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Molecular modelling of N-terminal region of human cardiac myosin light chain 2 induced during cardiac hypertrophy

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Studies on the expression of phosphorylatable myosin light chain 2 (MLC2) in the cardiac tissues obtained from patients with various cardiac anomalies have shown an increased expression of MLC2 in both atrial and ventricular biopsy tissues of patients with atrial septal defect and ventricular septal defect. Also, in an attempt to predict the three-dimensional structure and to identify the site of phosphorylation of the N-terminal region of this inducible human cardiac MLC2, a model has been proposed based on computer analysis. The proposed model has indicated that this phosphorylatable N-terminal region falls in the helical conformation and the possible site of phosphorylation could be serine 19, which is at the centre of the helix and might be involved in the conformational changes during muscle contraction.

MYOSIN, one of the major constituents of muscle fibre, consists of two myosin heavy chains (MHC), two alkali light chains (MLC1) and two regulatory phosphorylatable myosin light chains (MLC2)[1]. MLC2 has a characteristic hydrophobic head, as the N-terminal end, which binds to the actin during actomyosin complex formation. The regulation of myosin/actin interaction, crucial to force generation and enzymatic activity of myosin, is mediated by the regulatory light chains. MLC2 is phosphorylated by a Ca^{2+}-stimulated light chain kinase and dephosphorylated by phosphatase C[2]. Phosphorylation-induced changes of MLC2 are evident in smooth and skeletal muscles. Phosphorylation of smooth muscle MLC2 leads to contraction and dephosphorylation results in relaxation[3, 4]. In scallop adductor muscle, MLC2 inhibits actin-activated myosin ATPase activity and this inhibition is relieved upon binding of calcium to myosin[5]. Removal of skeletal muscle MLC2 is known to cause a decrease in V_{max} of actin-activated myosin ATPase and its potention depends[6, 7] upon phosphorylation of MLC2. In cardiac muscle, the precise role of phosphorylation of MLC2 in myosin/actin interaction and subsequent ATPase activity is not known. The amino acid sequences derived from chicken, rat and human cardiac MLC2 genes share significant homology in the region of phosphorylatable serine and in the basic N-terminal region but are divergent in the C-terminal region[8 – 10].

Recent studies have established that a significant increase occurs in MLC2 content and in the mRNA levels during myocardial hypertrophy in both rat and human[11–14]. Unlike MHC, changes in α-actin isoforms have also been observed both in rat and human during cardiac hypertrophy. Thus, study of the role of MLC2 in actin/myosin interactions may suggest the underlying pathogenetic mechanisms during cardiac hypertrophy. Presently, in order to see whether MLC2 expression is induced invariably in all cardiac anomalies leading to hypertrophy, the expression of MLC2 in cardiac tissues of patients with hypertrophic heart in various cardiac disorders has been studied. These studies have demonstrated that the expression of MLC2 is induced in both atrial and ventricular tissues of patients with various cardiac anomalies.

This along with the information of sequence homology of the N-terminal region of cardiac MLC2 of rat and human led us to make an attempt on the structural studies of the phosphorylatable N-terminal region of MLC2 in order to gain an insight into the role of phosphorylation

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tion of MLC2 in myosin–actin interaction and subsequent ATPase activity.

Materials and methods

Biopsy samples of cardiac muscle tissues from patients with atrial septal defect (ASD), ventricular septal defect (VSD), mitral valve stenosis and infective endocarditis were collected during surgical correction. Normal samples were collected at autopsy from healthy persons who died due to traffic accidents. Clinical diagnosis in patients was confirmed by 2D echocardiography and/or angiocardiography.

Probe used

The 769 bp EcoRI fragment corresponding to the 166 amino acid coding for human cardiac MLC2 cDNA[10].

Database used

Brookhaven Protein Data Bank (PDB)[15].

Software used

Genetics Computer Group (GCG) Version 6.2 (ref. 16) and in-house developed software.

Computer used

Microvax II.

RNA preparation

Total cellular RNA was isolated from cardiac tissues by the guanidinium thiocyanate method of Chomczynski and Sacchi[17]. The RNA was precipitated with isopropanol, washed twice with 80% ethanol and dissolved in DEPC-treated 2 mM EDTA (pH 8.0) solution.

Slot–blot hybridization analysis

From the total RNA isolated from each tissue 12 μg was taken in 6× SSC buffer, denatured and three different concentrations (2, 4 and 6 μg) were loaded onto the nylon membrane by using BRL Hybrid Slot–blot apparatus. RNA in the membrane was UV-cross-linked for 3 min in a UV transilluminator. The filter was prehybridized for 4 h at 42°C in 6× SSC, 50% formamide, 10% dextran sulphate and 5× Denhardt's solution. Hybridization was carried out in the same solution with the addition of ^{32}P-labelled human cardiac MLC2 cDNA probe. The filter was washed successively in 2× SSC with 0.1% SDS and 1× SSC with 0.1% SDS for 30 min at 42°C, followed by a stringent wash in 0.1× SSC with 0.1% SDS for 10 min at 42°C. The filter was then air-dried and autoradiographed at –70°C for 48 h.

Labelling

The 769 bp EcoRI fragment of human cardiac MLC2 was labelled with α-^{32}P-dCTP (3000 Ci/mmol) using Pharmacia Oligolabelling Kit.

3D model prediction

By using in-built programs like wordsearch[18], Gap[19], Fasta[20] and Bestfit, the sequence homology search was done for the first 25 amino acid residues of the N-terminal region of human cardiac MLC2, which read as follows:

'M A P K K A K K R A G G A N S N V F S M F E Q T Q'.

