Sequence Homology of the Ca\(^{2+}\)-dependent Regulator of Cyclic Nucleotide Phosphodiesterase from Rat Testis with Other Ca\(^{2+}\)-binding Proteins

(Received for publication, August 22, 1977)

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SUMMARY

A Ca\(^{2+}\)-dependent regulator protein of cyclic 3':5'-nucleotide phosphodiesterase (EC 3.1.4.17) has previously been isolated from rat testis and shown to be a heat-stable, Ca\(^{2+}\)-binding protein with a molecular weight of approximately 17,000. The Ca\(^{2+}\)-dependent regulator protein is also structurally similar to troponin-C, the Ca\(^{2+}\)-binding component of muscle troponin and Ca\(^{2+}\) mediator of muscle contraction. The present report describes a partial amino acid sequence of the Ca\(^{2+}\)-dependent regulator. The protein (148 amino acids) is 50% homologous with skeletal muscle troponin-C, but is 11 residues shorter than the muscle protein. The Ca\(^{2+}\)-dependent regulator protein has an NH\(_2\)-terminal sequence of acetyl-Ala-Asp-Glu, a COOH-terminal sequence of Thr-Ala-Lys and 1 residue of e-trimethyllysine located at position 115. All of these properties are distinct from those of other homologous Ca\(^{2+}\)-binding proteins. These properties may account for the biological specificities demonstrated by these proteins as compared to the Ca\(^{2+}\)-dependent regulator protein. Based on the sequence and a comparison of the Ca\(^{2+}\)-dependent regulator protein to other calcium-binding proteins, our data support the view that all of these molecules contain common sequences, especially at their proposed metal-binding sites.

The cellular role of Ca\(^{2+}\) is best understood regarding its regulation of muscle contraction. The Ca\(^{2+}\)-binding subunit component of the myofibril is troponin-C (TnC), which when complexed with troponin-I and -T, regulates the interaction of actin and the myosin cross-bridges in response to changes in the intracellular Ca\(^{2+}\) levels. Calcium also regulates a number of other cellular events, such as motility, secretion, division, and metabolic activity (1-6). Non-muscle cells contain a low molecular weight protein (calcium-dependent regulator) which binds Ca\(^{2+}\) with high affinity. Originally identified as a cyclic nucleotide phosphodiesterase activator protein (7, 8), the regulator has subsequently been shown to be structurally similar to skeletal muscle troponin-C (9-13). The amino acid sequences of rabit (14) and chicken (15) skeletal muscle and bovine cardiac troponin-C (16) are known. Based on a comparison of the sequence homologies of troponin-C to carp parvalbumin, a Ca\(^{2+}\)-binding protein with a known three-dimensional structure (17), a number of investigators (18-20) have predicted the location of the four sites which bind Ca\(^{2+}\) in troponin-C.

In the present communication, we report\(^2\) on the partial amino acid sequence of rat testis regulator protein and have compared it with skeletal muscle troponin-C. Although the regulator has physicochemical properties which are similar to troponin-C (13), there are differences in the two proteins. For example, the regulator has 1 residue of \(\varepsilon\)-trimethyllysine while skeletal troponin-C has a histidine at this position (21). In addition, the regulator has four equivalent Ca\(^{2+}\)-specific binding sites, whereas two of the four sites in troponin-C also bind Mg\(^{2+}\) (22). Finally, the molecular weight of the regulator is smaller than that of troponin-C by about 1000 (12, 13). The purpose of the present study was to determine the primary structure of the regulator and to relate this information with structure-function properties of the regulator and other calcium-binding proteins.

RESULTS

Isolation of Tryptic Peptides—Regulator (2 \(\mu\)mol) was treated with trypsin and tryptic peptides were fractionated on a column of Sephadex G-50 (Fig. 1). Four major zones of peptide were detected. Rechromatography of Zone A over the same column gave T-5. Acidification of Zone B to pH 1.6 precipitated peptide T-11. The acid-soluble peptides in Zone B were subjected to ion exchange chromatography to yield T-10 and a tryptic peptide consisting of T-2 + T-3; cleavage at the lysine residue in position 21 was only partial. Acidification of Zone C to pH 1.6 yielded an insoluble peptide, T-1. Ion exchange chromatography of the acid-soluble peptides of Zone C gave peptides T-2, T-3, T-4, T-7, and T-9. High voltage

\(^*\) This research was supported in part by grants from the American Heart Association (75-818) and the National Institutes of Health (H1-07503). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^\dagger\) National Institutes of Health Postdoctoral Fellow (HD-01925-93).

