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The Covalent Structure of *Acanthamoeba* Actobindin*

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Actobindin is a protein from *Acanthamoeba castellanii* with bivalent affinity for monomeric actin. Because it can bind two molecules of actin, actobindin is a substantially more potent inhibitor of the early phase of actin polymerization than of F-actin elongation. The complete amino acid sequence of 88 residues has been deduced from the determined sequences of overlapping peptides obtained by cleavage with trypsin, *Staphylococcus* V8 protease, endoproteinase Asp-N, and CNBr. Actobindin contains 2 trimethyllysine residues and an acetylated NH_2 terminus. About 76% of the actobindin molecule consists of two nearly identical repeated segments of ~33 residues each. This could explain actobindin's bivalent affinity for actin. The circular dichroism spectrum of actobindin is consistent with 15% α-helix and 22% β-sheet structure. A hexapeptide with sequence LKHAET, which occurs at the beginning of each of the repeated segments of actobindin, is very similar to sequences found in tropomyosin, muscle myosin heavy chain, paramyosin, and *Dictyostelium* α-actinin. A longer stretch in each repeated segment is similar to sequences in mammalian and amoeba profilins. Interestingly, the sequences around the trimethyllysine residues in each of the repeats are similar to the sequences flanking the trimethyllysine residue of rabbit reticulocyte elongation factor 1α, but not to the sequences around the trimethyllysine residues in *Acanthamoeba* actin and *Acanthamoeba* profilins I and II.

Eukaryotic cells contain many actin-binding proteins that regulate the polymerization of actin and the supramolecular organization of the microfilament system (for reviews, see Refs. 1-4). These proteins are generally classified as either G-actin- or F-actin-binding proteins. Among the best studied examples of G-actin-binding proteins are the profilins; they have been isolated from a large variety of cells including vertebrates (5, 6), invertebrates (7), and protozoa (8-10). The profilins form 1:1 complexes with monomeric actin in a simple equilibrium reaction (11, 12), thus "buffering" (11) the polymerization process by providing a regulatable (13, 14) pool of polymerization-competent monomers. When studied *in vitro*, the initial rate of actin polymerization, the rate of elongation of F-actin, and the concentration of F-actin formed at steady state are all diminished, as expected, in proportion to the amount of monomeric actin that is sequestered in the profilin-actin complex.

Recently, Lambooy and Korn (15, 16) purified another G-actin-binding protein, actobindin, from the cytoplasm of *Acanthamoeba castellanii*. Initially, based on the extent to which actobindin inhibited the rate of elongation of F-actin and its concentration at steady state, they concluded (15) that actobindin (which was then believed to be a homodimer of a 12,500-Da polypeptide) bound a single actin monomer in much the same manner as the profilins. Then, Lambooy and Korn (16) discovered that, in contrast to the profilins, actobindin is a much more potent inhibitor of the initial phase of actin polymerization than could be explained by its formation of a 1:1 complex with actin monomer. These latter observations gave rise to the hypothesis (16) that, again in contrast to the profilins, actobindin may interact with an early intermediate in the actin polymerization process (*e.g.* a dimer or trimer) in addition to its interaction with monomeric G-actin.

Recent sedimentation equilibrium studies (17) have established that actobindin is, in fact, a monomer of M_r ~9000 that binds two actin monomers at high concentrations of G-actin and one actin monomer at low concentrations of G-actin. These data provide the basis for a reasonable explanation of the complex effects of actobindin on the kinetics of actin polymerization (17).

To understand the details of the interactions of actobindin and actin at the structural level, we have determined its amino acid sequence by conventional amino acid sequencing methods. We report now that actobindin comprises a total of 88 amino acids in an unusually symmetrical structure with two nearly identical segments arranged in tandem. This internal repeat could account for actobindin's bivalent affinity for monomeric actin. Short segments within these internal repeat sequences are similar to sequences in several other actin-binding proteins.

