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QUARTERLY

Molecular cloning and expression in *Escherichia coli* of a gene coding for bovine S100A1 protein and its Glu32→Gln and Glu73→Gln mutants*

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Calcium binding S100A1 protein consists of two S100α subunits. On the basis of sequence homology to other S100 proteins it is believed that the binding loops are formed by amino-acid residues 19–32 and 62–73 of S100α polypeptide chain. In the oxidized form of the protein the subunits are linked covalently with each other by a disulphide bond between their Cys85 residues.

A synthetic gene coding for bovine S100α subunit was constructed and cloned into a derivative of pAED4 plasmid. The gene was expressed in *Escherichia coli* utilizing the T7 expression system. The expression products were purified and identified using mass spectrometry and by sequencing of their N- and C-termini. Three different forms (*a*, *b*, and *c*) of S100α were produced: with the native sequence, with the initiator methionine at the N-terminus, and with an additional alanine at the C-terminus as well as with the initiator methionine. The material was partly oxidized. Interestingly, only the homodimers of *a*, *b*, and *c* species were formed. The total yield of the protein was about 50 mg/l of culture.

Genes coding for Glu32→Gln and Glu73→Gln mutants of S100α were obtained by site-directed mutagenesis and expressed in the same system. In both cases similar mixtures of oxidized and reduced *a*, *b*, and *c* species have been obtained. The total yield of E73Q mutant is similar to that of the native protein and that of E32Q lower by about a half. As expected, the mutants of S100α subunits bind only one calcium ion.

S100 proteins represent one of major subfamilies of EF-hand calcium binding proteins (CaBPs). Various functions have been postulated for S100 proteins; they appear to be involved in cell cycle regulation, differentiation, and growth (for reviews see [1–5]). The proteins are expressed in a tissue- and cell-specific manner [4–5]. S100A1 was first

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Abbreviations: CaBP, calcium-binding protein of the EF-hand family; DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactoside; HPLC, high performance liquid chromatography; bHLH, basic helix-loop-helix fragment of myogenic proteins.

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found in nervous tissue, then in skeletal and heart muscle, kidney, and other tissues [4]. The cellular role of the protein is not clear but it seems very important since S100A1 is strongly conserved among mammalian species [6–8]. Previously fructose-1,6-bisphosphate aldolase [9], tubulin [10], and adenylate cyclase [11] were suggested as target proteins. In addition, S100A1 was postulated to inhibit microtubule assembly [10] and to stimulate Ca^{2+}-induced Ca^{2+} release from sarcoplasmic reticulum [12]. Recently, an interaction of S100A1 protein with substrates of protein kinase C has been observed, involving inhibition of the phosphorylation process [13, 14] as well as regulation of basic helix-loop-helix (bHLH) myogenic protein activities [13]. MyoD, a member of myogenic bHLH transcription factors activating muscle-specific genes, interacts with S100A1 protein, abundantly expressed in cardiac and skeletal muscles. This interaction results in inhibition of MyoD-bHLH phosphorylation by kinase C. S100A1 protein was found to be more efficient than calmodulin in antagonizing DNA binding to MyoD [13]. Binding to p53, a cell cycle regulator protein, and inhibition of p53 phosphorylation by kinase C was proved for S100B and postulated for S100A1 protein [14]. In skeletal muscle fibers S100A1 protein inhibits enzymatic activity of glycogen phosphorylase *a* in a calcium-independent manner that may be responsible for lack of fatiguability of slow-twitch fibers [15]. S100A1, like other proteins from the family, probably acts at various points in the Ca^{2+}-signal-transduction cascade by modulating activities of several target enzymes, as well as organization of cytoskeleton, but to date many of its functions remain unknown [5].