\(^\dagger\) Established Investigator of the American Heart Association (1972-1977).

\(^\dagger\) National Institutes of Health Research Career Development Awardee.

\(^\dagger\) The abbreviations used are: TnC, troponin-C; CDR, Ca\(^{2+}\)-dependent regulator protein; STnC, rabbit skeletal muscle troponin-C; CTnC, bovine cardiac troponin-C; MCBP, parvalbumin.
Electrophoresis of Zone D gave peptides T-6 and T-8. The amino acid composition of each tryptic peptide is shown in Table I.

Isolation of Cyanogen Bromide Fragments—Amino acid analysis of the CNBr digest of regulator without acid hydrolysis showed the presence of both free homoserine and homo-
serine lactone, indicating a Met-Met sequence. Chromatography of the CNBr digest is shown in Fig. 2; six zones of peptides were pooled. By amino acid analysis, Zone I con-
tained methionine and probably represents uncleaved mate-
rnal. Zone II contained three peptides, CNBr VI–VIII (Fig. 3), which were not further fractionated. Rechromatography of Zone III on Sephadex G-50 yielded a fragment which did not react in the Edman degradation, indicating that it represented the NH₂-terminal CNBr fragment of the regulator. High voltage paper electrophoresis at pH 3.7 of Zone IV gave CNBr-II and -III. CNBr-V and -X were obtained by paper chromatography of Zone V. CNBr-X was the only fragment which did not contain homoserine and represents the COOH-terminal peptide of regulator. Zone VI contained free homoserine. The amino acid compositions of each of the purified CNBr fragments are given in Table II.

Sequence of Regulator—The amino acid sequence of the tryptic peptides was determined by subtractive Edman degradations (25). Some of the peptides were degraded further with chymotrypsin and thermolysin, the derived peptides were isolated, and their sequences were determined (Fig. 3). Peptide T-1 was the only tryptic peptide resistant to Edman degradation and thus represents the NH₂-terminal peptide. Treatment of T-1 with thermolysin yielded four peptides (Fig. 3). Th-1 was ninhydrin-negative and did not react in the Edman degradation. Mass spectral analysis of Th-1 gave methyl ions at 100, 128, 172, 241, and 441, indicating a sequence of acetyl-Ala-Asp-Glu. The alignment of T-2 and T-3 was based on the chymotryptic peptides from a tryptic peptide which contained T-2 and T-3; no cleavage occurred at lysine residue as trimethyllysine has recently been described (21). The thermolytic peptides of T-10 are shown in Fig. 3. The alignment of T-9 and T-10 was based on the isolation and sequence of a tryptic peptide designated CNBr-VI-T-1 in Fig. 3 which was isolated from a tryptic digest of a mixture of CNBr-VI, -VII, and -VIII (Zone II, Fig. 2). This peptide was the only peptide to have NH₂-terminal lysine and, thus, represents the COOH-terminal part of CNBr-V. Chymotryptic digestion of T-9 yielded C-1 and C-2 (Fig. 3). T-10 contained an unknown amino acid. The identification of this residue as trimethyllysine has recently been described (21). The thermolytic peptides of T-10 are shown in Fig. 3. The alignment of T-9 and T-10 was based on homology to troponin-C. T-11 represented the COOH-terminal tryptic peptide of the regulator. Thermolytic digestion of T-11 gave five peptides (Fig. 3).

