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Calcium- and Magnesium-Dependent Conformational States of Calmodulin As Determined by Nuclear Magnetic Resonance†

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ABSTRACT: The effects of calcium and magnesium on the solution conformation of calmodulin have been studied by using 360-MHz proton nuclear magnetic resonance. Resonance assignments to tyrosine-99 and -138, histidine-107, ϵ-trimethyllysine-115, and a uniquely high-field shifted phenylalanine have been made in the spectra of the metal-free protein, the magnesium-saturated protein, and the calcium-saturated protein. These resonances have been monitored as calcium and magnesium are added to the metal-free protein. The calcium-induced conformational transition occurs in two steps. The first transition accompanies the binding of two calcium ions and affects the resonances of both tyrosines, the high-field phenylalanine, and the ϵ-trimethyllysine-115. The second transition is completed by the addition of a fourth calcium and is reflected by the resonances of the tyrosine-138 ortho protons and the phenylalanine. The magnesium-induced conformational transition affects the tyrosine-138 ortho protons and the phenylalanine. Changes in the main phenylalanine peaks due to magnesium binding are less dramatic than those associated with calcium binding. Tyrosine-99 is in the third of the four predicted calcium binding domains of calmodulin. The sensitivity of the tyrosine-99 resonances, only to the first calcium-induced conformational transition, suggests that the third domain is a high-affinity binding site. Tyrosine-138 is predicted to be near the fourth binding domain, where it would interact with other nonpolar amino acids in the interior of the protein. This is consistent with the observed chemical shift of the tyrosine-138 protons and their sensitivity to metal binding. The spectral characteristics of the metal ion dependent conformations of calmodulin are compared with those previously reported for skeletal troponin-C. The large sequence homology between the two proteins appears to result in regions of very similar tertiary structure.

A protein activator of brain nucleotide cyclic 3′,5′-phosphodiesterase was first isolated by Cheung (1970). The protein appears to be strongly conserved throughout its evolutionary development and is now referred to as calmodulin. Calmodulin was first isolated from brain and shown to activate nucleotide cyclic 3′,5′-phosphodiesterase only in the presence of calcium (Lin et al., 1974). It has since been isolated from heart (Teo et al., 1973), testes (Dedman et al., 1977), uterus (Grand & Perry, 1978), smooth muscle (Dabrowska et al., 1978), and skeletal muscle (Yagi et al., 1978). Calmodulin is also present in a wide variety of vertebrate (Drabikowski et al., 1978) and invertebrate (Waisman et al., 1975) species.

Numerous enzyme systems have been described which can be activated or modulated in a calcium-dependent manner by calmodulin. These include a detergent-solubilized preparation of adenylate cyclase (Cheung et al., 1975; Brostrom et al., 1975), calcium transport mediated by a Ca^{2+},Mg^{2+}–ATPase in erythrocytes (Jarrett & Penniston, 1978; Gopinath & Vincenzi, 1977), and calcium uptake in vesicles enriched in sarcoplasmic reticulum (Katz & Remtulla, 1978). An important class of calcium-dependent kinases which have calmodulin as a regulatory subunit includes the myosin light-chain kinase of smooth muscle (Dabrowska et al., 1978) and other tissues (Yagi et al., 1978; Waisman et al., 1978; Barylko et al., 1978; Dabrowska & Hartshorne, 1979), a kinase isolated from brain and other tissues capable of phosphorylating membrane proteins (Schulman & Greengard, 1978; DeLorenzo, 1979), and the phosphorylase kinase which can phosphorylate phosphorylase B (Cohen et al., 1978). These kinases are active only in the presence of calcium and calmodulin.

Calmodulin is a low molecular weight protein of ~16 500 (Vanaman et al., 1977). It lacks cysteine and tryptophan and contains 1 mol of the unusual amino acid ϵ-trimethyllysine. The sequences of calmodulin isolated from rat testes (Dedman et al., 1978), bovine brain (Vanaman et al., 1977), and rat uterus (Grand & Perry, 1978) appear to be almost identical. The physical characteristics of calmodulin are very similar to those of skeletal troponin-C (Stevens et al., 1976; Watterson et al., 1976), and a large degree of sequence homology exists between the two proteins (Vanaman et al., 1977; Dedman et

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al., 1978). This sequence homology can also be demonstrated for the carp MCBP,[1] whose crystal structure has been determined (Kretsinger & Nockolds, 1973), and MCBP has served as a model for high-affinity calcium binding proteins. By comparison to MCBP, four calcium binding regions can be predicted in the sequence of skeletal troponin-C (Collins et al., 1973; Weeds & McLachlan, 1974) and calmodulin (Vanaman et al., 1977; Dedman et al., 1978). This is consistent with the observation that both proteins can bind 4 mol of calcium. Troponin-C has two classes of calcium binding sites: two high-affinity sites which can competitively bind magnesium and two lower affinity, calcium-specific sites (Potter & Gergely, 1975). The calcium binding parameters of calmodulin have been studied by many investigators and while there is disagreement as to the relative number of high- and low-affinity sites, all studies indicate that 4 mol of calcium is bound per mol of calmodulin (Wolff et al., 1977; Dedman et al., 1977; Klee, 1977; Watterson et al., 1976; Lin et al., 1974).