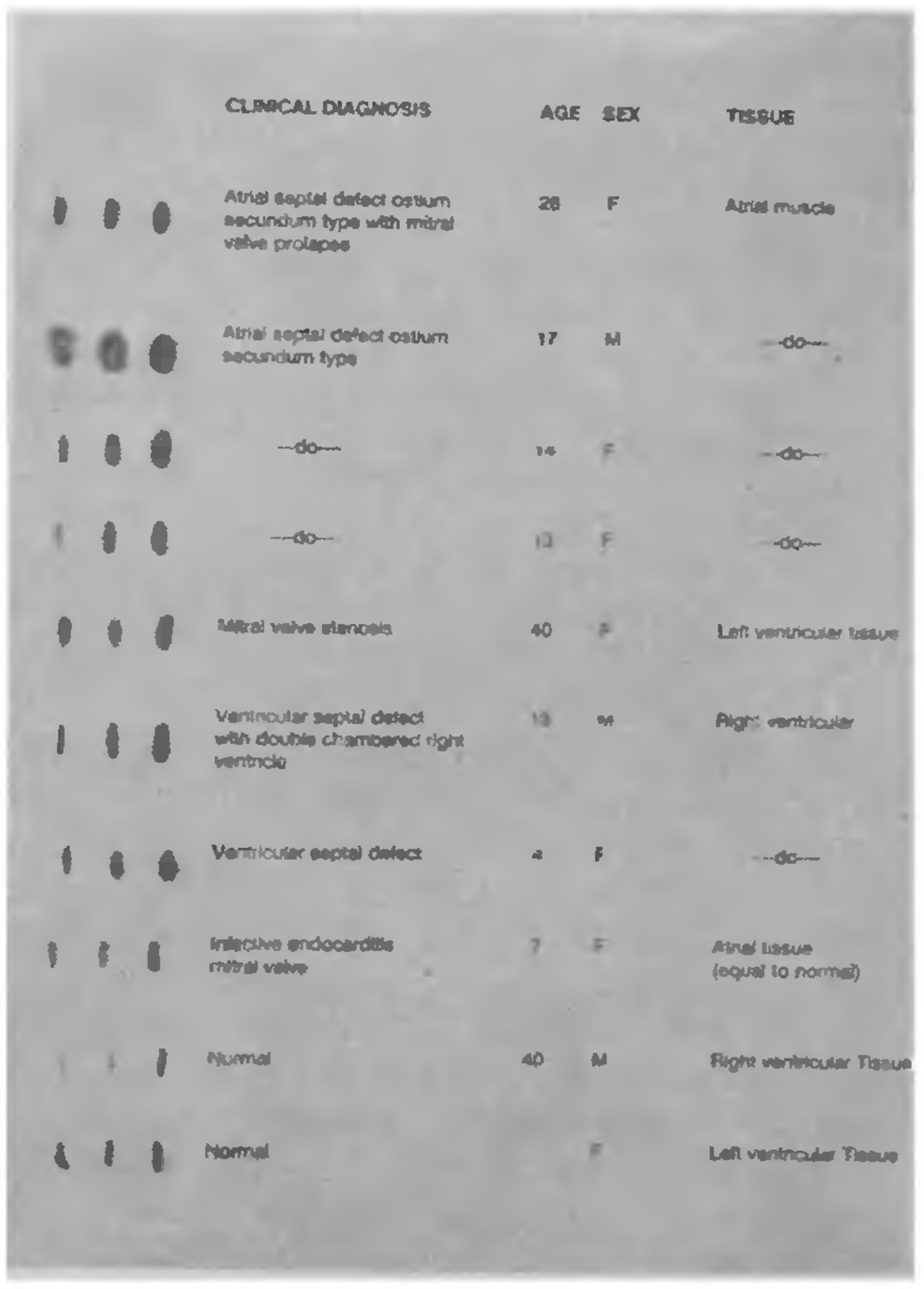


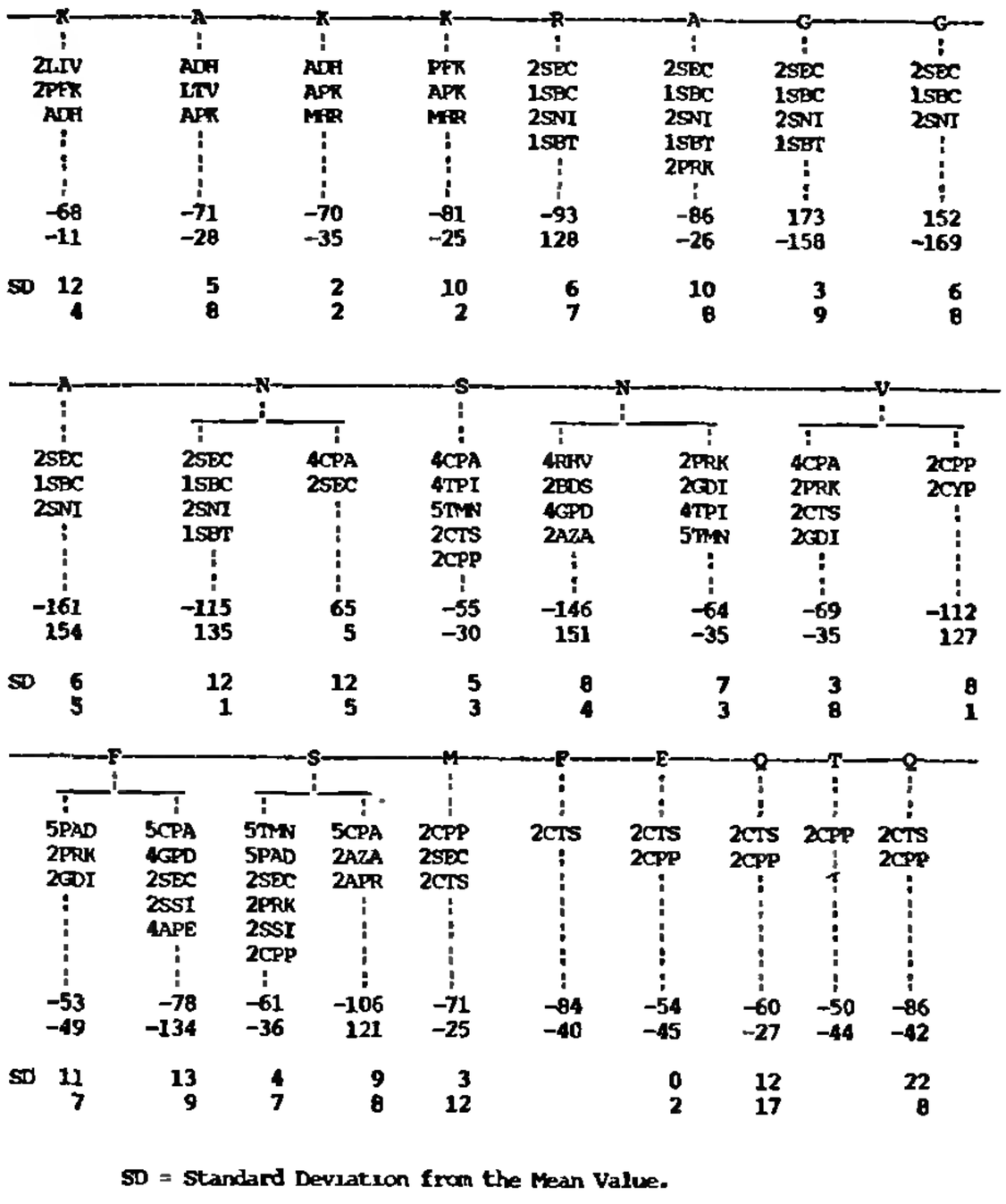
Figure 1. Total RNA isolated from atrial and ventricular biopsy tissues of patients with different cardiac anomalies were blotted as three different concentrations (3, 6 and 9 μg) and hybridized with ^{32}P-labelled human cardiac MLC2 cDNA probe. The filter was autoradiographed for 48 h at –70°C.