S100 proteins are small dimeric proteins which are characterized by two Ca^{2+}-binding motifs of the helix-loop-helix structure, separated by a central hinge region [1]. S100A1 protein is a homodimer, formed by two 93 amino-acid subunits [16] called S100α. Its molecular structure has not been determined. On the basis of sequence homology to other CaBPs of known structures [17] two EF binding sites are postulated; the first, classic one for the CaBPs, formed by residues 62–73 situated close to C-terminus, and the second site composed of 14 amino-acid residues 19–32, characteristic for S100 proteins.

S100α subunits contain cysteine residues at their C-termini (position 85). The protein can be found in two forms: the oxidized one, with both subunits linked together covalently by Cys85–Cys85 disulfide bond, and in the reduced form. Most probably the structures of the two forms are different. Moreover, the protein conformation changes upon calcium binding. Consequently, S100A1 can be expected to assume as many as four various structures. The main far-reaching goal of our research project is to determine these structures by NMR spectroscopy. For proteins of the size of S100A1 structural NMR studies are not feasible without using various heteronuclear experiments. Hence, it is necessary to produce the protein in bacteria where it can be easily labeled with ^{15}N and ^{13}C isotopes.

Another aim of our project is to study the mechanism of calcium binding to S100A1 and, in particular, to determine what is, if any, cooperativity of this process. The native protein, with its four binding sites is a too complicated system for such studies. Therefore, we decided to produce protein mutants with inactive either N- or C-terminal calcium binding sites.

The calcium binding loops of all known EF-hand proteins contain glutamate as the C-terminal residue. Substitution of glutamic acid by glutamine at this place results in drastic Ca^{2+} affinity decrease, as shown by Carlstrom & Chazin [18]. Therefore, we decided to obtain two mutants: Glu32→Gln and Glu73→Gln of S100A1 protein. Each of them should contain only a single active binding loop in each of its subunits.

In this paper we describe molecular cloning and expression, as well as purification and preliminary characterization of recombinant bovine S100A1 and its mutants E32Q and E73Q, produced in *E. coli*.

MATERIALS AND METHODS

Materials. All chemicals used were of the highest purity grade. Restriction enzymes,

T4 DNA ligase, T4 polynucleotide kinase and a dideoxy sequencing kit were from Amersham; carboxypeptidase A was from Sigma. Plasmid pUC19 was from Biolab; plasmid pAED4 was a generous gift from Professor P.S. Kim (Whitehead Institute, MIT). Oligonucleotides used for synthesis were purchased from the Center for Molecular and Macromolecular Research of the Polish Academy of Sciences (Łódź, Poland).

Plasmid construction. All plasmids were constructed using standard cloning procedures [19] and gene DNA sequences were confirmed by the dideoxy method.

The nucleotide sequence coding for S100A1 was programmed according to its respective amino-acid sequence [16] (Fig. 1a, b) with codon preferences established for *E. coli* [20]. The S100A1 structure gene was constructed from two gene fragments: fragment I corresponding to amino-acid residues 1–48 and fragment II corresponding to residues 45–93. Each of these parts was obtained from synthesis of 12 oligonucleotides; a ligation product of the expected size was cloned into *KpnI/SalI* sites of pUC plasmid. The whole gene of S100A1 was obtained by ligation of *FokI/SalI* gene fragment II with a synthetic linker (Fig. 2a) followed by insertion into the *XbaI* and *SalI* sites of the pUC vector with gene fragment I. To clone the gene to the *NdeI/Hin*dIII restriction sites of pAED4 plasmid [21] the gene was modified by changing the 5′ end of the coding sequence of the gene by ligation of synthetic oligonucleotides (Fig. 2b) and the *NcoI/Hin*dIII fragment of the gene. This plasmid utilizing the T7 expression system from pET3a plasmid [22] had been succesfully used previously for production of a number of recombinant proteins and peptides in *E. coli* [23–26].

The genes coding for Glu32→Gln S100A1 and Glu73→Gln S100A1 mutants were produced by oligonucleotide site-directed mutagenesis, as described by Kunkel *et al.* [27]. The sequence CAG was used as glutamine codon.