Chemical and physical studies have shown that the binding of calcium by calmodulin produces large changes in its solution conformation (Wolff et al., 1977; Kuo & Coffee, 1976; Klee, 1977; Liu & Cheung, 1976; Dedman et al., 1977; Walsh & Stevens, 1977; Richman & Klee, 1978) which are similar to those which have been observed to occur for skeletal troponin-C (Potter et al., 1976; Van Eerd & Kawasaki, 1972; Murray & Kay, 1972). The calcium-induced conformational change of troponin-C causes further changes in the conformation of the other troponin subunits, troponin-I and troponin-T (McCubbin et al., 1974; Mani et al., 1974), while calmodulin binds to nucleotide cyclic 3′,5′-phosphodiesterase (Teshima & Kakiuchi, 1974) and adenylate cyclase (Lynch et al., 1976) only in the presence of calcium. It is the calmodulin–enzyme complex which represents the activated enzyme.

Previous NMR studies on skeletal troponin-C have demonstrated large changes in conformation due to the binding of calcium and magnesium (Seamon et al., 1977; Levine et al., 1977a, 1978). These have also shown that specific changes due to binding of metals at the high- and low-affinity sites of troponin-C can be distinguished. A previous communication has reported qualitative similarities in the spectra of calmodulin and troponin-C in the absence of metals and in the presence of calcium and magnesium (Seamon, 1979). In this paper the calcium- and magnesium-dependent conformations of calmodulin have been studied as a function of metal ion to determine the conformational states of the protein which exist in solution. These results are compared to those previously reported for troponin-C in order to determine whether the large degree of sequence homology between the two proteins results in similar solution conformations. Finally, these results are examined with respect to the proposed structural model for calmodulin which is based on its observed sequence homology with carp MCBP and troponin-C.

Materials and Methods

Protein Purification. Brain calmodulin was isolated from bovine brain as the PAP-II protein of Isobe et al. (1977). A final purification of the protein was performed by chromatography on a 1.5 × 30 cm DEAE-Sephadex (A-50) column equilibrated with 0.1 M potassium phosphate, 0.2 M NaCl, and 1.0 mM EDTA, pH 7.1, and eluted with a 1.0-L linear salt gradient from 0.2 M NaCl to 0.5 M NaCl. The protein was judged to be at least 90% pure based on its electrophoretic pattern on 7.5% polyacrylamide gels electrophoresed with a Tris–glycine continuous buffer system (Calissano et al., 1969) and on $NaDodSO_4$–urea slab gels with a 10–25% polyacrylamide gradient and a 4–8 M urea gradient employing a Laemmli buffer system (Laemmli, 1970). No contaminating protein bands were seen when 50 μg of purified calmodulin was run on either of the gel systems. A crude preparation of calmodulin-deficient phosphodiesterase prepared from bovine brain (Watterson et al., 1976) was stimulated 3.5-fold by the purified calmodulin and required 25 ng for half-maximal activation when assayed as described by Davis & Daly (1979).

Preparation of Protein Samples. Ca^{2+}-free samples were prepared by dialyzing 1.0 mL of calmodulin (~15 mg/mL) for 24 h against 1.0 L of 0.1 mM EGTA, pH 7.5. This was followed by dialyzing the protein against two changes of distilled deionized water. The free calcium level of the distilled deionized water was less than 5×10^{-7} M. The protein solution was then lyophilized and dissolved in 0.5 mL of 0.2 M KCl–D_2O. The calcium content was measured by atomic absorption and was found to be less than 0.2 mol of calcium per mol of calmodulin. The calcium- and magnesium-saturated samples were prepared by dialyzing calmodulin against 0.1 mM $CaCl_2$ and 10 mM $MgCl_2$–0.1 mM EGTA, respectively. The calcium and magnesium titrations were performed by adding aliquots of 0.5 M $CaCl_2$ or 1 M $MgCl_2$ to solutions of the metal-free protein. After each addition the pH of the protein solution was adjusted to 7.5.

Analysis of Total Calcium and Protein. Total calcium and protein were determined for each sample by the following procedure. Five microliters of each sample was removed and added to 95 μL of deionized water. Protein was determined in two 10-μL aliquots (containing 20–30 μg of protein) of this solution by using the Bio-Rad protein assay with a standard curve made by using purified calmodulin. The rest of the solution (80 μL) was diluted to a total volume of 400 μL with a final concentration of 1% $LaCl_3$–0.2% HCl. Calcium was determined in triplicate on 0.1-mL aliquots of the solution on an Instrumental Laboratories Model 112 atomic absorption spectrometer.

pH Titrations. The pH titrations were performed by adding 0.4 M KOD or 0.1 M DCl to the samples. pH values were measured on a Corning Model 112 pH meter equipped with a Radiometer GKS73041 electrode, and pH values reported are not corrected for deuterium isotope effects.

NMR Spectra. Proton spectra were taken at 360 MHz on a Nicolet NT-360 spectrometer operating in the Fourier transform mode with quadrature detection at the Purdue Biochemical Magnetic Resonance Laboratory. Typical spectra were obtained by using a 9-μs pulse (~90°), a sweep width of ±2500 Hz, an acquisition time of 0.82 s, and digital filtering of 0.5 Hz. Each spectrum is the sum of ~512 free induction decays. The residual HDO peak was continuously saturated, and decoupling experiments were performed with continuous irradiation such that decoupling effects and Overhauser effects could be observed. Spectra were taken at 23 °C, and chemical shifts were measured from an internal standard of TSP and are accurate to ±0.005 ppm.

Results

Spectral Assignments. The spectra of calmodulin in the absence of cations, in the presence of 0.1 mM free calcium, and in the presence of 10 mM free magnesium are shown in

[1] Abbreviations used: MCBP, muscle calcium binding protein; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TSP, (trimethylsilyl)-propionic acid.