EXPERIMENTAL PROCEDURES[1]

RESULTS

Actobindin, isolated according to a previously described procedure (15), was further purified by chromatography on a C_4 reverse-phase column. Unsuccessful attempts to obtain an NH_2-terminal sequence of the intact protein by Edman degradation provided convincing evidence that the NH_2 terminus was blocked.

Actobindin was then cleaved with CNBr at its single methionine residue (Table I). Reverse-phase HPLC[2] of the CNBr cleavage products yielded a single component (CNBr-actobin-

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[1] Portions of this paper (including "Experimental Procedures," Figs. 1 and 3, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin.

din), which eluted with the same retention time as intact actobindin (Fig. 1, *A* and *B*). CNBr-actobindin was also indistinguishable from intact actobindin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In addition, except for the absence of the single methionine residue, the amino acid composition of CNBr-actobindin was essentially identical to that of the uncleaved protein (Table I). The NH_2 terminus of CNBr-actobindin was not blocked, and it was successfully sequenced through 30 cycles (Table II). These data established that the single methionine residue of actobindin is at or close to its NH_2 terminus (see below).

To determine the complete amino acid sequence, aliquots (~200 μg each) of intact actobindin were digested with several different proteases, and the respective peptide mixtures were separated by reverse-phase HPLC (Fig. 1, *C–E*). Each of the purified peptides was then subjected to gas-phase sequence analysis (Table II). All of the peptide sequences could be aligned in a single unique sequence (Fig. 2, *upper*).

The sequence of the amino acids at the blocked NH_2 terminus of actobindin was deduced by comparing the amino acid composition of an acid hydrolysate of the NH_2-terminal V8 protease peptide (residues 1–4) with the NH_2-terminal sequence of CNBr-actobindin. Methionine was the only residue detected in the V8 protease peptide that was not observed in the NH_2-terminal sequence of CNBr-actobindin. This identified methionine as the blocked NH_2-terminal residue.

The sequence at the COOH terminus of actobindin was confirmed by amino acid analysis of the COOH-terminal V8 protease peptide (residues 84–88). Lysine, alanine, and glutamic acid (derived from the COOH-terminal glutamine) were present at a molar ratio of 1.8:2.0:1.0, which is consistent with the COOH-terminal sequence of -Lys-Ala-Ala-Lys-Gln.

At the steps in the sequencing cycles of peptides Asp-N(22) and Asp-N(67) (Table II) that corresponded to positions 35 and 72 of the native protein, respectively, modified phenylthiohydantoin-derivatives were obtained whose elution positions in the conventional on-line phenylthiohydantoin-derivative analysis system were close to that of phenylthiohydantoin-arginine. As actobindin contains 2 trimethyllysine residues (15) and none of the other residues in any of the peptides that were sequenced was found to be modified, we assigned the trimethyllysines to positions 35 and 72.