Expression and purification of recombinant proteins. *E. coli* strain BL21(DE3) pLys S cells were transformed with pAED4 plasmid bearing either the gene coding for S100A1 protein or for one of its Glu32→Gln or Glu73→Gln mutants, as described by Studier *et al.* [28]. The cells taken from overnight cultures were grown at 37°C in Luria broth containing 100 mg/ml ampicillin and 30 mg/ml chloramphenicol. The cells were induced at A_{590} = 1.0 with 0.4 mM IPTG, harvested after 3 h, and centrifuged. The pellet was suspended in 20 mM Tris, pH 7.5,

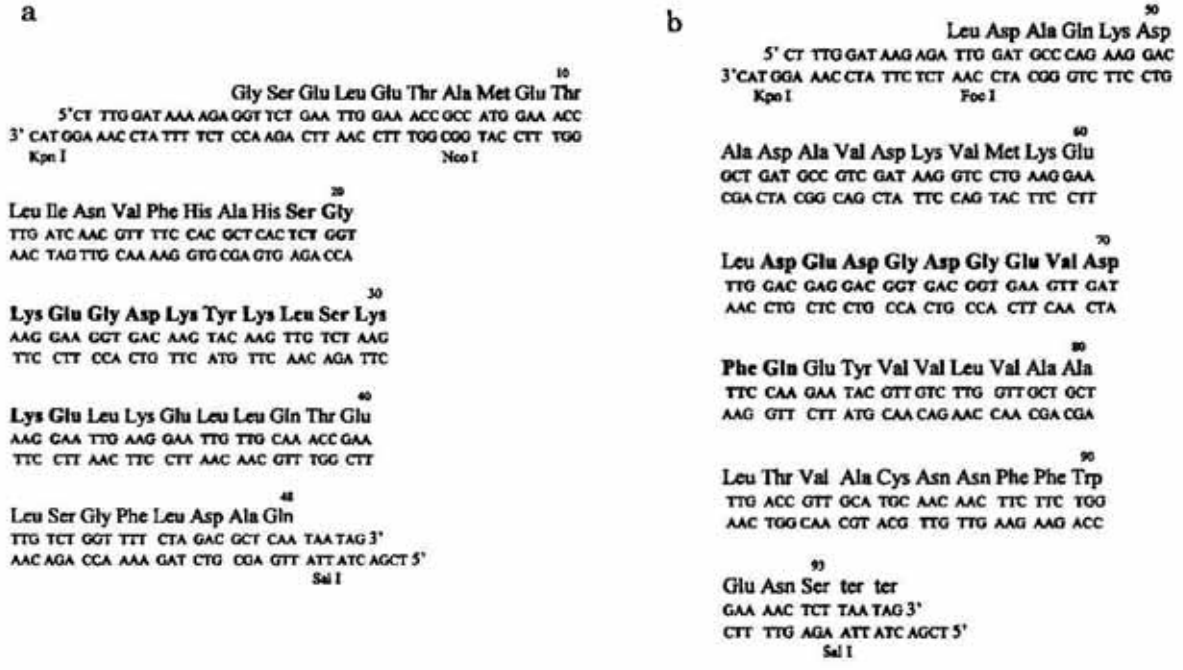

Figure 1. Amino-acid and gene sequences of fragment I (a) and fragment II (b) of S100α subunit.

50 mM $CaCl_2$, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF) sonicated, and centrifuged at 4°C.

The first step of protein purification was carried out on a Phenyl-Sepharose column [29]. The protein solution, after centrifugation, was applied to the column buffered with 50 mM Tris, pH 7.5, 0.1 M $CaCl_2$, washed with the same buffer and eluted with 50 mM Tris, pH 7.5, 5 mM EDTA. The fraction containing the protein, identified by its absorption spectrum, was stored and lyophilized.