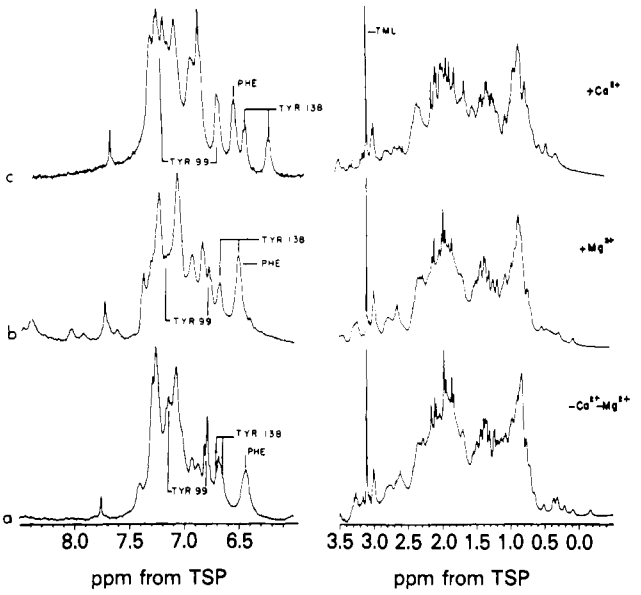

FIGURE 1: Aliphatic and aromatic regions of the spectrum of calmodulin: [calmodulin] $\simeq 1.5 \times 10^{-3}$ M, 0.2 M KCl, pH 7.5. (a) Ca^{2+}, Mg^{2+}-free; (b) $[Mg^{2+}]_{free} = 0.01$ M; (c) $[Ca^{2+}]_{free} = 2 \times 10^{-4}$ M. Resonances assigned to tyrosine-99 and -138, ϵ-trimethyllysine-115, and a uniquely shifted phenylalanine residue are labeled.

Table I: Chemical Shifts of Tyrosine-99 and -138 of Calmodulin

	chemical shifts (ppm from TSP)			
	Tyr-99[a]		Tyr-138	
sample[b]	δ_{ortho}	δ_{meta}	δ_{ortho}	δ_{meta}
$-Ca^{2+}$, $-Mg^{2+}$	6.82	7.31	6.70	6.72
$+Mg^{2+}$	6.82	7.31	6.59	6.72
$+2Ca^{2+}$	6.76	7.29	6.65	6.36
$+4Ca^{2+}$	6.76	7.29	6.55	6.36

[a] The ortho protons correspond to the protons ortho to the hydroxyl group, and the meta protons are meta to the hydroxyl group. [b] The $-Ca^{2+}$, $-Mg^{2+}$ sample corresponds to calmodulin with less than 0.2 mol of calcium per mol of protein. The $+Mg^{2+}$ sample corresponds to calmodulin in the presence of 10 mM Mg^{2+}. The $+2Ca^{2+}$ and $+4Ca^{2+}$ samples correspond to calmodulin with a mole ratio of calcium/calmodulin of 2 and 4, respectively. All samples were at pH 7.5 in 0.2 M KCl.

Figure 1. Binding studies indicate that all four of the calcium binding sites of calmodulin will be saturated at 0.1 mM free calcium (Wolff et al., 1977; Dedman et al., 1977). In the presence of 10 mM free magnesium, binding sites with the ability to bind magnesium with a $K_D \leq 10^{-3}$ M should be ~90% occupied. Assignments of peaks in the aromatic region to the two tyrosines, 99 and 138, are indicated and are summarized in Table I. These are based on the following experimental data and rationale. The resonances in the metal-free state and the calcium-saturated state were assigned by pH titrations in the alkaline region as previously reported (Seamon, 1979). The peak at 6.82 ppm assigned to the ortho protons of tyrosine-99 shifts upfield and exhibits a pK of ~10.3 in both the calcium-saturated and metal-free states, in agreement with the report that tyrosine-99 has a pK of 10.1 in the absence of calcium (Richman & Klee, 1978). The assignment of the tyrosine-99 resonances in the magnesium-saturated state was made by observing the resonances as magnesium was added to the metal-free protein, as described later.

The tyrosine-138 resonance at 6.57 ppm in the spectrum of calcium-saturated calmodulin shifts upfield only at pH values greater than 11.3 (Seamon, 1979). Since it has been

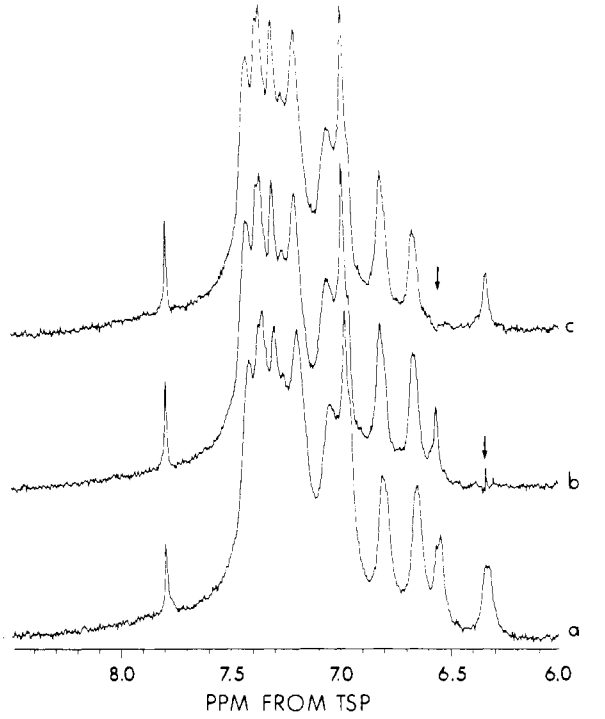

FIGURE 2: Decoupling of the tyrosine-138 resonances of calmodulin: [calmodulin] $\simeq 1.5 \times 10^{-3}$ M, 0.2 M KCl, 2×10^{-4} M $CaCl_2$, pH 7.5. (a) Control spectrum with no irradiation; (b) irradiation at 6.36 ppm (meta protons); (c) irradiation at 6.57 ppm (ortho protons). The positions of the peaks irradiated in (b) and (c) are indicated by arrows. The nomenclature for the tyrosine ring protons is explained in Table I.