The PDB coordinates were extracted for the required proteins and their respective ϕ and ψ angles were calculated using in-house developed Fortran programs. The figures were drawn using GKS Pluto programme.

Results

Increased synthesis of messengers for ventricular MLC2 has been observed in the pressure-overloaded, hypertrophied rat heart[11, 12]. An increased level of MLC2 messages has been observed in the left human atria of patients with mitral valve stenosis and pressure overload[14]. These reports prompted us to analyse the level of MLC2 mRNA in the biopsied tissues obtained from patients with ventricular and atrial septal defect and with *mitral valve stenosis. The total RNA isolated from the* atrial and ventricular tissues was blotted onto a nylon membrane and hybridized with ^{32}P-labelled human ventricular MLC2-specific cDNA probe. The results show (Figure 1) a very high intensity of hybridization signals in atrial and ventricular biopsy tissues obtained from patients with ASD or VSD compared to that of normal samples. In a 17-year-old patient with defined hypertrophy of left atria, the messengers level for MLC2 was very high. All other atrial samples obtained from females of different groups showed almost equal intensity of signal, except for a 7-year-old female, who had a mitral valve defect due to infective endocarditis. Similar results were obtained in ventricular samples of patients with VSD.

The above observations clearly demonstrate that the induction of MLC2 is a common event during myocardial hypertropy in both rat and human and may play an important role in myosin/actin interaction and subsequent ATPase activity. In a recent publication[10] one of the present authors has elucidated the amino acid sequence of human cardiac MLC2 based on the nucleotide sequence and compared it with the amino acid sequence of rat MLC2. This comparison has indicated that they share 92.2% sequence homology with the conservation of their N-terminal regions. Further, for the serines present at positions 15th and 19th, which may bring about the conformational changes upon phosphorylation in the N-terminal region of human cardiac MLC2, a theoretical three-dimensional structure was predicted.



SD = Standard Deviation from the Mean Value.

Figure 2. *Conformational tree showing conformational preference* of amino acids in the best aligned proteins

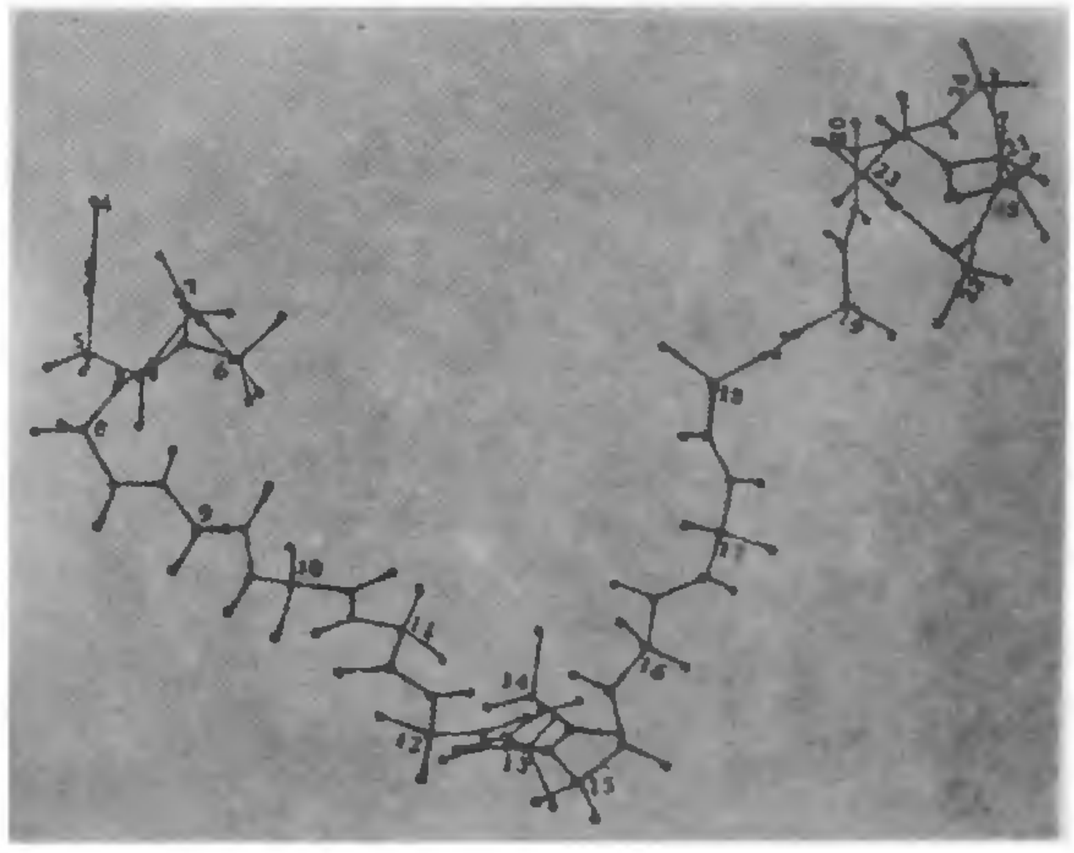


Figure 3. Conformational model 1 Backbone atoms alone are shown and C_α's are numbered according to the sequence