The proteins were purified on a semi-prepa-

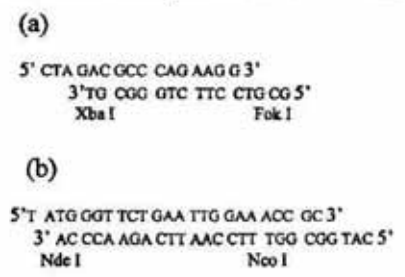

Figure 2. Nucleotide sequences: (a) synthetic DNA linker to construct the S100α gene from two gene fragments (I and II); (b) synthetic DNA linker to change a beginning of the gene and to introduce *Nde*I restriction site.

rative Vydac C18 column in acetonitrile/water gradient in the presence of 0.1% trifluoroacetic acid.

Mass spectrometry. Mass spectra were obtained with a MALDI TOF apparatus at Whitehead Institute, MIT. The samples of lyophilized proteins were suspended in 70% acetonitrile before the measurements.

Determination of amino-acid sequence. Automated Edelman degradation of proteins was carried out on an Applied Biosystems 473A protein sequencer with on-line detector in the laboratory of Professor M. Żylicz, Department of Molecular Biology, University of Gdańsk (Poland) The sequencing was made only for the first 27 amino-acid residues.

C-Terminal residues of the proteins were identified by carboxypeptidase A digestion as described by other authors [30–32]. Dansylated digestion products were analyzed on Vydac C18 column in acetonitrile/water gradient in the presence of trifluoroacetic acid (0.1%).

Spectroscopic methods. UV absorption spectra were obtained with a Varian Cary 3E spectrophotometer. Relative content of aromatic residues in HPLC fractions was determined by fourth derivative analysis of their UV spectra using a self-made PC program.

^{31}P NMR measurements were made with a Varian 200 MHz instrument at the Institute of Organic Chemistry, Polish Academy of Sciences, Warsaw.

RESULTS

All studied recombinant proteins can be effectively isolated on a Phenyl-Sepharose column. They are absorbed in the presence of calcium and are eluted by the EDTA containing buffer (see Materials and Methods), according to the procedure described earlier by Lucas & Watterson [33]. This proves that all these proteins bind calcium ions with a concomitant increase of their hydrophobicity characteristic for all CaBPs.

Although the material always is eluted from Phenyl-Sepharose as a single peak it is not homogeneous. A typical HPLC chromatogram of the material obtained from the bacteria transformed with the plasmid containing the gene coding for native S100α subunit is shown in Fig. 3. As many as 6 different species can be distinguished corresponding to peaks *aa*, *bb*, *a*, *b*, *cc*, and *c*. The fourth derivative analysis of the absorption spectra of the isolated species have shown that all of them have the same composition of aromatic amino-acid residues Trp:Tyr:Phe = 1:2:5 as expected for native S100α subunit. Tryptophan fluorescence measurements (Goch, G. & Bierzyński, A., to be published) have proved that all these species bind, as expected, two calcium ions per one tryptophan residue.

Species *aa*, *bb*, and *cc* disappear when incubated with DTT. The reduction products are *a*, *b*, and *c* species (Fig. 4). Similar experiments made separately with each isolated species *aa*, *bb*, and *cc* (not shown) have proved that *a*, *b*, and *c* are the respective products of their reduction. In aerobic conditions the content of *a*, *b*, and *c* species drops slowly with time and the content of *aa*, *bb*, and *cc* species increases (Fig. 5). Some other peaks also appear, as that preceding peak *aa*