reported that tyrosine-138 has a pK of 11.9 in the calcium-saturated calmodulin (Richman & Klee, 1978), this resonance was tentatively assigned to either the ortho or meta protons of tyrosine-138. The resonance at 6.36 ppm due to the other ring protons of tyrosine-138 in the spectrum of calcium-saturated calmodulin was identified by decoupling experiments as shown in Figure 2. Irradiation of either tyrosine-138 resonance produces a sharpening of the other resonance due to decoupling and a decrease in the integrated intensity of the resonance due to negative Overhauser effects. The low-field tyrosine-138 resonance in the spectrum of the Ca^{2+}-saturated calmodulin exhibits an appreciable upfield shift as the tyrosine ring is deprotonated, suggesting that this resonance is due to the protons ortho to the hydroxyl group. This is consistent with the observation that irradiation of the upfield tyrosine-138 resonance (the meta proton resonance) produces a larger NOE for the downfield tyrosine-138 resonance (the ortho proton resonance) than the NOE observed for the upfield resonance upon saturation of the downfield resonance. The assignment of the meta proton resonances appearing at higher field than the ortho proton resonances is the reverse of model tyrosine compounds (Karplus et al., 1973; Martin & Morlino, 1965); however, this same type of reversed assignment has also been made for tyrosine-109 of the CB-9 fragment of troponin-C (Birnbaum & Sykes, 1978) and tyrosine-21 of the bovine pancreatic trypsin inhibitor (Snyder et al., 1975). The resonances in the spectra of the metal-free protein were assigned by pH titration, and the assignments for the magnesium-saturated protein were made by observing the resonances throughout the course of metal titrations of the metal-free protein, as described later. Tyrosine resonance assignments were also confirmed by spectra of calmodulin in which both tyrosines were nitrated and spectra of calmodulin in which only tyrosine-99 was nitrated (K. Seamon, unpublished experi-

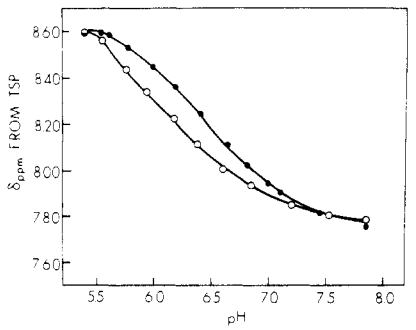

FIGURE 3: Chemical shift of the H-2 proton of histidine-107 of calmodulin as a function of pH: [calmodulin] $\simeq 1.5 \times 10^{-3}$ M, 0.2 M KCl, pH 7.5. The titration was carried out in the absence of calcium and magnesium (●) and in the presence of 2×10^{-4} M free calcium (○). The titration data were fit to a modified form of the Hill equation as described by Edwards & Sykes (1978). A least-squares fit to the data gave values of $pK_a = 6.10$ and $n = 0.97$ for the titration in the presence of calcium and $pK_a = 6.48$ and $n = 0.97$ for the titration in the presence of EGTA.

ments). These derivatives were produced by the method of Richman & Klee (1978).

The single sharp resonance at 7.79 ppm in the spectra is assigned to the H-2 proton of the single histidine of calmodulin. The H-4 proton of the histidine appears at 6.91 ppm and was assigned by using a Carr–Purcell pulse sequence (Campbell et al., 1975). The histidine H-2 and H-4 proton resonances were monitored as a function of pH between 5 and 8 and exhibit single monoprotic titration curves with pK values of 6.10 and 6.48, respectively, in the presence and absence of Ca^{2+} (Figure 3). The H-2 resonance in the spectrum of the Ca^{2+}-saturated protein exhibited substantial broadening during the pH titration accompanied by a noticeable cloudiness of the protein solution as the pH neared 5.5, presumably due to protein precipitation. This broadening was also observed during the titration of the Ca^{2+}-free sample; however, a subsequent narrowing of the resonance was observed as the pH neared 5.5. No noticeable cloudiness of the Ca^{2+}-free sample was observed at any of the pH values, suggesting that the Ca^{2+}-saturated protein precipitates at a higher pH than the Ca^{2+}-free protein.

Calmodulin does not contain any tryptophan residues, and therefore the resonance at 6.48 ppm in the spectra of the metal-free protein (Figure 1a) is assigned to a uniquely shifted phenylalanine residue (labeled PHE in Figure 1). This resonance was also assigned in the spectra of the magnesium- and calcium-saturated protein by observing the resonance throughout the course of metal titrations. The large peak at 7.35 ppm in the spectrum of the metal-free protein is due to phenylalanine ring protons. The peaks between 6.90 and 7.40 ppm in the spectra of the magnesium-saturated and calcium-saturated protein are due to phenylalanine ring protons and the meta proton resonances of tyrosine-99.