Table 1. Number (n) of coordinates containing proteins matched to each fragment

Sl No	Fragments	n
1	M A P K K A K K R A	10
2	R A G G A N	9
3	G G A N S N V F S	16
4	N S N V F S M	10
5	S N V F S M F E Q T Q	11

For this purpose, the first 25 amino acid residues of the N-terminal region of human cardiac MLC2 were compared with the amino acid sequences of 560 proteins in Brookhaven Protein Data Bank (PDB)[15]. Only a few fragments showed maximum similarities with the existing proteins. Interestingly, these fragments do contain superimposable regions. Such fragments are shown in Table 1. Some of the fragments in Table 1 do show per-

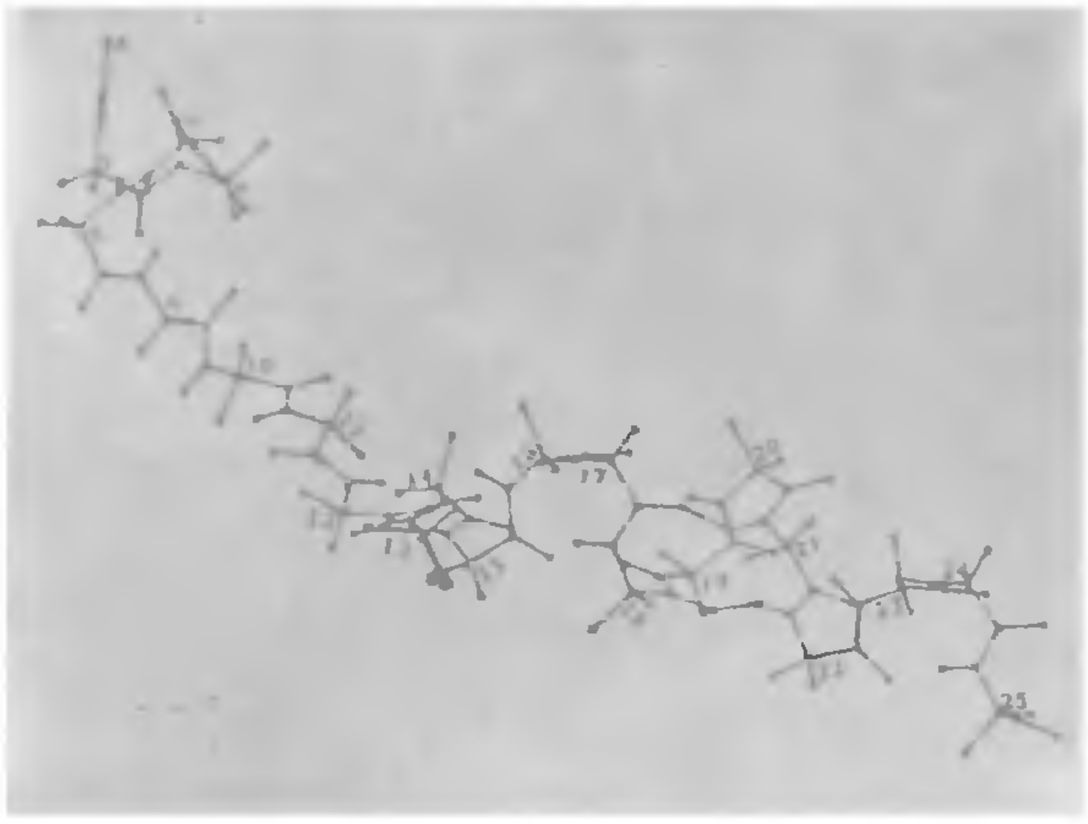


Figure 4. Conformational model 2. Backbone atoms alone are shown and C_α's are numbered according to the sequence.