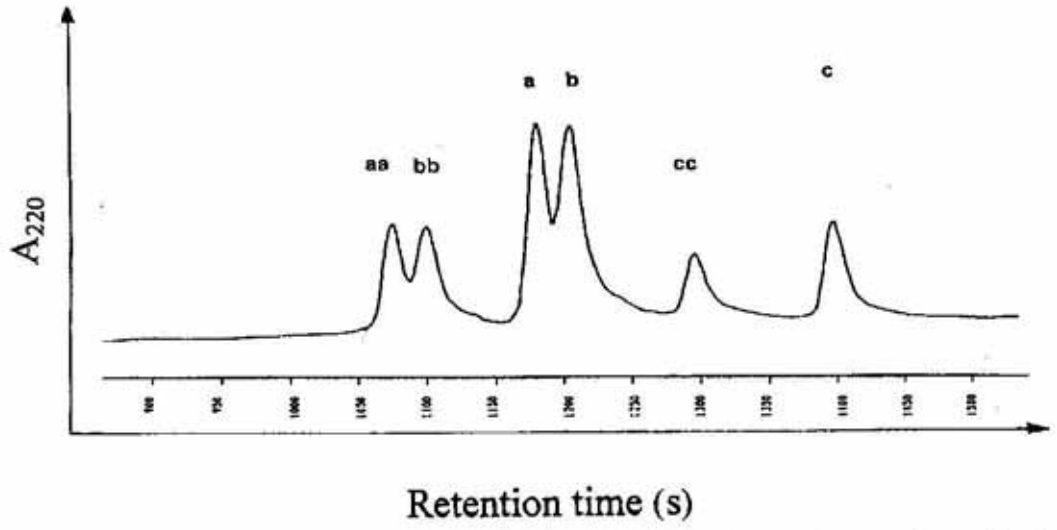

Figure 3. HPLC chromatogram of the material obtained from the bacteria transformed with the plasmid containing the gene coding for the native S100α subunit.

Acetonitrile gradient 0.5%/min; absorbance was monitored at 220 nm. The proteins were eluted within the gradient range 45–52% of acetonitrile.

or another one in between peaks *bb* and *a*, but they are very small. It is clear, then, that the bacteria produce three different kinds of S100α subunits (*a*, *b*, and *c*) and at least part of them in the form of homodimers linked covalently by disulphide bridges.

Mass spectroscopy and sequencing of N- and C-termini of isolated *a*, *b*, and *c* species

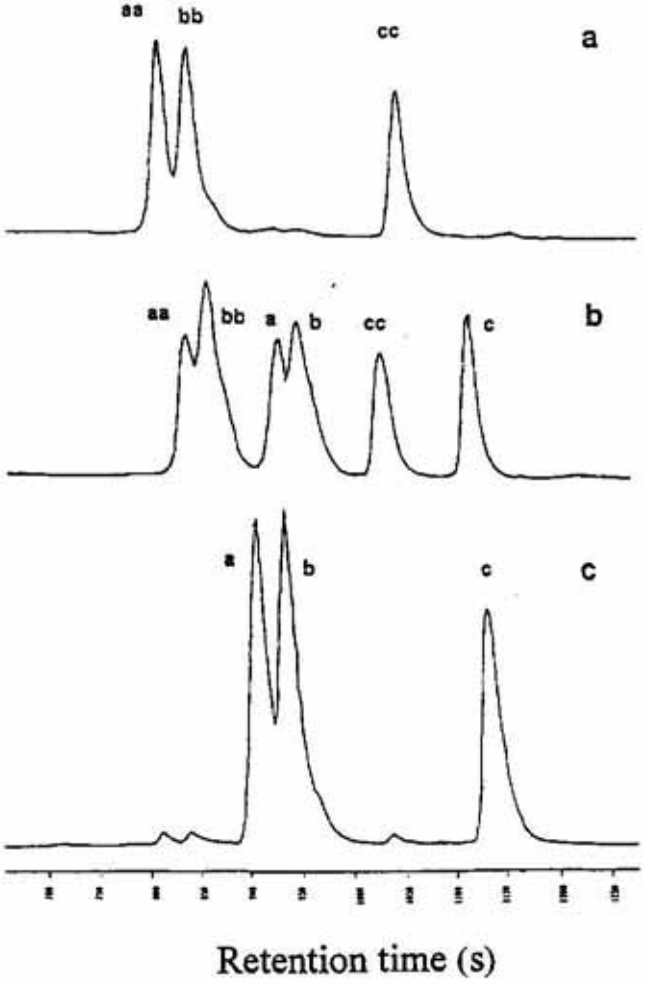

Figure 4. HPLC chromatograms of the protein reduced with dithiothreitol (DTT).

