grundström et al., 1985) to assemble a synthetic gene encoding the known amino acid sequence of the minor A form of bovine ICaBP. The artificial gene was efficiently expressed

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[1] Abbreviations: ICaBP, intestinal calcium binding protein; IPTG, isopropyl β-D-thiogalactopyranoside; SDS, sodium dodecyl sulfate; kb, kilobase(s); EDTA, ethylenediaminetetraacetic acid.

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in *Escherichia coli*, and the structure of the protein produced was demonstrated to be identical with that of the same protein obtained from bovine intestines.

Materials and Methods

Bacterial Strains and Plasmids. M13 phages were propagated on *E. coli* strain JM103 (Messing et al., 1981). The plasmids pTAC12, used for the construction of the expression vector, and pMMB24, used as source of the *lacIQ* gene, have been described previously (Amann et al., 1983; Bagdasarian et al., 1983). *E. coli* strains W3110 (*lon*+, from Bachman, *E. coli* Genetic Stock Center, Yale University) and SG20043 (*lon*−; Trisler & Gottesman, 1984) were used in the plasmid expression experiments.

Synthesis and Ligation of Oligonucleotides. Oligonucleotides were synthesized by a rapid microscale method utilizing the phosphotriester method with cellulose disks as solid supports (Matthes et al., 1984). The oligonucleotides were purified on denaturing polyacrylamide gels and phosphorylated according to Grundström et al. (1985). Oligonucleotides were cloned into linearized M13 vectors with the "shotgun ligation" technique as previously described (Grundström et al., 1985) except that only 0.25 pmol of each phosphorylated oligonucleotide was pooled and lyophilized before ligation.

DNA Techniques. Transformation of *E. coli* was performed as described by Hanahan (1983). Preparation of single-stranded M13 DNA and dideoxy sequencing were according to Sanger et al. (1977, 1980) as modified with ^{35}S labeling and salt gradient gels by Biggin et al. (1983). Digestions with restriction endonucleases were according to the suggestions of the manufacturers, and standard cloning techniques were used throughout.

SDS–Polyacrylamide Gel Electrophoresis of Protein Extracts. Cells were grown at 37 °C in LB media (Bertani, 1951). IPTG was added to a final concentration of 0.5 mM at an A_{600} of 0.5, and after 2 h of growth, the cells were harvested by centrifugation and boiled for 5 min in a buffer containing SDS and urea (Lugtenberg et al., 1975). The proteins were separated on 10–20% acrylamide–bis(acrylamide) (30:0.8) gradient gels essentially as described by Laemmli (1970).

Purification of Bovine ICaBP. The protein was purified as previously described by Hitchman et al. (1973). The minor A form was obtained by trypsin treatment as described by Fullmer et al. (1975).

Purification of ICaBP from E. coli. A 4.5-L sample of strain SG20043 containing the plasmid expressing the ICaBP, pICB1, was induced with IPTG and harvested as described above. The cells were sonicated, and the extract was fractionated on a DEAE-Sephedex A-25 column essentially as described by Hitchman et al. (1973) for the purification of porcine ICaBP. The fractions containing ICaBP (detected by SDS–polyacrylamide gel electrophoresis) were pooled and lyophilized. The material was resuspended in 10 mL of either 0.1 M NH_4HCO_3, pH 8.0, or 8 M urea (see below) and loaded on a 3 × 200 cm Sephadex G-50 Superfine (Pharmacia Fine Chemicals) column equilibrated with 0.1 M NH_4HCO_3, pH 8.0 (room temperature, flow rate 35 mL/h, fraction size 7 mL). Fractions with pure ICaBP were pooled and lyophilized.

CNBr Cleavage. Sixteen milligrams of ICaBP was dissolved in 5 mL of 70% HCOOH and flushed with N_2; 21 mg of CNBr was added, and the material was incubated at room temperature overnight. Water (50 mL) was added, and the solution was evaporated to dryness, dissolved in 1 mL of water, and fractionated on a 1.4 × 200 cm Sephadex G-50 Superfine column (Pharmacia Fine Chemicals) equilibrated with 0.1 M NH_4HCO_3, pH 8.0.

NMR Analysis. All proton NMR spectra were obtained at a radio frequency of 361.79 MHz on a Nicolet 360 WB NMR spectrometer. The instrumental settings used were the same as reported elsewhere (Thulin et al., 1984).