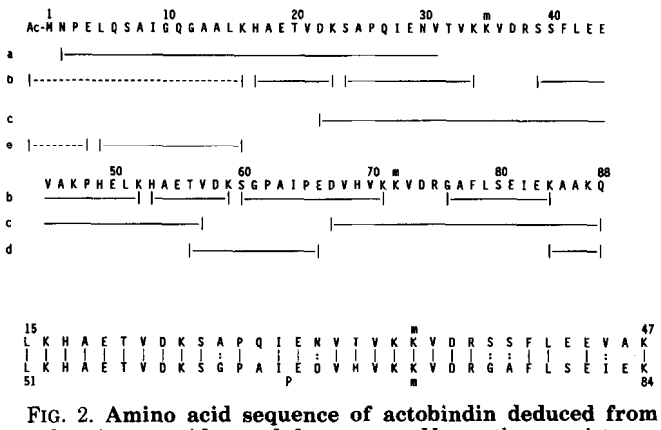

FIG. 2. **Amino acid sequence of actobindin deduced from overlapping peptides and fragments.** *Upper*, the complete sequence in one-letter notation. *Ac*, acetyl group; $\overset{m}{K}$, N^{ϵ}-trimethyllysine. Peptides which were sequenced are indicated by *solid lines* below the sequence. NH_2-terminal blocked peptides which could not be sequenced are indicated by *broken lines*. Only the peptides necessary to provide unambiguous overlaps are identified; the sequences of other peptides were consistent with these data. *Row a*, sequence of CNBr-actobindin; *row b*, sequences of tryptic peptides; *row c*, sequences of Asp-N peptides; *row d*, sequences of *Staphylococcus* aureus V8 protease peptides; *row e*, sequences of V8 protease peptides derived from the blocked NH_2-terminal tryptic peptide. The HPLC chromatograms of the purifications of the peptides in *rows a–e* are shown in Fig. 1, *B–F*, respectively. *Lower*, the sequence of residues 15–84 is aligned to illustrate the nearly identical internal repeat. Identical amino acids are connected by *solid lines* and conservative substitutions by *dotted lines*.

Three lines of evidence indicate that the deduced sequence of actobindin (Fig. 2, *upper*) accounts for the complete polypeptide. (i) The amino acid composition calculated from the sequence data is almost identical to that obtained by direct analysis of actobindin and CNBr-actobindin (minus methionine) (Table I); (ii) the sequences of all of the peptides produced by proteolysis, including those not shown, are accommodated by the sequence; and (iii) the M_r of 9682 calculated from the sequence agrees well with the value from sedimentation equilibrium (~9000). In addition, plasma desorption mass spectrometry of native actobindin and proteolytic fragments derived from it established the presence of an NH_2-terminal acetyl group.[3]

The most striking feature of the actobindin sequence is that 76% of the polypeptide is contained within two tandemly repeated, nearly identical segments of 33 and 34 amino acids (Fig. 2, *lower*); 24 of the amino acids in residues 15–47 and residues 51–83 are identical, and 5 others are conservative substitutions. Secondary structure algorithms (19, 20) predict an α-helix configuration for residues 3–14 and an α-helix-β-sheet-α-helix configuration for the two repeats (residues 33–38 and 70–75 in β-sheet and the rest of the repeat sequences in α-helix). However, the circular dichroism spectrum of actobindin (Fig. 3) is consistent with the presence of only 15% α-helix and 22% β-sheet structure. This would leave a major part of the molecule as either β-turn or coiled structure.

DISCUSSION

The symmetry in the actobindin sequence is likely to be reflected in the tertiary structure and function of the molecule. For instance, each of the repeated segments could constitute an actin-binding domain (possibly individually influenced by the nonrepetitive flanking sequences), which would explain the ability of actobindin to bind two molecules of monomeric actin (17). This hypothesis has been verified by identification of the side chains involved in chemical cross-linking between actin and actobindin.[4] Moreover, the demonstration (17) that actobindin associates with two actin monomers at high concentrations of G-actin and one actin monomer at low concentrations of G-actin is consistent with the observations (16) that actobindin inhibits the initial rate of actin polymerization much more effectively than it inhibits the rate of elongation of F-actin or the final concentration of F-actin.

Comparison of the actobindin sequence with the sequences of other actin-binding proteins reveals some interesting relationships (Fig. 4). The sequence of the first 6 residues in the two actobindin repeats is very similar to sequences in tropomyosin, muscle myosin heavy chain, paramyosin, and *Dictyostelium* α-actinin (Fig. 4*A*). Sequence similarities also occur among actobindin, mammalian profilins, and the two *Acanthamoeba* profilin isoforms (Fig. 4*B*). Although *Acanthamoeba* actin, profilin, and actobindin all contain trimethyllysine residues, there is no similarity among the sequences surrounding the trimethyllysines of these three amoeba proteins (Fig. 4*C*). However, the sequences around the 2 trimethyllysines of actobindin are remarkably similar to the sequences flanking

[3] B. R. Beavis, B. T. Chait, M. R. Bubb, and E. D. Korn, unpublished results.