The aliphatic regions of the spectra of calmodulin in the absence of cations, in the presence of magnesium, and in the presence of calcium exhibit many spectral similarities and differences (Figure 1). The single sharp resonance at 3.12 ppm (labeled TML) is assigned to the N-methyl protons of the single residue of ϵ-trimethyllysine. The trimethylamino protons would not be coupled to any other protons and would appear as a singlet with 9 proton intensity. Furthermore, this resonance is in the chemical-shift region where choline group resonances have been reported. There is no large shift or broadening of this resonance in the spectrum of either the magnesium- or the calcium-saturated calmodulin (a small shift is seen in the presence of calcium which will be discussed in more detail later), suggesting that the ϵ-trimethyllysine side chain is not buried or hindered in its rotation but is probably on the surface of the protein in a solvent environment. Numerous resonance peaks are seen in the region between –0.3 and 0.7 ppm which are due to ring current shifted aliphatic protons most probably attributed to close interactions between aliphatic protons and aromatic phenylalanine rings. Specific spectral differences in this region are evident between the three states of calmodulin. The resonance at 0.37 ppm in the spectrum of the metal-free protein exhibits a doublet character with a coupling of 6 Hz, which suggests that this resonance is due to a (alanine, valine, leucine, isoleucine, or threonine) methyl group.

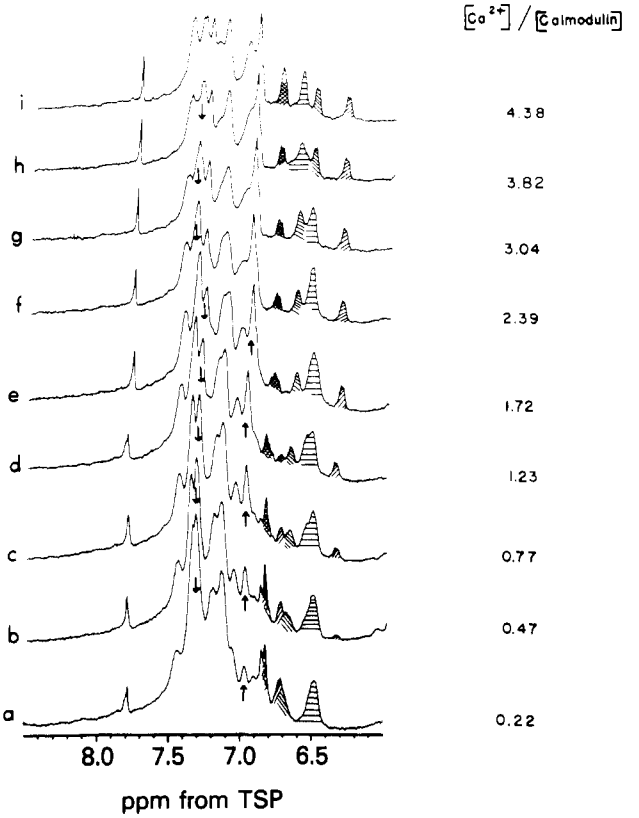

FIGURE 4: Aromatic region of the spectrum of calmodulin as a function of added Ca^{2+}: [calmodulin] $\simeq 1.5 \times 10^{-3}$ M, 0.2 M KCl, pH 7.5. Ca^{2+} was added sequentially to metal-free calmodulin, and total protein and total calcium were measured in each sample as described under Materials and Methods. The ratio of total calcium/total protein is indicated for each spectrum. Changes in phenylalanine intensity are indicated by arrows, and the tyrosine resonances and upfield phenylalanine resonance are individually shaded.

Calcium Titration. A calcium titration of calmodulin was carried out by adding stoichiometric amounts of calcium to metal-free calmodulin. Calmodulin binds 4 mol of calcium per mol of protein; however, it is not clear whether the binding occurs at one or two sets of binding sites. The calcium-induced spectral changes will therefore be discussed in terms of the molar ratio of calcium/calmodulin instead of in terms of the fractional occupancy of binding sites as was done for troponin-C (Seamon et al., 1977).

The aromatic region of the spectrum of calmodulin as a function of the calcium/calmodulin mole ratio is shown in Figure 4. Two distinct conformational transitions are observed to occur as a consequence of calcium binding. These are indicated by the following spectral changes. (1) The tyrosine-138 meta resonance in the spectrum of metal-free calmodulin (6.72 ppm in Figure 4a) progressively decreases in intensity and cannot be distinguished at a molar ratio of

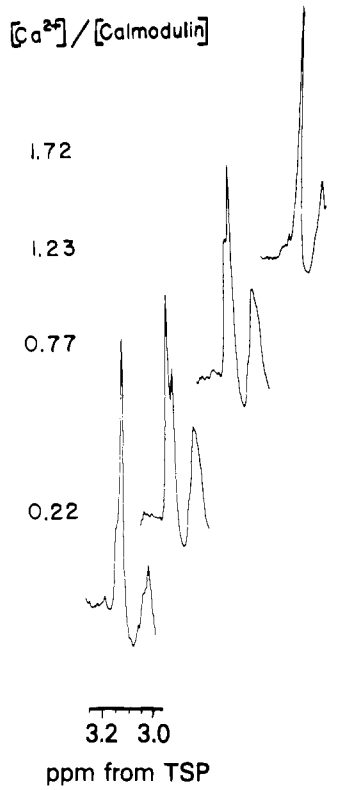

FIGURE 5: Effect of Ca^{2+} on the *N*-methyl resonance of ϵ-trimethyllysine-115. Spectra were taken from the Ca^{2+} titration depicted in Figure 4. The calcium/protein ratio is indicated to the left of each spectrum. The spectra are shown displaced to the right for ease of presentation and cover the identical chemical-shift range as indicated for the bottom spectrum.