Results

Synthesis of a Gene Encoding Bovine Intestinal Calcium Binding Protein. The amino acid sequence of the minor A form of the bovine intestinal calcium binding protein (ICaBP) has been published (Fullmer & Wasserman, 1981). To produce ICaBP in *E. coli*, a gene encoding this protein was designed. The known amino acid sequence and the nucleotide sequence designed are displayed in Figure 1. Codons mainly found to be preferentially utilized in highly expressed *E. coli* genes (Grantham et al., 1980) were used, but a few moderately used *E. coli* codons were also utilized when restriction enzyme sites were to be introduced or avoided. The coding region was designed to start with an ATG initiation codon and end with two consecutive stop codons, TAA and TAG. A terminator of transcription with the DNA sequence of the *rrnC* terminator (Young, 1979) was introduced behind the coding sequence in order to obtain independence from the existence of terminators in different expression vectors. The gene was constructed from 34 overlapping oligonucleotides as shown in Figure 1.

The entire DNA sequence was analyzed for direct and inverted sequence repetitions by computer programs (Harr et al., 1986), and the overlaps were designed to minimize possibilities for alternative ligations. The oligonucleotides were assembled into five DNA segments in different M13 derivatives using the "shotgun ligation" technique of Grundström et al. (1985). The DNA segments constructed are those delimited by the restriction enzyme sites *Eco*RI, *Pst*I, the upstream *Sac*I, *Bgl*II, the downstream *Sac*I, and *Xba*I in Figure 1. The DNA segments were cloned into the corresponding restriction enzyme sites of the phages M13mp18, M13mp18, M13mp19, M13mp18, and M13tg130 (Norrander et al., 1983; Kieny et al., 1983), respectively. Progeny phages were isolated from all five ligations, and the nucleotide sequence of the DNA segments constructed was determined by using dideoxy sequencing. From two of the ligations, the first sequence screening identified phages with a difference relative to the sequence designed. The first difference, from C to T in the nucleotide at the 5' end of oligonucleotide 5 (Figure 1), only alters a TTC codon to a TTT codon, both being phenylalanine codons frequently used in *E. coli* genes, and therefore, no further screening for the designed sequence was performed. The second difference, from G to T in the position corresponding to the seventh nucleotide from the 5′ end of oligonucleotide 31 (Figure 1), represents a change in the *rrnC* terminator, but since preliminary tests showed that the constructed DNA segment functioned for our purpose (see below) further DNA sequence screening was not performed. The complete gene was constructed from the assembled DNA segments in two steps. First the *Eco*RI/*Pst*I DNA segment and the *Pst*I/upstream *Sac*I segment (Figure 1) were cloned between the *Eco*RI and *Sac*I restriction enzyme sites of the M13tg130 derivative carrying the downstream *Sac*I/*Xba*I segment. A phage with the wanted structure was isolated, and the two *Sac*I/*Bgl*II segments were cloned into the *Sac*I restriction enzyme site of this phage, completing the gene.

Construction of an Expression Vector for ICaBP. An expression plasmid based on pTAC12 (Amann et al., 1983) was chosen because this plasmid carries a strong inducible hybrid promoter, the tac promoter, constructed from the −35 part of