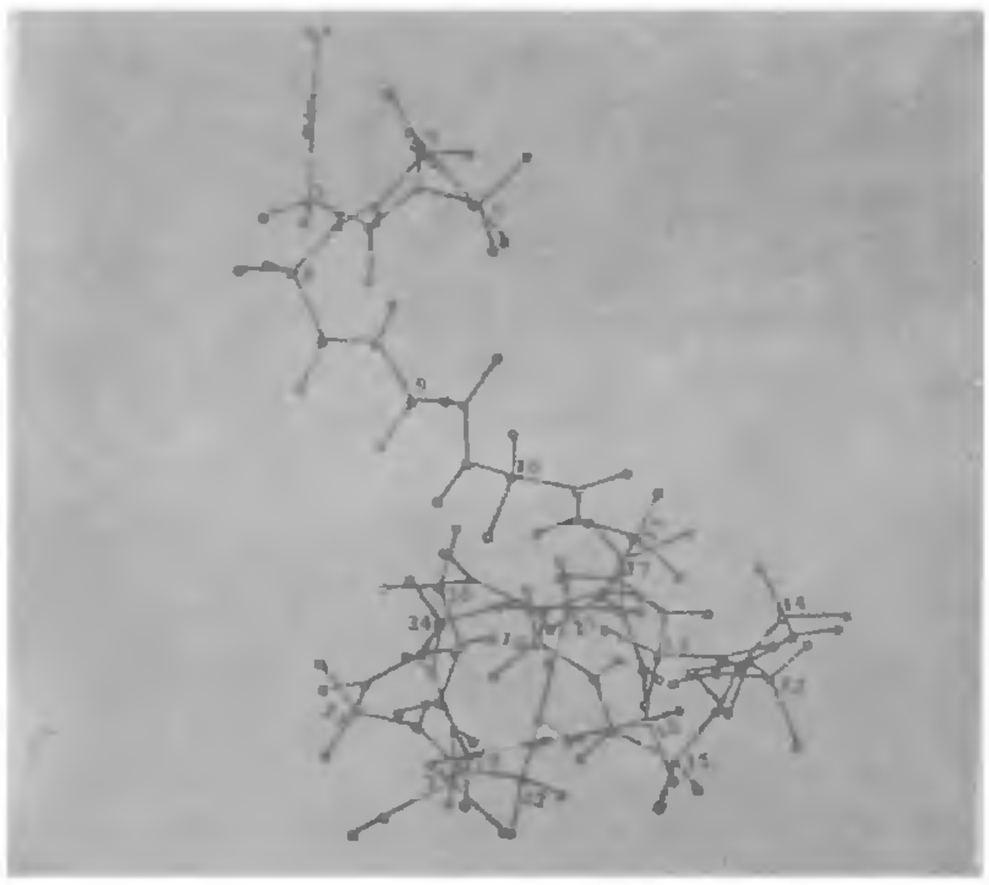


Figure 5. Conformational model 3. Backbone atoms alone are shown and C_α's are numbered according to the sequence.

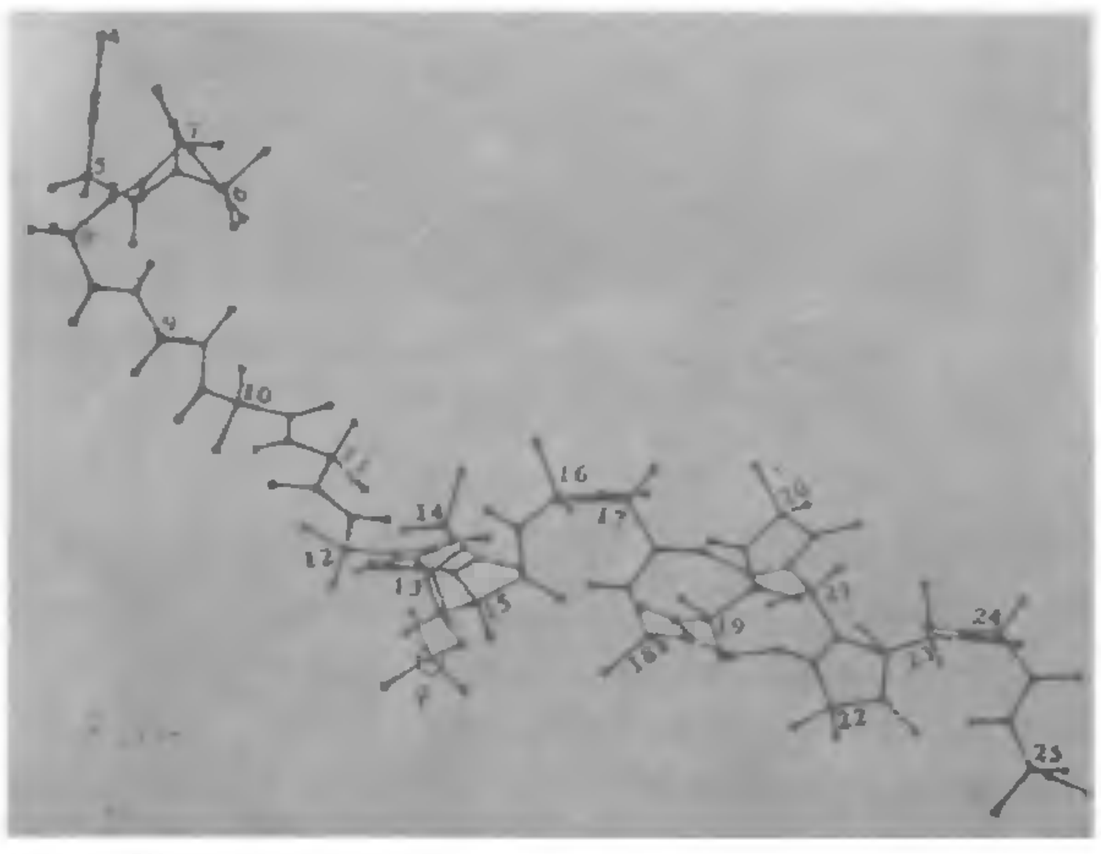


Figure 6. Phosphate group on S15 in model 2. The phosphate group is indicated as P.