[4] K. Vancompernolle, J. Vandekerckhove, M. R. Bubb, and E. D. Korn, unpublished results.

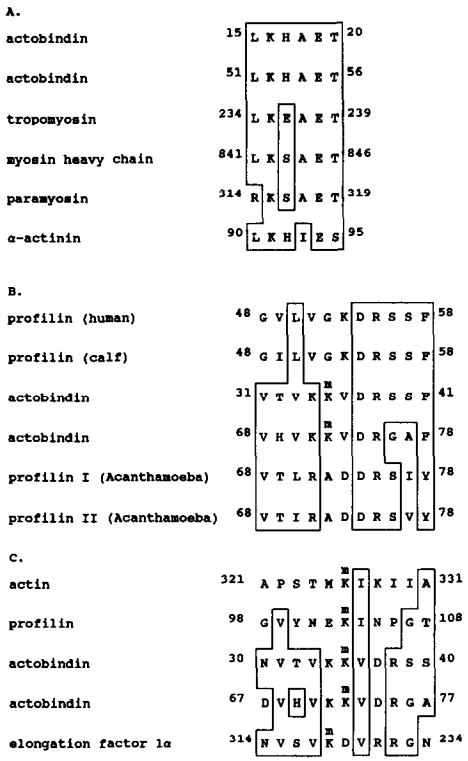

FIG. 4. **Amino acid sequence similarities between actobindin and other proteins.** *A*, comparison of actobindin with other actin-binding proteins: tropomyosin (22), muscle myosin heavy chain (23), paramyosin (24), and *Dictyostelium* α-actinin (25); *B*, comparison of actobindin with profilins (26–30); *C*, The amino acid sequences surrounding the trimethyllysine residues in *Acanthamoeba* actin (31), profilins (29, 30), actobindin, and rabbit reticulocyte elongation factor 1α (32). Positions containing identical amino acids or conservative substitutions are *boxed*.

the trimethyllysine in rabbit reticulocyte elongation factor 1α (Fig. 4*C*).

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SUPPLEMENTAL MATERIAL TO
THE COVALENT STRUCTURE OF ACANTHAMOEBA ACTOBINDIN

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EXPERIMENTAL PROCEDURES

Proteins and enzymes - Acanthamoeba actobindin was purified according to previously described procedures (15). The proteases used were trypsin and endoproteinases Asp-N from Boehringer Mannheim FRG, and Staphylococcus aureus V8-protease from Miles Laboratory Inc, Nappervilla, IL, USA. CNBr was purchased from Merck, Darmstadt, FRG. All other chemicals were analytical grade unless specified otherwise.

Protein cleavages - HPLC-purified actobindin in aliquots of ~200 µg was dissolved in 500 µl of 0.1 M Tris-HCl, pH 8.5, and digested with proteases using an enzyme to substrate ratio of 1:50 (weight/weight). Digestions were carried out at 37 °C for either 2 h (trypsin), 4 h (endoproteinase Asp-N) or 24 h (V8-protease) and terminated by the addition of TFA to a final concentration of 5%. CNBr-cleavage was carried out in 1 ml of 70% formic acid containing 1% CNBr. The reaction was allowed to proceed in the dark at room temperature for 24 h and was terminated by evaporating the solvent to dryness under nitrogen in a hood. The residue was redissolved in 500 µl of 0.1% TFA for HPLC.