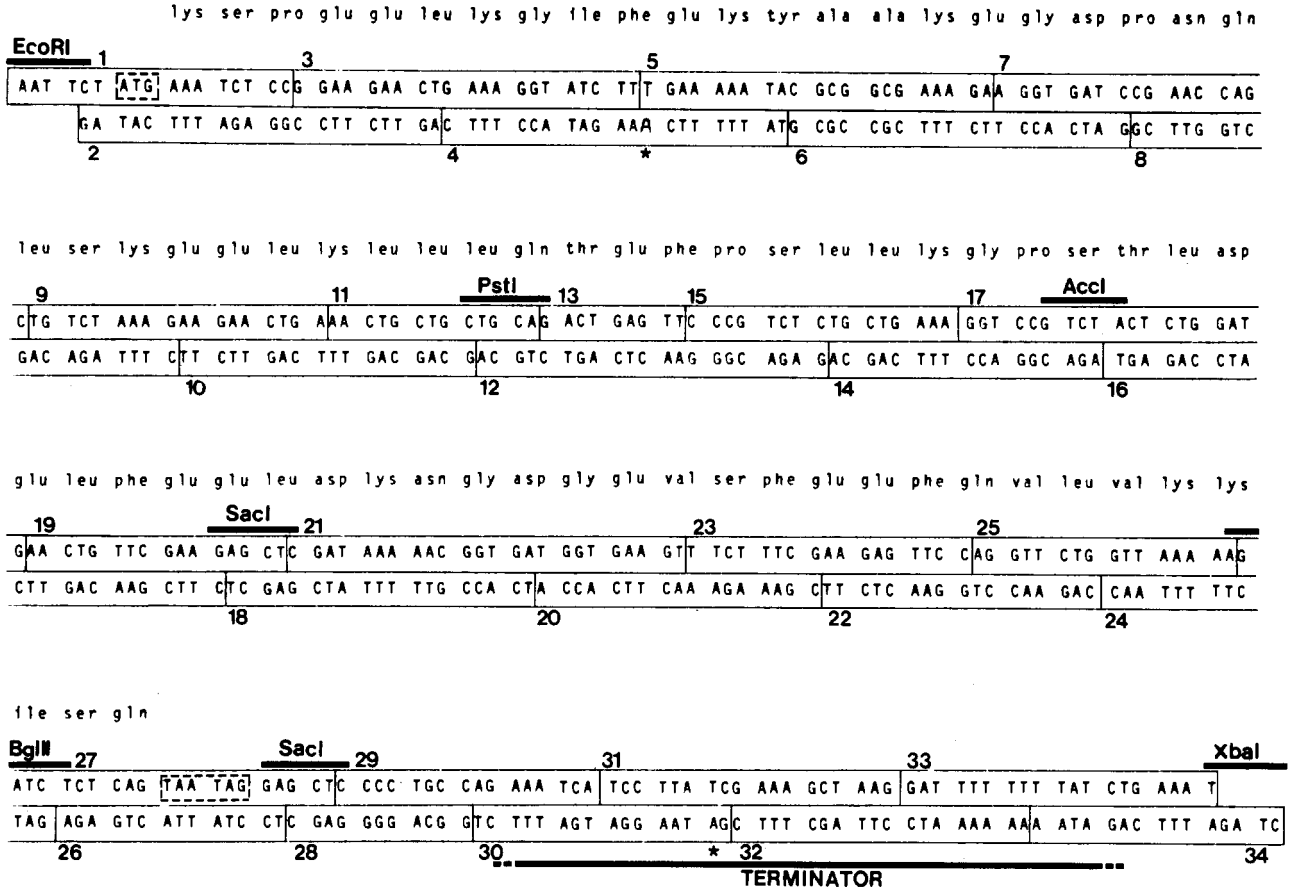

FIGURE 1: Amino acid sequence of the minor A form of the bovine ICaBP (Fullmer & Wasserman, 1981) and the nucleotide sequence designed for the synthetic gene. The limits of the 34 different oligonucleotides used for constructing the gene are indicated as well as recognition sites for several restriction endonucleases. The initiation codon and the two stop codons are boxed, and the *rrnC* transcription terminator is underlined. The two nucleotide alterations obtained relative to the designed sequence are indicated with asterisks.

the *trp* promoter and the −10 part of the *lac* promoter (de Boer et al., 1982). A polylinker containing several restriction enzyme sites was introduced directly after the tac promoter (Figure 2). A ribosome binding site with high homology to the 3′ end of 16S ribosomal RNA and with the most commonly found distance between this homology and the initiation codon (Shine & Dalgarno, 1974; Steitz, 1979) was designed. Divergences from the sequence with the highest homology to strong ribosome binding sites were introduced at two positions (indicated in Figure 2) in order to avoid stem structures in the ribosome binding site because such stems have been shown to severely affect initiation of translation (Iserentant & Fiers, 1980; Hall et al., 1980; Munson et al., 1984). Two oligonucleotides giving rise to the designed ribosome binding site were cloned in front of the assembled ICaBP gene in the polylinker of the expression vector (Figure 2). The plasmid obtained was denoted pICB0. To keep the tac promoter in a repressed state on a multicopy plasmid such as pICB0 in the absence of the inducer IPTG, the *lac* repressor has to be overproduced (Amann et al., 1983). Therefore, a 1.7 kb DNA fragment carrying *lacIQ*, a *lac* repressor overproducing gene, was cloned into the *Xba*I site after the terminator (Figure 2). The plasmid finally obtained was denoted pICB1.