200 μM S100α in 20 mM Tris, pH 8.5, without DTT (a). The same sample after 1 h (b) and 24 h (c) incubation with 20 mM DTT at room temperature. Separation conditions as in Fig. 3.

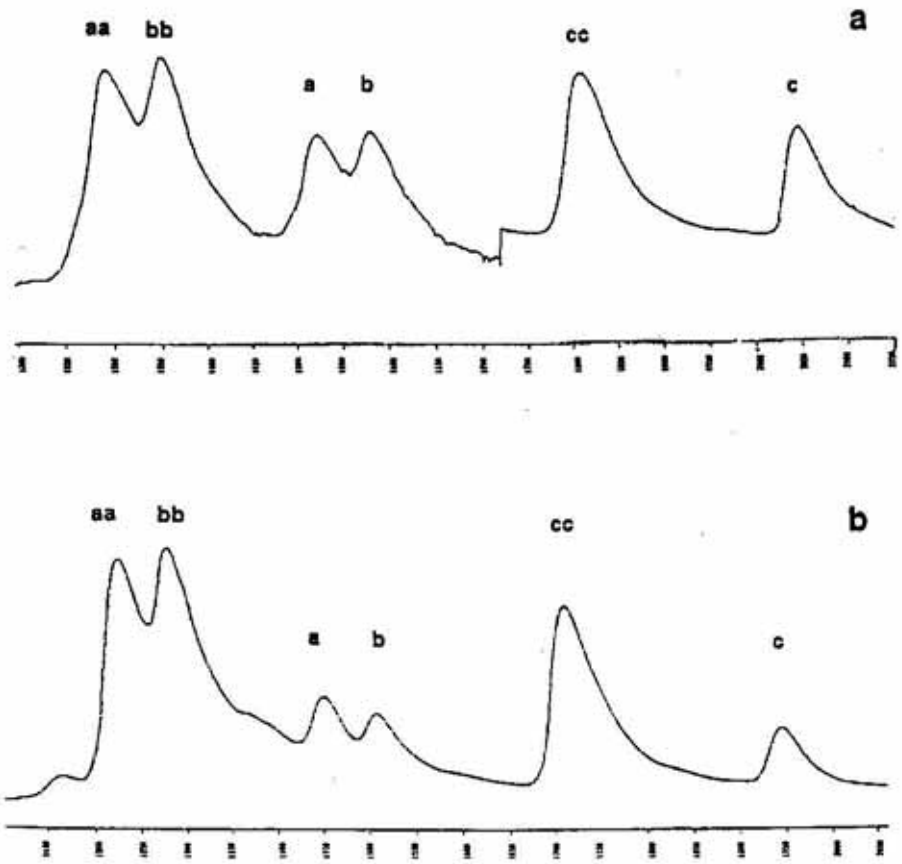

Figure 5. HPLC chromatograms of S100α oxidized in aerobic conditions.

a, 2 mM S100α in 20 mM Tris, pH 6.5, freshly prepared; b, the same solution after 24 h incubation at 5°C. Acetonitrile gradient 0.25%/ min; other separation conditions as in Fig. 3.

revealed the differences between them. Whereas the sequence of the first 27 amino-acid residues of *a* species is exactly the same as that of bovine S100A1 protein, two other expression products — *b* and *c* — contain an additional N-terminal methionine residue. The molecular mass of *b* product determined by mass spectrometry is 10520 ± 10 Da — in excellent agreement with the value calculated from the sequence of bovine S100α subunit plus one methionine residue (10520 Da). The mass of *c* product is larger than that by 75 Da (10595 ± 10 Da). At first we suspected that *c* is a phosphorylation product of *b* species. Nevertheless, ^{31}P NMR measurements have not detected any traces of phosphorus in the analyzed material.