HPLC procedures - TFA and acetonitrile were HPLC-grade solvents from Carlo Erba, Milano, Italy. The peptide separations were performed on a 0.46 X 25-cm, C4 reverse-phase column (Vydac Separations Group, Hesperica, CA, USA) using a Waters HPLC system (Millipore, Milford, MA. USA) consisting of a gradient controller (model 680), two pumps (model 510) and a fixed wavelength UV detector (model 411) measuring at 214 nm. The column was equilibrated in 0.1% TFA (solvent A). A gradient was applied 5 min after sample loading with a linear increase (1% per min at a flow rate of 1 ml per min) of solvent B (0.1% TFA and 70% acetonitrile). Fractions containing peptides were collected manually in Eppendorf tubes and dried in a Speed-Vac Concentrator (Savant Instruments, Inc., Farmingdale, NY, USA).

Other procedures - Acid hydrolysis for amino acid analyis was carried out using standard conditions and amino acids were quantified as their phenylthiocarbamoyl derivatives by HPLC (18). Amino acid sequences were determined with a gas phase sequenator (model 470, Applied Biosystems, Inc., Foster City, USA) equipped with an on-line PTH-amino acid analyzer (model 120A). The dried peptide was redissolved in 100 µl of 1% TFA in 30% acetonitrile and an aliquot was loaded on a precycled polybrene-coated glass fibre.

TABLE I

The amino acid composition of actobindin

The three sets of data are from: A. the acid hydrolysate of native actobindin (15); B. the sequence determination (Fig. 2, upper); and C. the acid hydrolysate of CNBr-actobindin. Amino acid compositions are expressed in mol %. Values for trimethyllysine were not determined (n.d.) for CNBr-actobindin. The values in column C were calculated assuming the same contents of trimethyllysine and methionine as in column B.

Amino Acid	A	B	C
		mol %	
Aspartic acid + asparagine	8.0	7.9	8.2
Threonine	3.4	3.4	3.2
Serine	6.3	6.8	6.2
Glutamic acid + glutamine	15.9	15.9	16.1
Proline	5.9	5.7	6.0
Glycine	6.1	4.5	5.0
Alanine	12.9	12.5	12.6
Valine	9.9	10.2	10.0
Cysteine	0	0	n.d.
Methionine	0.9	1.1	0
Isoleucine	4.4	4.5	4.3
Leucine	5.9	5.7	6.1
Tyrosine	0.2	0	0
Phenylalanine	2.2	2.3	2.1
Trimethyllysine	1.8	2.3	n.d
Lysine	9.7	10.2	10.0
Histidine	4.3	4.5	4.2
Arginine	2.4	2.3	2.5
Tryptophan	0	0	