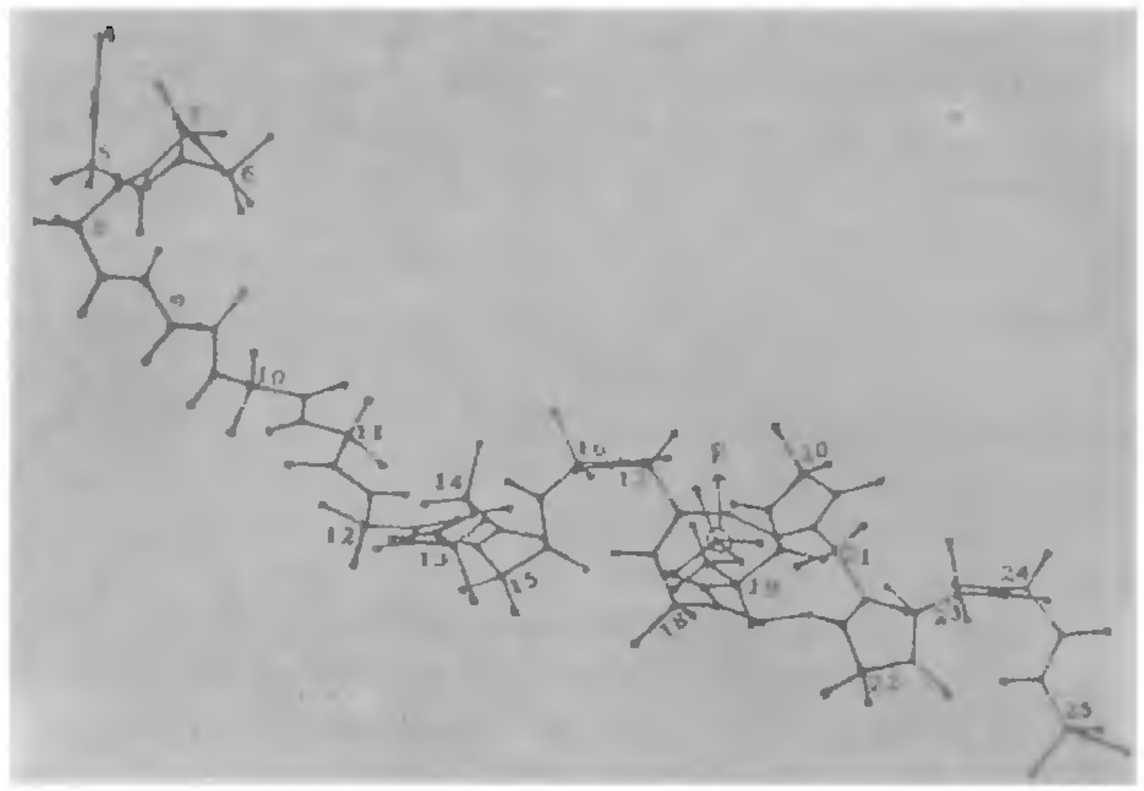


Figure 7. Phosphate group on S19 in model 2. The phosphate group is indicated as P.

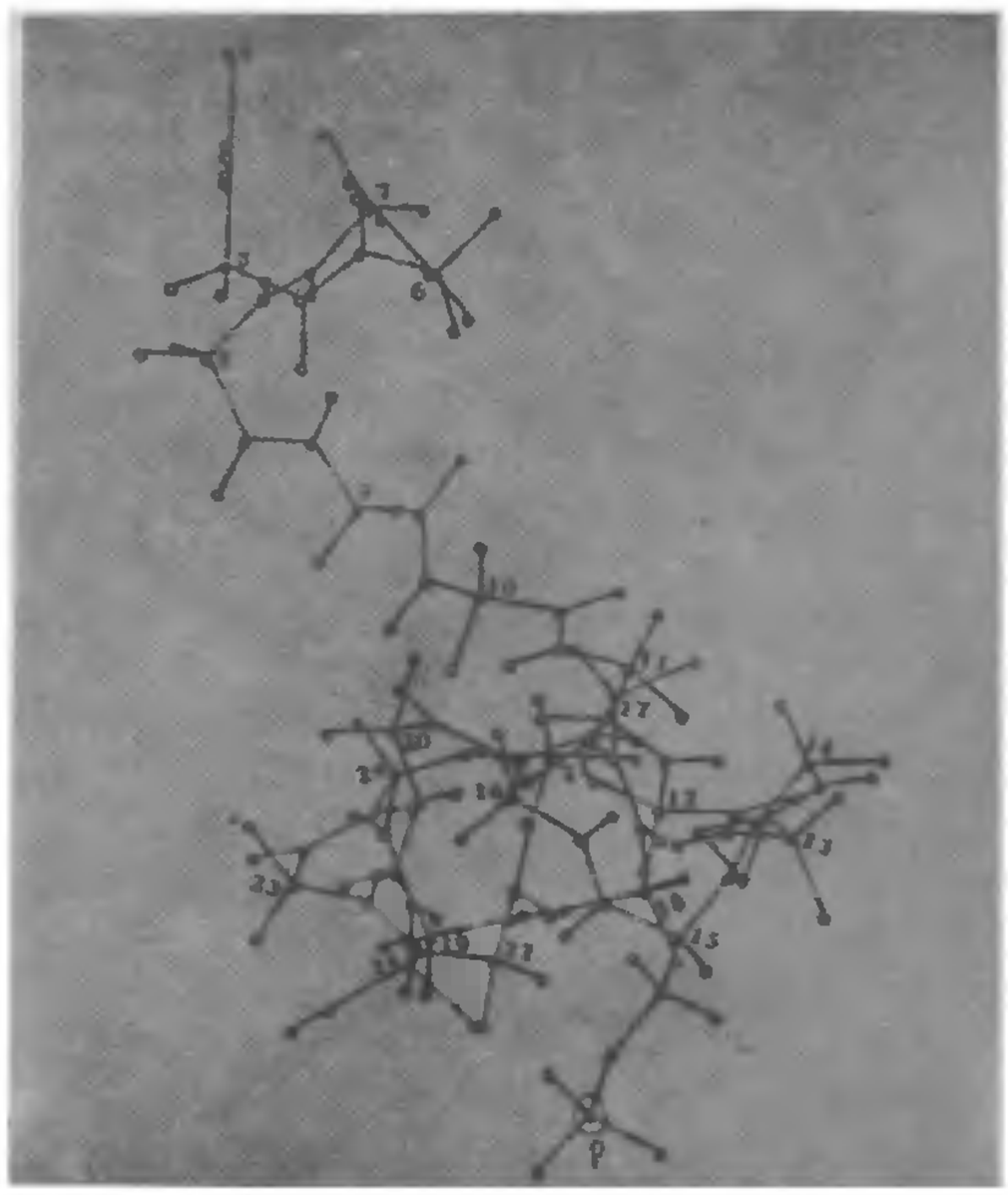


Figure 8. Phosphate group on S15 in model 3. The phosphate group is indicated as P.