Expression of ICaBP in E. coli. The *lon* protease has been shown to decrease the stability of many foreign proteins in *E. coli* [see Goff & Goldberg (1985) and references cited therein]. For this reason, total protein extracts from plasmid-containing derivatives of the *lon*−strain SG20043 and, as a control, the *lon*+ strain W3110 were separated by SDS–polyacrylamide gel electrophoresis (Materials and Methods). Bacteria with a pICB1 plasmid derivative lacking the ICaBP gene (Figure 3, lanes B and E) did not give rise to any band of the same mobility as that of purified ICaBP (lane A), neither from their chromosomes nor from the plasmid. In contrast, there is a significant expression of a protein with the same mobility as purified ICaBP from the pICB1-containing strains induced with IPTG (lanes D and G). This protein is not produced when the same bacteria are not induced with IPTG (lanes C and F). The level of expression is approximately 3 times higher from the *lon*− than from the *lon*+ strain (cf. lanes D and G; the same difference was seen with other *lon*+ strains; data not shown). Figure 3 indicates that the higher level of expression represents approximately 1–2 wt % of total *E. coli* protein. However, when the bacteria were grown at a lower growth rate (in minimal media with acetate as the carbon and energy source), the proportion of ICaBP to total protein was about 3 times higher than in the rich media (data not shown).

Purification and Characterization of ICaBP. The protein extract from 4.5 L of IPTG-induced *lon*− bacteria was fractionated by chromatography on a DEAE-Sephadex A-25 column (Materials and Methods). The elution profile obtained is shown in Figure 4A. The fractions containing ICaBP were pooled and loaded on a gel filtration column (Materials and Methods). The elution profile obtained from this column is shown in Figure 4B. SDS–polyacrylamide gel electrophoresis of the pooled fractions from the two columns indicates that the protein obtained from the second column was less than 1% contaminated with other proteins (Figure 4C, lane 4). The yield was 33 mg of ICaBP from 3.4 g of crude protein extract, representing 1 wt % of total *E. coli* protein. The amino acid composition of the protein obtained was determined and compared to that calculated from the known sequence of the

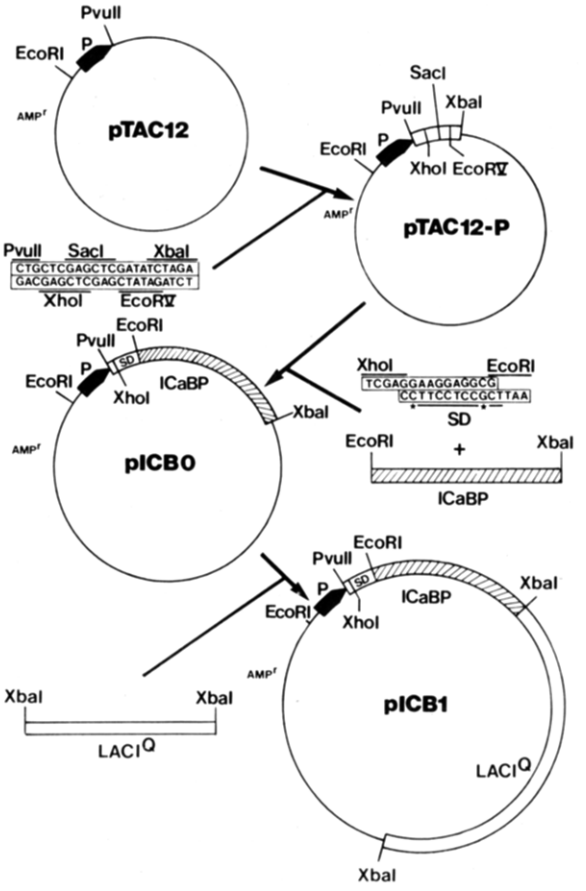

FIGURE 2: Construction of the ICaBP expression vector pICB1. Two oligonucleotides with recognition sequences for several restriction endonucleases, indicated in the figure, were cloned into the *Pvu*II site of the plasmid pTAC12. The resulting plasmid, pTAC12–P, was digested with *Xho*I and *Xba*I, and the ICaBP gene was inserted together with two oligonucleotides giving rise to a ribosome binding site, designated SD in the figure. This plasmid, denoted pICBO, was digested with *Xba*I, and a 1.7 kb *Eco*RI fragment with *Xba*I linkers (^{5}'CTCTAGAG^3'), carrying a constitutive mutant of the *lac* repressor gene (*lacIQ*), was inserted. The plasmid obtained was denoted pICB1. The tac promoter is indicated with P in the figure, and the two asterisks in the sequence of the ribosome binding site show where changes have been made from the "consensus" ribosome binding site in order to avoid base pairing with the 5′ end of the gene.