Discussion

The amino acid sequence of rat testis regulator was aligned with that of rabbit skeletal muscle troponin-C as shown in Fig. 4. The two proteins are clearly similar in that approximately 50% of the residues represent homologous sequences. Many of the substitutions, in fact, are conservative replace-
ments. Regulator, however, is 7 residues shorter than tropo-
nin-C at the NH₂ terminus and a single residue shorter at the COOH terminus. Based on sequence homology to troponin-C, there is a deletion of 3 residues at positions 85–87. Overall, these changes leave the regulator 11 amino acids smaller
than troponin-C, which supports the $M_r = 1000$ difference reported previously by our laboratory (12, 13). Regulator also contains, at position 115, an unusual basic amino acid, identified as e-trimethyllysine (21); the homologous position in troponin-C represents the sole histidine residue. The physiological importance for the presence of this rare amino acid is unknown. These differences in primary sequence may reflect the marked differences in biological, structural, and metal-binding properties reported earlier by our laboratory (12, 13). Each protein is a poor substitute for the other in regulating cyclic nucleotide phosphodiesterase or actomyosin ATPase (12). They also have distinct differences in their structural conformation as determined by circular dichroism and tryptophan fluorescence (13). In addition, troponin-C contains three classes of metal-binding sites (22), while testis regulator contains four equivalent Ca$^{2+}$-specific sites (13).

Based on the homology between parvalbumin and troponin-C, Kretsinger and Barry (28) have constructed a model of the predicted three-dimensional structure of troponin-C, including the four metal-binding sites. Again, due to sequence homology, the Ca$^{2+}$-binding sites of testis regulator can be predicted (18). Compiled in Fig. 5 are the metal-binding sites of various Ca$^{2+}$-binding proteins. The $X$, $Y$, and $Z$ notation represents the octahedral binding coordinates located in the EF helical loop required for high affinity metal binding (28). The most outstanding features are the aspartate located at the $X$ and $Y$ coordinates and glutamate at the $-Z$ coordinate. In addition, glycine is invariant between the $Y$, $Z$ and the $Z$, $Y$ calcium coordinate residues (Fig. 5). This latter characteristic appears to be unique for the lower affinity, Ca$^{2+}$-specific sites (18). The exception to these observations is bovine troponin-C site 1, which has leucine and alanine, respectively, at the $X$, $Y$ positions and glutamate replacing the invariant glycine between the $Y$, $Z$ positions. As proposed by vanEerd and Takahashi (16), this site is defective and the protein binds just one Ca$^{2+}$ ion specifically (18).

The Ca$^{2+}$-dependent regulator has been found in essentially all eukaryotic cells examined and is in considerable excess compared to that necessary to regulate cyclic nucleotide phosphodiesterase (29, 30). The protein is furthermore found in high quantities in secretory tissues, including adrenal medulla, testis, platelets, and brain (13, 29, 31-33). It has also been shown to regulate, via Ca$^{2+}$, several enzyme systems including phosphodiesterase (34, 35), adenylate cyclase (36, 37), skeletal muscle actomyosin ATPase (12), and recently erythrocyte membrane Ca$^{2+}$-ATPase (38, 39). Wang and Desai (40) have isolated an additional unidentified protein which binds regulator in a Ca$^{2+}$-dependent manner. Calcium is well known to be involved in regulating cellular motility, secretion, division, and metabolic activity (1-6). It has been estimated that the Ca$^{2+}$ levels increase from $10^{-8}$ to $10^{-6}$ M during cell activation (4). Regulator ($K_{d, Ca^{2+}} \sim 10^{-6}$ M) also displays significant conformational changes and subsequent phosphodiesterase activation within these calcium concentrations (13). In addition, using monospecific antibody to testis regulator, our laboratory has localized this protein within cells by indirect immunofluorescence. Regulator fluorescence is associated with filamentous cables in the cytoplasm, the mitotic apparatus, and the cytoplasmic space of late telophase. Collectively, these observations suggest that the regulator plays a fundamental cellular role as a Ca$^{2+}$ receptor or mediator of Ca$^{2+}$-stimulated events.

Acknowledgments—We are grateful to Drs. D. M. Desiderio and Vijay Mahajan for performing the mass spectral analysis of the NH$_2$-terminal tripeptide.

* J. M. Welch, J. R. Dedman, B. R. Brinkley, and A. K. Means, manuscript in preparation.