fect matches (fragments 2 and 4) and some other fragments have more than 60% match with the rest of the amino acids having complementarity (fragments 1, 3 and 5). This table is restricted only to proteins having crystal structure coordinates. In a protein structure, the Ramachandran's peptide conformational angles (ϕ, ψ) play a crucial role in determining the three-dimensional structure[21, 22]. The Ramachandran angles were computed for the best aligned fragments from the existing crystal structure data. Since the first four amino acids M, A, P

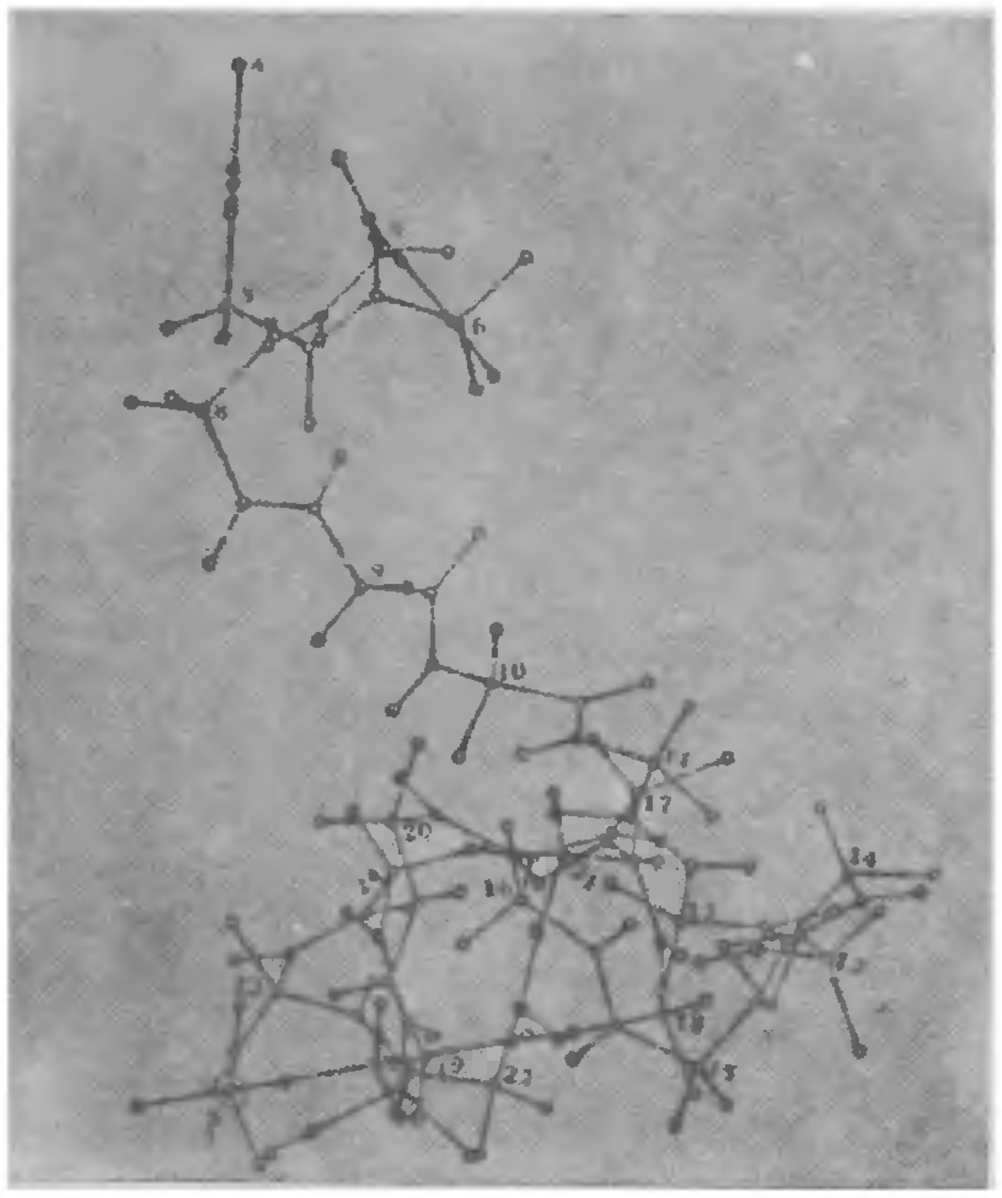


Figure 9. Phosphate group on S19 in model 3 The phosphate group is indicated as P

and K do not have sufficient data from sequence alignment for valid interpretation, and because of their occurrence at the N-terminal tail end, these four amino acids are left as a loose fit in our modelling.

In the model building, the conformational angles were calculated (based on the sequence similarity) from the PDB data files, which contain cartesian coordinates of the protein atoms. The proteins which contain maximum sequence similarity were used in the calculation of (ϕ, ψ) angles and the conformational tree is constructed based on the predominancy of the occurrence of (ϕ, ψ) angles together with the corresponding proteins. This is shown in Figure 2. The results indicate that most of the amino acids do favour a single conformation, with the exception of five amino acids: N14, N16, V17, F18 and S19. These five amino acids favour two different conformations which lead to the possibility of generating 2^5 models (32 models) for the backbone. Though the number of possible models is 32, a few models were selected based on the dominant conformational preference and favourability of regular structures such as helical and β-sheets. Such models are shown in Figures 3 to 9. Model 1 (Figure 3) has two helices (5–8, 20–25) and two sheets (8–12, 15–20) flanked in between by a turn (12–15). A cursory look at the conformational tree (Figure 2) indicates that though N16, V17, F18 and S19 favour two conformational angles, one of them always falls in the helical region (ϕ value in the range $-61 \pm 8°$ and ψ value in the range $32 \pm 7°$). If this is considered as favoured conformation then the region of amino acids S15 to Q25 will tend to form a long stretch of helical structure. This is shown as model 2 (Figure 4). In this model the residues 8th to 12th tend to fall on a sheet structure, but N14 has two possible conformational preferences and hence can orient the helical structure in two different ways with respect to the β-sheet (Figures 4 and 5). Figure 5 corresponds to conformational model 3. The interesting feature of this model is that the serine 19 (S19) falls in the middle of the helical structure and serine 15 (S15) at the base of the helix. Since models 2 and 3 have a long stretch of helical structure, which will lead to structural stability, one can conclude that models 2 and 3 will give the preferred structure for MLC2 rather than model 1.