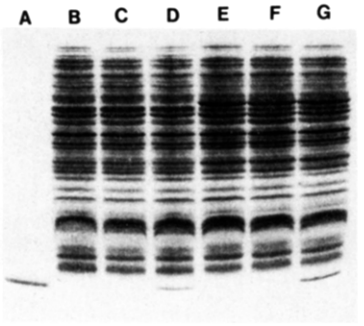

FIGURE 3: SDS–polyacrylamide gel of protein extracts from *E. coli*. Lane A, 1.5 μg of purified ICaBP from bovine intestine; lane B, strain W3110 with a derivative of plasmid pICB1 lacking the ICaBP gene, +IPTG; lane C, W3110 with pICB1, –IPTG; lane D, W3110 with pICB1, +IPT6; lane E, SG20043 with a derivative of plasmid pICB1 lacking the ICaBP gene, +IPTG; lane F, SG20043 with pICB1, –IPTG; lane G, SG20043 with pICB1, +IPTG.

minor A form of bovine ICaBP (Table I). The quantities found for the different amino acids are close to those expected except for the presence of a methionine residue not existing in the bovine protein. Amino acid sequencing of the 22 N-terminal residues showed that the sequence was as designed

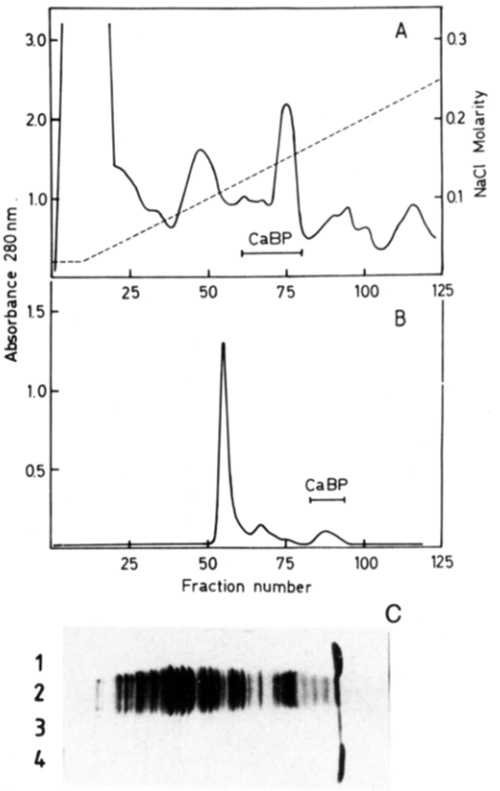

FIGURE 4: (A) Separation of *E. coli* extract using a 3 × 20 cm DEAE-Sephadex A-25 column. The initial buffer was 0.02 M imidazole, 20 mM NaCl, and 1 mM EDTA, pH 7.0, and elution was with a linear NaCl gradient with collection of 10-mL fractions. The fractions pooled are indicated with a horizontal line and CaBP in the figure. (B) Separation of pooled fractions from the DEAE-Sephadex column by gel filtration on a Sephadex G-50 superfine 3 × 200 cm column in 0.1 M NH_4HCO_3, pH 8.0, with collection of 7-mL fractions. The pooled fractions are indicated CaBP in the figure. (C) SDS–polyacrylamide 10–20% gradient gel: (lane 1) pure minor A form of ICaBP from bovine source; (lane 2) crude *E. coli* extract; (lane 3) pooled fractions from DEAE-Sephadex A-25 column; (lane 4) pooled fractions from Sephadex G-50 column.

Table I: Amino Acid Composition of ICaBP Produced in *E. coli*[a]

amino acid	mol/8500 g	amino acid	mol/8500 g
Met	0.9 (0)	Asp/Asn	7.0 (6)
Thr	2.1 (2)	Ser	5.6 (6)
Glu/Gln	16.7 (17)	Pro	3.5 (4)
Gly	5.5 (5)	Ala	2.7 (3)
Val	3.7 (3)	Ile	2.3 (2)
Leu	11.5 (12)	Tyr	1.5 (1)
Lys	10.7 (12)	Phe	4.5 (5)

[a] Residues per molecule based on the designed sequence for the synthetic gene (see Figure 1) are given in parentheses.

(Figure 1) and that the initiator methionine was indeed retained at the N-terminus of the protein.