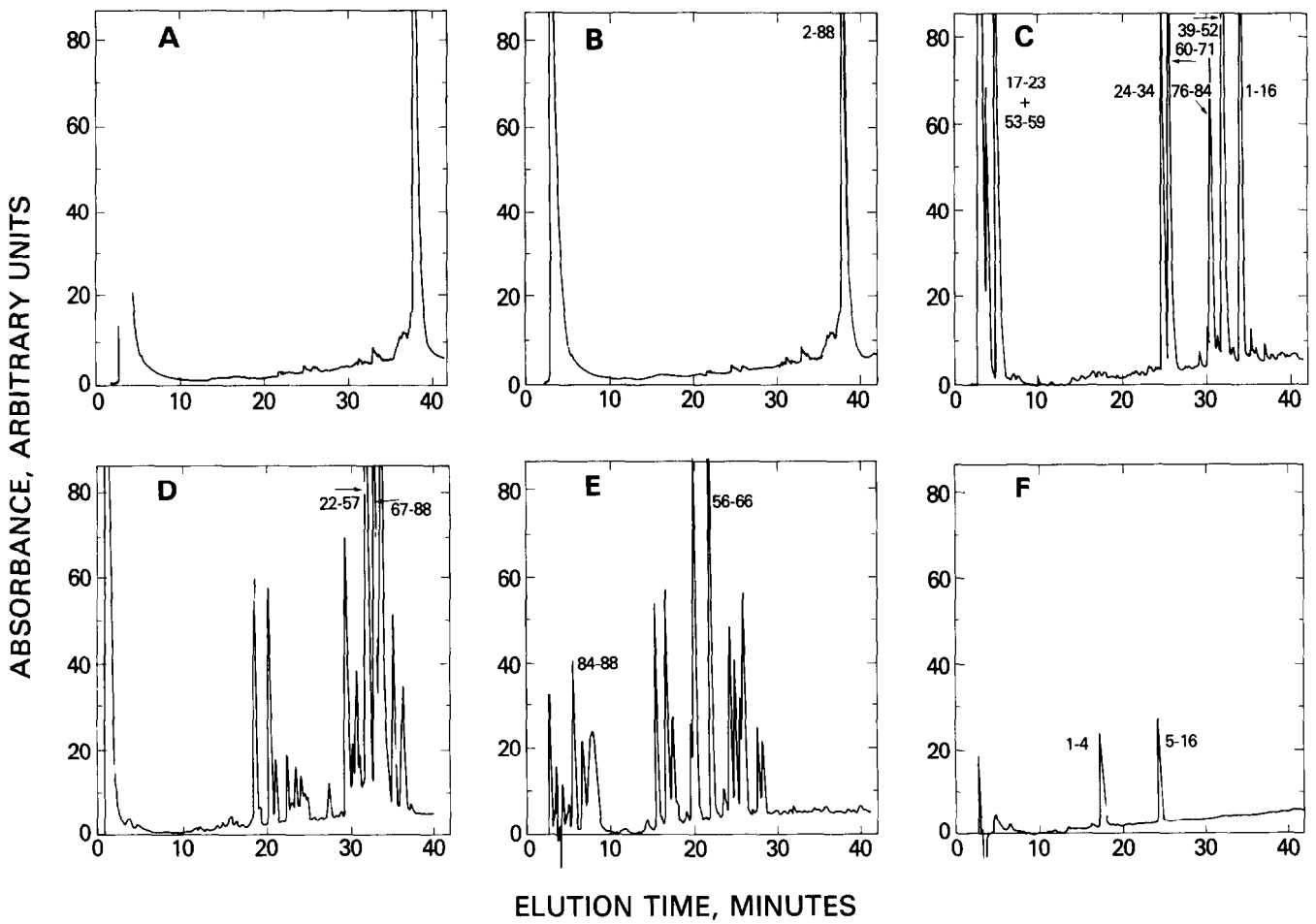

Fig. 1. **Elution profiles of the reverse-phase HPLC separations of the CNBr-fragment and proteolytic peptides used to establish the sequence of actobindin.** A. intact actobindin, B. the CNBr-fragment, C. the tryspin digest, D. the endoproteinase Asp-N digest, E. the Staphylococcus V8-protease digest, F. the digest of the blocked NH_2-terminal tryptic peptide (peptide 1-16 in panel C) with the V8-protease. The peptides whose sequences were used to establish the sequence of actobindin are identified by the positions of their first and last residues in the actobindin sequence (Fig. 2). The same conditions were used for all of the separations (see "Experimental Procedures").

TABLE II

Amino acid sequences of CNBr-actobindin and the proteolytic fragments used to determine the actobindin sequence

The peptides derived from cleavage with endoproteinase Asp-N, trypsin and Staphylococcus V8-protease are identified as Asp-N, T and V8, respectively. The number in parentheses identifies the position of the first residue of each peptide in the sequence of actobindin (Fig. 2). The purifications of the peptides are illustrated in Fig. 1. The yield in pmol of the PTH-amino acid at each step in the Edman cycle follows the amino acid (40% of each sample was applied to the sequenator).