In order to study the effects of phosphorylation of S15 and S19 on the structural modification, phosphate groups were incorporated into models 2 and 3 without stereochemical constraint to the backbone. This is shown in Figures 6–9. This model indicates that the phosphate group added to S15 just hangs outside and no way can it push the side-chains of the nearer amino acids apart. In the case of S19, though the added phosphate group, being at the centre of the helix, does not have stereochemical clash with the backbone, there is a possibility that it can push some of the side-chains nearer to it. Thus, phosphorylation of MLC2 at S19 rather than at S15 may be involved in the conformational changes during contraction and relaxation cycle.

Discussion

The changes in the levels of ventricular MHC isoforms that occur during the development and chronic overloading of heart in rats have been accounted for by the changes in the levels of respective mRNAs[23]. The previously reported increase in the rate of incorporation of ^{3}H-leucine into the phosphorylated form of MLC2 in pressure-overloaded hypertrophic hearts in rats[11] has also been shown to be due to the increase in the levels of MLC2 mRNA[12]. A similar increase in the mRNA levels of skeletal α-actin occurs in adult rat heart after aortic stenosis[24]. Although distinct MLC2 isotypes have previously been reported in human adult right and left atria, the left atrium contains an additional ventricular type MLC2. Stenosis of the atrial valve causes marked elevation of left atrial pressure and subsequent atrial hypertrophy. In patients with such a condition of stenosis of left atrium valve, significant amount of ventricular MLC2 isoform has been identified[14]. Cummins[14] observed an apparent transition in terms of MLC2 composition from atrial to ventricular type in the left atrium of patients with mitral valve stenosis and subsequent hypertrophy. Such a transition has also been observed in developing human heart[25].

Cummins' observation is substantiated by the present observation of increased ventricular MLC2 transcripts not only in the atrial samples from patients with atrial septal of ostium secundum type, but also in the left ventricular samples from patients with mitral valve stenosis. In fact, except in the case of infective endocarditis, in all other defective hearts the level of MLC2 transcripts is elevated both in ventricular and atrial samples. This suggests an activation of MLC2 gene in the heart under various defective conditions with resultant hypertrophy, which may either be an adaptive response or a pathological phenomenon. The functional significance of such an activation of the regulatory (MLC2) gene in various diseased hearts is not clear.

The observation of uniform expression of MLC2 in hypertrophic hearts along with the sequence similarity in rat, human and chicken in the N-terminal region suggests that MLC2 may have a uniform structural dependence for a presumed function of contractile regulation during actomyosin interaction. MLC2 has a characteristic N-terminal end which binds to the actin in the actin/myosin ATPase complex. Electron microscopic observations have shown that both the N- and C-terminals of MLC2 are located in close proximity to each other on the heavy chain[26]. Using cysteine mutants of recombinant MLC2, it was established that the bulk of MLC2 is located within a region about 3 nm long at the head rod junction[27, 28]. Recently, Wadgaonkar *et al.*[10] have identified a highly conserved central domain of 20 amino acids in human cardiac MLC2 which apparently participates in the MLC2/MHC interaction. However, the role of MLC2 in muscle contraction is not yet fully understood. It has been reported that in smooth muscles MLC2 phosphorylation increases the ATPase activity[3, 4]. However, in skeletal muscles, phosphorylation of MLC2 leads to downward modulation in the actomyosin turnover rate[29]. The regulatory light chain is required for folding of the smooth muscle myosin. Phosphorylation of smooth muscle MLC2 at serine 19 by MLC kinase favours unfolding of the myosin tail[30]. Amino acid comparison analysis of rat and human cardiac MLC2 reveals the significance of preserving the 15th and 19th serine in the N-terminal region, which may be involved in the conformational changes during the contraction cycle. Although rat cardiac MLC2 has few more serines at positions 14th and 2nd, this may remain unimportant as these positions are changed to (A) and (N) in human cardiac MLC2. The phosphorylation of MLC2, that occurs at the N-terminal serines, may either be specifically restricted to the 15th and 19th serines or the phosphorylation of serines at the 14th and 2nd position may not be of functional significance. This is further substantiated by the fact that in chicken too the serine at position 14 is changed to (N) as in the case of human[8–10]. The conformational changes which may be induced by the phosphorylation of serines at positions 15th and 19th may be quite different from each other, as the serine residue at the 15th position is internal and that at the 19th is an external residue.

An interesting finding of our study is the conformational preference of amino acids when they occur in a particular sequence. A set of five amino acids spanning the region of phosphorylation showed ambiguity in conformational preference. They preferred either β-sheet or α-helical conformation. But the preference for α-helix by these amino acids leads to a long stretch of helical structure for MLC2 and hence the favoured conformation (models 2 and 3). Adding a phosphate group to S15 (base of the helical structure) may not allow the side-chains of the nearer amino acids to be pushed off. On the other hand, a phosphate group added to S19 can push the side-chains of some of the neighbouring amino acids because of its occurrence at the centre of the long stretch of helical structure. By comparing the conformational requirement of myosin light-chain kinase (MLCK), the question of whether the addition of phosphate group is to S15 or to S19 was resolved based on the generated model of the long stretch of helical conformation. MLCK is known to have three functional regions, viz. connecting peptides 774–788, pseudosubstrate 787–807 and calmodulin binding region 796–813. Among these regions, the amino acid residues 769–807 tend to perform a dual function as calmodulin as well as pseudosubstrate binding sites and this region tends to form a helical structure[31]. Our model predicts that MLC2 tends to fall in the helical conformation like the pseudosubstrate. Our proposed models do indicate that the most probable candidature for phosphorylation is S19, which is present at the centre of the helix and might be involved in the conformational changes during muscle contraction.

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