### Table 1: Ca$^{2+}$-Specific Sites

| Protein | $X$ | $Y$ | $Z$ | Reference |
|---------|-----|-----|-----|-----------|
| CDR-I   | Asp-Lys-Asp-Gly-Asp-Gly-Thr-Ile-Thr-Thr-Lys-Glu | 20 | 31 | Present Comm. |
| CDR-II  | Asp-Asp-Asp-Gly-Asp-Gly-Thr-Ile-Thr-Thr-Lys-Glu | 56 | 67 | Present Comm. |
| CDR-III | Asp-Lys-Glu-Asp-Glu-Thr-Ile-Thr-Thr-Lys-Glu | 93 | 104 | Present Comm. |
| CDR-IV  | Asp-Ile-(Asp,Glu,Asp,Glu,Asp)-Val-Asp-Thr-Glu | 129 | 140 | Present Comm. |
| STnC-I  | Asp-Ala-Asp-Glu-Gly-Gly-Glu-Thr-Asp-Lys-Glu | (14) | |
| STnC-II | Asp-Ala-Asp-Glu-Gly-Glu-Thr-Asp-Lys-Glu | (14) | |
| CnC-I   | Leu-Glu-Ala-Asp-Glu-Gly-Glu-Glu-Asp-Lys-Glu | (16) | |
| CnC-II  | Asp-Glu-Asp-Glu-Ser-Thr-Asp-Lys-Glu | (16) | |

### Table 2: Ca$^{2+}$-Mg$^{2+}$ Sites

| Protein | $X$ | $Y$ | $Z$ | Reference |
|---------|-----|-----|-----|-----------|
| STnC-I  | Asp-Glu-Asp-Glu-Glu-Thr-Asp-Lys-Glu | (14) | |
| STnC-IV | Asp-Glu-Asp-Glu-Glu-Thr-Asp-Lys-Glu | (14) | |
| CnC-I   | Asp-Glu-Asp-Glu-Glu-Thr-Asp-Lys-Glu | (16) | |
| CnC-IV  | Asp-Glu-Asp-Glu-Glu-Thr-Asp-Lys-Glu | (16) | |
| MCBP-CD | Asp-Glu-Asp-Glu-Glu-Thr-Asp-Lys-Glu | (17) | |
| MCBP-CD | Asp-Glu-Asp-Glu-Glu-Thr-Asp-Lys-Glu | (17) | |

*Fig. 5. Comparison of proposed Ca$^{2+}$-binding sequences of different Ca$^{2+}$-binding proteins. The roman numerals represent the order of the Ca$^{2+}$-binding sites beginning at the NH$_2$-terminus (16).*
Supplemental Material to

346 Sequence Homologies in Ca2+-binding Proteins

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EXPERIMENTAL PROCEDURE

Methods of 32P-CID were isolated from rat liver by the method described by Proskauer and co-workers. Column chromatography was performed on Sephadex G-25. The purity of the proteins was determined by electrophoresis in a polyacrylamide gel. The proteins were then subjected to further electrophoresis in a SDS-polyacrylamide gel. The gels were stained with Coomassie Blue and the protein bands were visualized. The bands were then excised and the proteins were subjected to further analysis.

Table 1. Sequence composition of tropomyosin peptides

| Peptide | Sequence |
|---------|----------|
| Peptide A | KVQKQLPQF |
| Peptide B | KVQKQLPQF |

**Figures**

Figure 1: Chromatography of the trypsin digest of the Ca2+-binding protein from rat liver. The protein was dissolved in 0.1 M phosphate buffer pH 7.0 and incubated at 37°C for 24 hours. The digestion of the protein was monitored by SDS-PAGE.

Figure 2: Western blots of tropomyosin peptides resolved by SDS-PAGE. The blots were stained with Coomassie Blue, and the protein bands were visualized. The bands were then excised and subjected to further analysis.

**Tables**

Table 2. Details of sequence composition of tropomyosin peptides

| Peptide | Sequence |
|---------|----------|
| Peptide C | KVQKQLPQF |
| Peptide D | KVQKQLPQF |

The data in Table 2 were obtained from the analysis of the peptides resolved by SDS-PAGE. The details of the sequence composition of tropomyosin peptides are shown in Table 2. The peptides were subjected to further analysis to determine their amino acid composition.
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*J. Biol. Chem.* 1978, 253:343-346.

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