Both the UV absorption spectrum (Figure 5) and the ^{1}H NMR spectrum (not shown) of the protein purified from *E. coli* by the two chromatographic steps described above were different from the corresponding spectra of the bovine ICaBP, presumably due to the presence of a low molecular weight contaminant (see below). This contaminant strongly adhered to the *E. coli* produced protein and could be removed neither by extensive dialysis nor by a phenyl-Sepharose column found to remove impurities attached to cardiac troponin C (data not shown). However, chromatography of denatured protein could be utilized for removing the impurity because bovine ICaBP renatures correctly after denaturation. Addition of 8 M urea before gel filtration as above yielded a protein with a UV

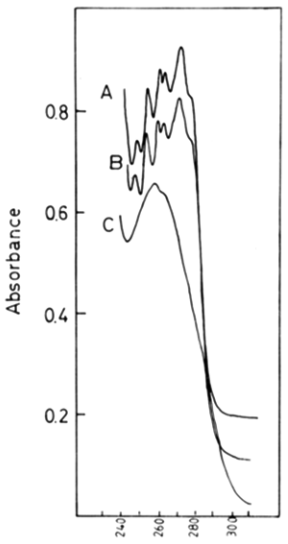

FIGURE 5: Ultraviolet absorption spectra of (A) native bovine ICaBP, (B) minor A form of ICaBP from *E. coli* purified with urea treatment, and (C) minor A form of ICaBP from *E. coli* purified without urea treatment.

FIGURE 6: SDS–polyacrylamide 10–20% gradient gel. Lane 1, purified *E. coli* ICaBP; lane 2, CNBr-cleaved purified *E. coli* ICaBP (minor A form of ICaBP); lane 3, native bovine ICaBP; lane 4, trypsin-cleaved native bovine ICaBP (minor A form of ICaBP).

absorption spectrum indistinguishable from that of the native bovine protein (cf. spectra A and B in Figure 5).

To compare the structures of the minor A forms of ICaBP from *E. coli* and from the bovine source, the extra N-terminal methionine present in the former was removed by CNBr treatment, and the two N-terminal amino acids of the latter were removed by trypsin treatment (Materials and Methods). The absence of other methionine residues in the sequence rendered the CNBr cleavage highly specific as indicated by the SDS gel of Figure 6 and the 360-MHz ^{1}H NMR spectra of Figure 7. The NMR spectra clearly show that the methionine was removed since the sharp signal at about 2 ppm from the S–CH_3 group is missing in the spectrum of CNBr-treated protein from *E. coli* (cf. spectra C and D in Figure 7). Likewise, after the trypsin treatment required to produce the minor A form of ICaBP from the bovine source, the signal at about 2 ppm from the *N*-acetyl methyl group of the native protein is absent (cf. spectra A and B in Figure 7). Comparison of the ^{1}H NMR spectra of the minor A forms of ICaBP from *E. coli* and the bovine source shows that the two proteins have the same structure (cf. spectra B and D in Figure 7; also see Discussion).

Discussion

The results presented show that it was feasible to assemble a synthetic gene for bovine ICaBP on the basis of the known primary structure and express the protein in *E. coli* with a good yield. The gene was assembled by a microscale "shotgun ligation" of oligonucleotides as recently described by Grundström et al. (1985). In this method, enrichment for correctly ligated oligonucleotides is achieved through biological selection instead of polyacrylamide gel purifications. The method, previously employed for the construction of mutants, is shown here to be very convenient also for the construction of DNA segments for subsequent assembly into a complete gene. The gene synthesis could be accomplished with as little as 0.25 pmol of each oligonucleotide.

The assembly of the gene from "DNA building blocks" bordered by restriction endonuclease sites will greatly facilitate construction of amino acid substitutions in the ICaBP because only the DNA segment containing the nucleotide substitutions desired has to be reassembled from oligonucleotides for each mutation. The chemical synthesis of a calmodulin gene also possessing regularly spaced restriction endonuclease sites has recently been described (Roberts et al., 1985).

The yield of ICaBP was 1–2% (w/w) from a strain deficient in the protease encoded by the *lon* gene, a level about 3 times higher than from *lon*+ strains. The *lon*-deficient strain was used because this mutation has been shown to increase the stability of eukaryotic proteins expressed in *E. coli* [see Goff & Goldbert (1985) and references cited therein].