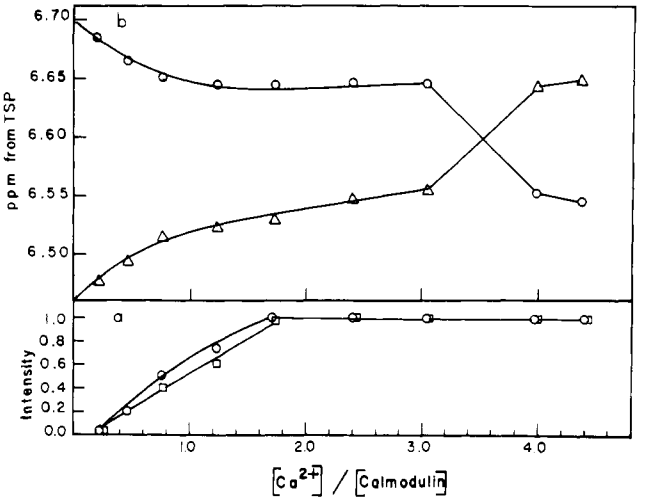

FIGURE 6: Spectral monitors of the Ca^{2+}-induced conformational transitions of calmodulin. Spectral parameters (taken from Figures 4 and 5) are plotted as a function of the ratio of calcium/calmodulin. (a) The normalized intensity of the *N*-methyl group of ϵ-trimethyllysine-115 (□) and the tyrosine-138 meta proton resonance at 6.36 ppm (○); (b) the chemical shifts of the tyrosine-138 ortho proton resonance (○) and the high-field shifted phenylalanine resonance (△). That part of the phenylalanine intensity which shifts under the tyrosine-99 ortho proton resonance is not plotted since an accurate determination of its chemical shift was difficult due to overlapping resonances.

calcium/calmodulin greater than 1.72 (Figure 4a–e). This decrease occurs in parallel with an increase in intensity of the tyrosine-138 meta resonance which appears at 6.36 ppm in the spectrum of calcium-saturated calmodulin. Neither the intensity nor the chemical shift of this resonance is affected by the addition of calcium in excess of a molar ratio of calcium/calmodulin of ~2 (Figure 4e–i). (2) The tyrosine-138 ortho resonance in the spectrum of metal-free calmodulin (6.70 ppm in Figure 4a) shifts upfield to 6.64 ppm as the first two calciums are added (Figure 4a–e). A further shift to 6.57 ppm is observed as the third and fourth calciums are added (Figure 4f–i). (3) The ortho proton resonance of tyrosine-99 (6.82 ppm in Figure 4a) exhibits a broadening and upfield shift as the calcium/calmodulin molar ratio increases to 1.23 (Figure 4a–d) with a subsequent narrowing and further shift upfield as the calcium/protein molar ratio increases to 2.39 (Figure 4e and 4f). No further changes in chemical shift or line width are evident as the third calcium is added. The apparent broadening of the resonance as a fourth calcium is added is due to a phenylalanine resonance which appears at the same chemical shift. (4) The high-field phenylalanine resonance (6.49 ppm in Figure 4a) broadens as the first calcium is added to calmodulin (Figure 4a–d) and then exhibits a narrowing of the resonance with a resulting downfield shift to 6.55 ppm as the molar ratio of calcium/calmodulin increases to 2.39 (Figure 4d–f). A further shift downfield is observed as the third and fourth calcium ions are added (Figure 4f–i) with some of the phenylalanine intensity shifting under the tyrosine-99 ortho proton resonance. (5) There is an increase in phenylalanine intensity at 6.95 ppm as the first two calciums are added with no further change as the third and fourth calciums are added. This is accompanied by a decrease in intensity of the main peak of phenylalanine intensity at 7.30 ppm. As the third and fourth calcium ions are added, a decrease in phenylalanine intensity at 7.32 ppm can also be observed. (6) As the molar ratio of calcium/calmodulin increases to 2.23, the resonance at 3.12 ppm assigned to the *N*-methyl protons of ϵ-trimethyllysine-115 decreases in intensity in parallel with the increase in intensity of a peak 6.5 Hz upfield at 3.10 ppm (Figure 5). This resonance does not show any change in chemical shift or line width as the third and fourth calcium ions are added. This spectral change is indicative of a slow conformational transition between calcium-free and calcium-bound calmodulin. Based on the separation between the two ϵ-trimethyllysine resonances of 6.5 Hz, we can calculate an upper limit of 40 s^{-1} for the rate constant of the transition.

In the first stage of calcium binding, corresponding to the initial binding of two calcium ions, the spectral behavior of the tyrosine-99 ortho protons, the tyrosine-138 meta protons, and the *N*-methyl protons of ϵ-trimethyllysine-115 is indicative of a slow conformational transition between two stable conformations of the protein. The spectral changes accompanying the addition of the third and fourth calcium ions, the downfield shift of the high-field phenylalanine peak, the upfield shift of the tyrosine-138 ortho proton peak, and the changes in the main phenylalanine peak, indicate that a second conformational transition occurs as the third and fourth binding sites are occupied.