Cycle	CNBr-actobindin	Asp-N(22)	Asp-N(67)	T(17)&T(53)	T(24)	T(39)	T(40)	T(76)	T(1)-V8	V8(56)
1	Asn 556	Asp 852	Asp 498	His 703	Ser 849	Ser 548	Ser 403	Gly 1719	Leu 203	Thr 576
2	Pro 321	Lys 891	Val 471	Ala 654	Ala 1270	Ser 409	Gly 765	Ala 1628	Gln 190	Val 620
3	Glu 376	Ser 373	His 285	Glu 344	Pro 802	Phe 615	Pro 691	Phe 1502	Ser 81	Arp 498
4	Leu 340	Ala 637	Val 410	Thr 300	Gln 765	Leu 669	Ala 792	Leu 1533	Ala 125	Lys 476
5	Gln 328	Pro 504	Lys 485	Val 204	Ile 623	Glu 673	Ile 549	Ser 763	Ile 143	Ser 148
6	Ser 178	Gln 471		Asp 165	Glu 617	Glu 601	Pro 522	Glu 1114	Gly 123	Gly 271
7	Ala 287	Ile 433	Val 346	Lys 136	Asn 611	Val 354	Glu 476	Ile 911	Gln 113	Pro 115
8	Ile 242	Glu 407	Asp 329		Val 351	Ala 448	Asp 453	Glu 713	Gly 111	Ala 175
9	Gly 286	Asn 388	Arg 152		Thr 428	Lys 436	Val 399	Lys 503	Ala 101	Ile 101
10	Gln 232	Val 362	Gly 273		Val 193	Pro 306	His 157		Ala 95	Pro 88
11	Gly 295	Thr 196	Ala 271		Lys 143	His 211	Val 293		Leu 89	Glu 48
12	Ala 251	Val 320	Phe 254			Glu 261	Lys 191		Lys 45	
13	Ala 201	Lys 214	Leu 257			Leu 170				
14	Leu 195		Ser 162			Lys 101				
15	Lys 212	Val 271	Glu 146							
16	His 105	Asp 247	Ile 152							
17	Ala 228	Arg 212	Glu 128							
18	Glu 103	Ser 103	Lys 147							
19	Thr 75	Ser 92	Ala 156							
20	Val 121	Phe 115	Ala 123							
21	Asp 96	Leu 119	Lys 100							
22	Lys 108	Glu 103	Gln 36							
23	Ser 56	Glu 109								
24	Ala 84	Val 97								
25	Pro 59	Ala 111								
26	Gln 32	Lys 102								
27	Ile 42	Pro 42								
28	Glu 26	His 51								
29	Asn 24	Glu 55								
30	Val 24	Leu 50								
31		Lys 53								
32		His 25								
33		Ala 54								
34		Glu 42								
35		Thr 11								
36		Val 8								

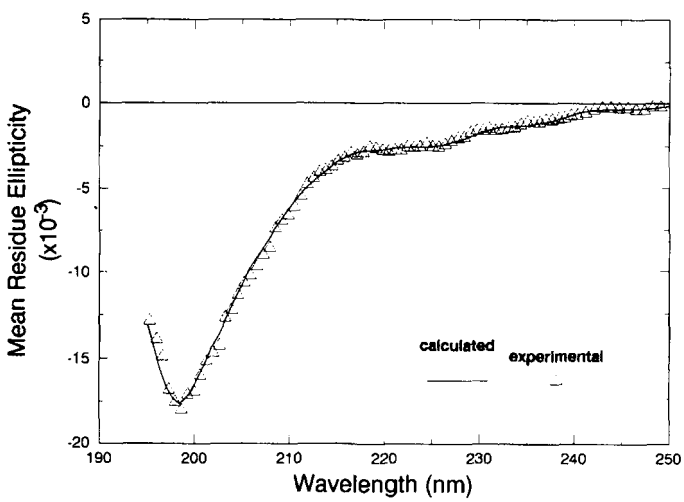

Fig. 3. **The circular dichroism spectrum of actobindin.** The sample (0.1 mg/ml) was dissolved in 10 mM potassium phosphate, pH 7.5, at 23 °C and the light path was 0.1 cm. The triangles show the experimental data and the line shows the fitted spectrum (21) corresponding to 15% α-helix and 22% β-sheet structure.