The small size of ICaBP simplified its purification from the *E coli* protein extract because virtually all other proteins are larger (see Figure 3) and could be efficiently removed by gel filtration (Figure 4). However, the initial ICaBP preparation from *E. coli* was found to contain considerable amounts of some unidentified contaminant(s) that could not be removed either by gel filtration or by extensive dialysis. The contaminant(s) gave rise to a very strong signal at about 2 ppm in the ^{1}H NMR spectrum (data not shown). From the narrowness of this NMR signal, it seems likely that the contaminant(s) has (have) relatively low molecular weight(s). Similar adhering contaminants have also been observed by other groups attempting to produce eukaryotic proteins in *E. coli* (E. Chiancone et al., unpublished results). In our case, it was possible to remove the contaminant through gel filtration after denaturation of the protein, which could indicate that the contaminant is trapped during the initial folding of the protein at the time of synthesis. If such strongly adhering contaminants are common when foreign proteins are expressed in *E. coli*, this will be a very serious problem, especially for proteins that do not renaturate correctly after denaturation as bovine ICaBP does. It is notable that the impurity(ies) was (were) only detected by analysis of UV spectrum and by high-resolution ^{1}H NMR, making these techniques essential for analyses of the purity of proteins produced.

Before the solution structures of the ICaBP produced in *E. coli* and from the bovine source could be compared by high-resolution ^{1}H NMR, the N-terminal amino acids of the primary products had to be removed (see above). The removal of the two N-terminal amino acid residues (acetyl-Ser-Lys-) from native bovine ICaBP should result in the disappearance of resonances directly attributable to protons on these amino acids and possibly also in minor changes in shifts of amino acids immediately adjacent to those removed. Furthermore, resonance shifts could arise as a result of more extended tertiary structure changes (Wüthrich et al., 1982; Jardetzky & Roberts, 1981). It is evident that the overall appearance of the spectra from the two proteins is very similar (cf. spectra A and B in Figure 7), indicating a close structural resemblance of the proteins. The most prominent difference between the

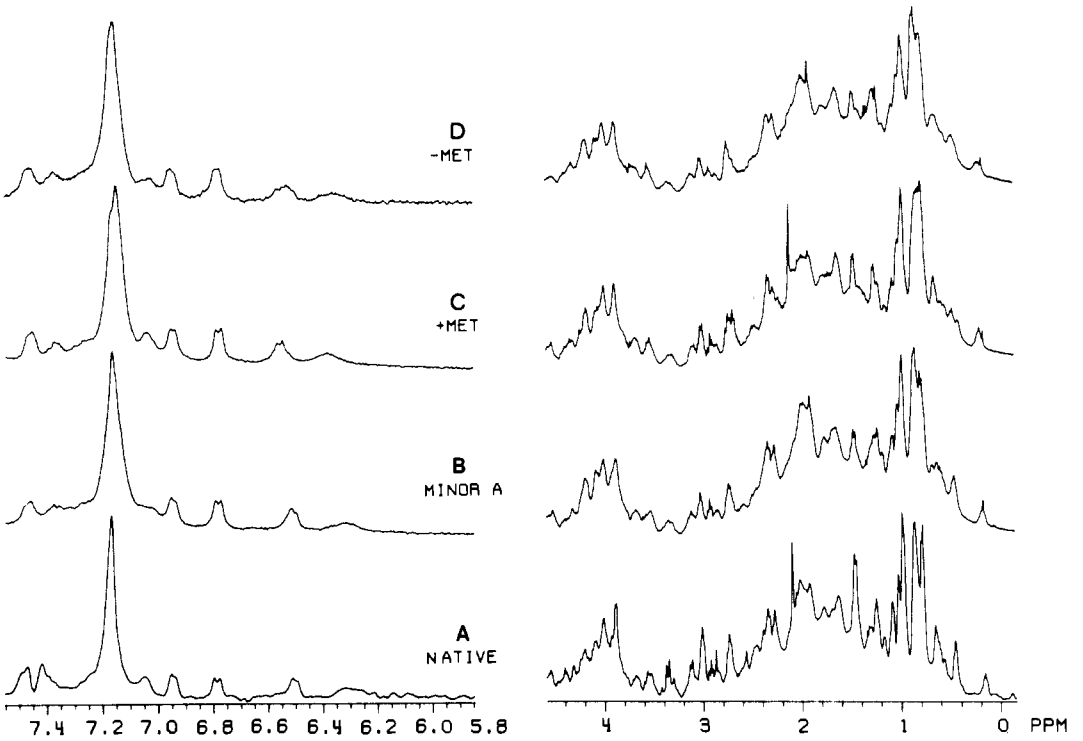

FIGURE 7: 360-MHz ^{1}H NMR spectra of Ca^{2+}-saturated ICaBP derivatives at 0.5–1 mM concentration, pH 6.0. (A) Native bovine ICaBP; (B) minor A form of native bovine ICaBP; (C) intact *E. coli* produced protein purified with urea treatment; (D) *E. coli* produced protein purified with urea treatment and with the methionine cleaved off.