These calcium-induced spectral changes are summarized in Figure 6, where spectral parameters are plotted as a function of the molar ratio of calcium/calmodulin. The first stage conformational change due to the binding of two calcium ions is indicated by the increase in the intensity of the tyrosine-138 meta proton resonance and the increase in the intensity of the upfield *N*-methyl resonance of ϵ-trimethyllysine-115 (Figure 6a). The chemical shifts of the tyrosine-138 ortho proton resonance and the high-field phenylalanine resonance reflect not only the conformational transition due to the binding of the first two calcium ions but also a further conformational

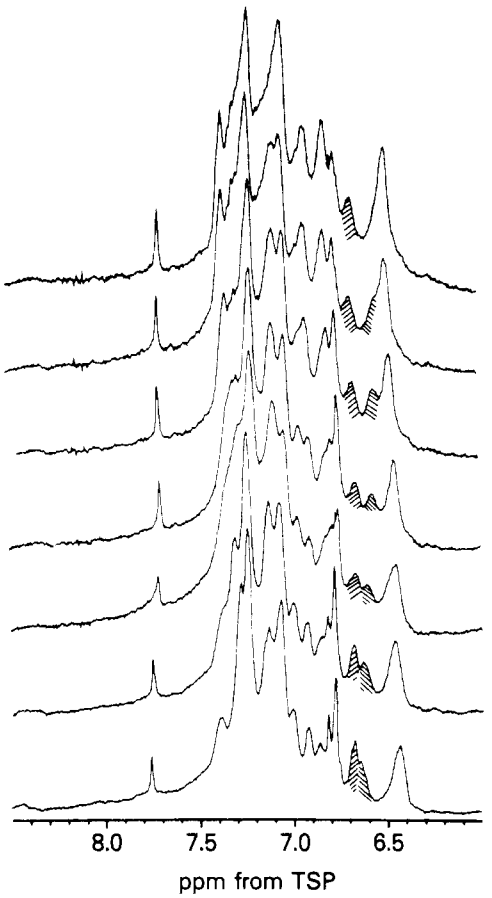

FIGURE 7: Aromatic region of the spectrum of calmodulin as a function of added Mg^{2+}: [calmodulin] $\simeq 1.5 \times 10^{-3}$ M, 0.2 M KCl, pH 7.5. Mg^{2+} was added in increments to metal-free calmodulin. The spectra are displayed from the bottom of the figure to the top in order of increasing magnesium, ranging from 0 to 10 mM free magnesium.

transition as the third and fourth calcium ions are added (Figure 6b). The crossing of the tyrosine-138 and the phenylalanine resonances appears to be quite abrupt; however, a close examination of the spectra (Figure 4f–i) reveals that the intensities and line widths of the resonances are changing throughout the addition of the third and fourth calcium ions.

Magnesium Titration. The spectrum of calmodulin was examined as a function of added magnesium ranging between 0 and 10 mM free magnesium (Figure 7). A detailed analysis of the magnesium-induced spectral shifts was not performed due to the high concentration of magnesium used and also because of the contradictory results which have been reported for magnesium binding to calmodulin (Wolff et al., 1977; Dedman et al., 1977). It can be seen that the sequential addition of magnesium results in the same spectral characteristics as the magnesium-saturated calmodulin (Figure 1b). Specific spectral changes due to the binding of magnesium are indicated by the upfield shift of the tyrosine-138 ortho proton resonance and by the downfield shift of the high-field phenylalanine resonance. Both resonances appear as one peak with the same chemical shift in the magnesium-saturated spectrum. The tyrosine-138 meta proton resonance does not seem to be affected by magnesium binding. Small changes are evident in the region where the ortho protons of tyrosine-99 appear, but these cannot specifically be attributed to changes in that particular resonance. The main phenylalanine peak at 7.35 ppm decreases in intensity with respect to the phenylalanine peak at 7.12 ppm until the two peaks are approximately of equal intensity in the magnesium-saturated spectrum.

Discussion

Changes in the solution conformation of calmodulin upon binding calcium have been detected by CD (Liu & Cheung, 1976; Wolff et al., 1977; Klee, 1977; Walsh et al., 1978), increases in tyrosine fluorescence (Dedman et al., 1977; Dabrowska et al., 1978), differences in the reactivity of amino acid side chains (Walsh & Stevens, 1977, 1978), and spectral differences of nitrotyrosine derivatives of calmodulin (Richman & Klee, 1978; McCubbin et al., 1979). The advantage of using ^1H NMR to study the solution conformation of calmodulin is that large conformational transitions as well as more subtle changes in the environment of specific amino acid residues will be reflected by perturbations of the protein's proton resonances.

Resonances have been assigned to the ortho and meta protons of tyrosine-99 and -138 of calmodulin. The pK of tyrosine-99 is 10.1 in the presence of calcium and 10.4 in the absence of calcium (Richman & Klee, 1978), which are similar to the pK for a solvent-exposed tyrosine ring (10.1). The chemical reactivity of tyrosine-99 to acetylation and nitration is consistent with the ring being freely exposed to solvent in the presence or absence of calcium (Richman, 1978). The chemical shift of the ortho protons of tyrosine-99 is similar to that of model tyrosine compounds, and the binding of ~ 2 mol of calcium by calmodulin causes the resonance to shift upfield ~ 20 Hz. This indicates that the tyrosine-99 ring is being affected by calcium binding; however, the small spectral shift is not characteristic of a substantial change in its physical surroundings.