Since the 91th residue of S100α is glutamate, a residue very resistant to digestion by carboxypeptidase A, it is easy, using this enzyme, to cleave off specifically the C-terminal residues of the protein following Glu91. In all three species the asparagine and serine residues have been found, as expected, after the carboxypeptidase A digestion, but in *c* species also an additional residue, namely an alanine, has been identified. The molecular mass of this residue — 71 Da — corresponds to the mass spectroscopy results.

The expression products of bacteria transformed with plasmids bearing genes coding for both of S100α subunit mutants are not homogeneous either, and their HPLC chromatograms look very similar to that of the material obtained from the bacteria containing the gene coding for native S100α subunit, although all 6 peaks are eluted at a slightly higher acetonitrile concentration. The reduction-oxidation experiments also confirmed, in both cases, that *a*, *b*, and *c* species are the reduction products of *aa*, *bb*, and *cc* species, respectively.

Fluorescence measurements have shown that each of the mutants binds, as expected, only one metal ion per one subunit of the protein (Goch, G. & Bierzyński, A., to be published).

After the final purification the total yield of all three forms of S100α subunit has been estimated spectrophotometrically to be about 50 mg/l of culture. In the case of Glu32→Gln and Glu73→Gln mutants this value is lower: 25 mg/l and 45 mg/l, respectively.

DISCUSSION

The final yield of S100A1 protein is as high as the yield of rat S100A4 (also known as p9Ka, CAPL, calvasculin) obtained by Gibbs *et al.* [34] and it is higher than the usually obtained yield of other proteins (see for example [26, 35–37]). For unknown reasons the yield of the mutant Glu32→Gln S100A1 is lower by about a half, although the vector and all the expression conditions are identical. Similar differences in yields of two closely homologous S100 proteins expressed in the same *E. coli* system were observed by Pedrocchi *et al.* [36].

Many results of this work are difficult to interpret. One of them is the heterogeneity of S100α subunit and its mutants produced by the bacteria. It has been found previously that recombinant proteins are expressed in *E. coli* in two forms — with and without the initiator methionine at their N-termini [38]. S100B protein has been obtained in an *E. coli* expression system as a mixture of both these forms [35]. Therefore, the presence of species *a* and *b* in our material is quite understandable. Nevertheless, we cannot explain why, apart of them, the protein containing the additional alanine residue at its C-terminus (*c* species) is produced. We do not know either, why this protein is not processed by the bacteria and all its molecules contain the initiator methionine.

In the material purified from the bacteria we always found a mixture of oxidized and reduced S100A1 protein, albeit in various proportions, changing uncontrollably from one preparation to another. *In vitro* the oxidation process is rather slow as shown in Fig. 5. It is very unlikely, then, that the protein is oxidized during the purification process. Apparently, the protein is oxidized either within the bacterial cytoplasm, in spite of its reductive potential, or immediately after the sonication, in aerobic solution. In any case, either the bacterial membrane or some bacterial enzymes seem to be involved in this process.

Another intriguing problem is why only the homodimers of subunits *a*, *b*, and *c* are formed. Some traces of heterodimers appear during the oxidation *in vitro*, as indicated by the presence of the additional small peaks in HPLC chromatograms (see Fig. 5) but in the material isolated from the bacteria only the homodimers have been detected. One may speculate that the homodimers are formed preferentially because of favorable hydrophobic interactions between the N-terminal methionines (in species *b* and *c*) as well as between the C-terminal alanines (in *c* species) of the subunits. For further discussion of this phenomenon much more has to be known about structural properties of S100A1 protein and about its oxidation mechanism.

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