two spectra is the sharp signal at about 2 ppm in the spectrum from native ICaBP (spectrum A in Figure 7). This resonance must be assigned to the N-terminal acetyl blocking group. Some small differences in the two spectra are discernible, but it is difficult to predict which of these should be attributable to the NMR signals from the amino acids removed since their chemical shifts in the protein most likely differ from those in a random-coil protein or in an isolated amino acid. However, the amino acids removed should not have any resonances in the aromatic region ($\delta > 6$) or in the aliphatic methyl region ($\delta < 1.2$). Thus, differences in these specific regions can be used to assess possible tertiary structure changes. There are such spectral differences in the aliphatic methyl region, but they are very minor as compared to shift changes observed to accompany the unfolding of globular proteins (Wüthrich, 1976; Jardetzky & Roberts, 1981). The same is true for the region of aromatic proton resonances where in fact the largest difference is a selective broadening of the resonance at about 6.5 ppm in minor A (spectrum B). This difference in line width is most likely due to a dynamic process proceeding at slightly different rates in the two proteins. Thus, removal of the two N-terminal amino acids from native bovine ICaBP only leads to minor changes in the tertiary structure of the protein.

When the spectra from ICaBP produced in *E. coli* are compared, before and after the CNBr treatment creating the minor A form (spectra C and D in Figure 7), the most obvious difference is the disappearance of a resonance at about 2 ppm, in this case due to the *S*-methyl group in the methionine removed. The two spectra are almost identical in the aromatic region, and in the aliphatic methyl region there are only minor differences. With the same argumentation as above, we therefore conclude that the N-terminal methionine does not significantly affect the tertiary structure of the protein. The ^{1}H NMR spectra of the minor A forms of ICaBP from the bovine source (spectrum B) and from *E. coli* (spectrum D) are more similar than the two other pairs just discussed. This is particularly evident in the aliphatic region, where the spectra are virtually superimposable. The very minor differences between these two spectra can be accounted for by the presence of small amounts of impurities which may originate from the dialysis bag and always are present in various amounts (for example, the sharp signals at about 2.3 ppm). Although precautions have been taken, it is also virtually impossible to analyze two protein preparations with exactly the same instrumental conditions as regarding B_0 homogeneity, sample temperature, etc. [see Neuhaus et al. (1985)]. Taken together, the results from UV spectroscopy, electrophoretic mobility studies, partial amino acid sequence determination, and ^{1}H NMR spectroscopy show beyond reasonable doubt that the minor A form of bovine ICaBP produced in *E. coli* is identical with the minor A protein obtained from bovine intestines.

The present work represents the first steps toward the analysis of a number of intestinal calcium binding proteins modified in those parts of the molecule that are assumed to play an important role for the structure and function of the protein. Obviously, such regions are the EF- and pseudo-EF-hand domains and those parts that are involved in the interactions which have been found between the two calcium binding domains (Vogel et al., 1985). The preparation of such proteins (or "muteins") and characterization of their properties by different biophysical techniques are in progress.

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Registry No. **1**, 103691-73-2; **2**, 103691-89-0; **3**, 103691-88-9; **4**, 103691-84-5; **5**, 103691-87-8; **6**, 103691-83-4; **7**, 103691-70-9; **8**, 103691-77-6; **9**, 103691-79-8; **10**, 103691-78-7; **11**, 103691-59-4; **12**, 103691-85-6; **13**, 103669-09-6; **14**, 103691-71-0; **15**, 103691-63-0; **16**, 103710-46-9; **17**, 103691-80-1; **18**, 103691-81-2; **19**, 103691-64-1; **20**, 103691-69-6; **21**, 103691-90-3; **22**, 103691-72-1; **23**, 103691-74-3; **24**, 103669-10-9; **25**, 103691-65-2; **26**, 103691-60-7; **27**, 103691-86-7; **28**, 103669-11-0; **29**, 103691-62-9; **30**, 103691-66-3; **31**, 103691-75-4; **32**, 103691-76-5; **33**, 103691-82-3; **34**, 103691-61-8; DNA (ox in-

testinal calcium binding protein specifying), 103750-96-5; protein (ox intestinal calcium binding), 78337-48-1.

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