Tyrosine-138 is in a quite different environment than tyrosine-99, as reflected by its high pK of 11.9 in both the absence and presence of calcium (Richman & Klee, 1978). The reactivity of tyrosine-138 to chemical modifying agents is calcium dependent and suggests that the tyrosine ring is less accessible in the presence of calcium (Richman, 1978). The ring proton resonances of tyrosine-138 reflect its unique environment in both the absence and presence of calcium such that four stable conformations of calmodulin can be defined by the chemical shift of the tyrosine-138 resonances (Table I). The sensitivity of the tyrosine-138 resonances to calcium binding suggests that the environment of the tyrosine ring is being changed due to two calcium-induced conformational transitions. The first transition affects both sets of protons, which could be explained by a substantial burying of the ring due to a large conformational change. The ortho and meta protons reflect different rates for this first transition, with the ortho protons exhibiting a relatively fast rate and the meta protons exhibiting a slower rate. This could indicate that tyrosine-138 is reflecting two environmental changes within the first transition; the ortho protons are affected by the relatively fast exchange of calcium at a binding site while the meta protons are affected by a conformational change involving a large portion of the polypeptide chain. The second calcium-induced conformational transition affects only the protons ortho to the hydroxyl group and probably represents a more localized change in the environment around tyrosine-138.

A calcium titration of metal-free calmodulin indicates that there are three unique solution conformations of calmodulin: the metal-free protein, the protein with two calcium ions bound, and the fully saturated protein with four calcium ions bound. The spectral changes associated with the first conformational transition corresponding to the binding of the first two calcium ions indicate that a large number of aromatic and aliphatic groups are being affected by the transition. These

are similar to calcium-induced spectral changes which have been observed in other calcium binding proteins (Seamon et al., 1977; Levine et al., 1977a; Birnbaum & Sykes, 1978). The second conformational transition attributed to the binding of the third and fourth calcium ions is also indicative of a transition affecting a number of nonpolar residues of the protein. Recently, CD studies of native calmodulin (Wolff et al., 1977; Klee, 1977; Walsh et al., 1978) and spectral studies on nitrated calmodulin (McCubbin et al., 1979) have provided evidence that two conformational transitions can be distinguished due to the binding of calcium at high- and low-affinity sites and are consistent with the two-step conformational transition reported here. The free calcium concentration is not measured in the NMR experiments, and therefore apparent binding constants cannot be reported for the transition. However, it appears that the binding of two calcium ions is responsible for the first transition, which would correspond to the binding of calcium at two high-affinity sites. The further conformational transition is then related to the binding of calcium at a third and fourth binding site, which would represent lower affinity binding sites.

The magnesium binding to calmodulin does not induce as large a conformational change as does calcium binding. The magnesium-induced NMR spectral changes are observed in the presence of 0.2 M KCl, indicating that there is a specific magnesium conformational change that is not due to just ionic strength effects. The resonance shifts accompanying this change are similar to some of the calcium-induced resonance shifts; however, the characteristics associated with the large calcium-induced conformational transition are not seen. This indicates that magnesium may be binding at the calcium binding sites of calmodulin without inducing the subsequent large change in conformation.

The sequence of calmodulin has been determined and exhibits considerable homology with skeletal troponin-C and carp MCBP. Carp MCBP binds 2 mol of calcium in two sites. Each binding site contains a large number of free carboxyl or carbonyl groups available for metal coordination, and immediately adjacent to each binding site are regions of α-helical structure containing a number of nonpolar amino acid residues (Kretsinger & Nockolds, 1973). Four such binding regions can be identified in the sequence of skeletal troponin-C (Collins et al., 1973) and calmodulin (Vanaman et al., 1977). Tyrosine-99 of calmodulin and tyrosine-109 of skeletal troponin-C occupy positions in a calcium binding loop, the S_3 loop, homologous to phenylalanine-57 of carp MCBP which participates in the binding of calcium through its peptide carbonyl. Both tyrosines exhibit similar pK values, chemical reactivities, and NMR spectral behavior, suggesting that in the presence or absence of Ca^{2+} neither tyrosine ring is removed from a solvent environment. This is not inconsistent with the suggestion that the two tyrosines are participating in the binding of calcium through their peptide carbonyl groups with their phenolic rings directed toward the solvent away from the binding site. The sensitivity of the tyrosine-99 ortho proton resonance of calmodulin only to the binding of the first two calcium ions suggests that the S_3 loop represents a high-affinity binding site. Similar behavior has been observed for tyrosine-109 of skeletal troponin-C (Levine et al., 1977a), and it has recently been shown that the S_3 loop of troponin-C corresponds to a high-affinity binding site (Sin et al., 1978; Nagy et al., 1978; Leavis et al., 1978). The assignment of the S_3 loop of calmodulin as a high-affinity binding site is also supported by the observation that ϵ-trimethyllysine-115 is only sensitive to the binding of the first two calcium ions. ϵ-Trimethyllysine-115 occupies a position in a region of the sequence which links the S_3 and S_4 binding loops of calmodulin together. If the S_3 and S_4 binding sites of calmodulin were high-affinity binding sites, then it might be expected that the behavior of the ϵ-trimethyllysine-115 resonance would only reflect the first conformational transition.

The sequence homology predicts that tyrosine-138 of calmodulin occupies a position in a helical region connected to the S_4 binding loop and interacts with other nonpolar residues in the interior of the protein. The sensitivity of the tyrosine-138 resonances to both calcium-induced conformational transitions might then be representative not only of the initial binding of calcium at the S_4 loop but also of the formation or structuring of the nonpolar core of calmodulin as a consequence of calcium binding. The homologous position in skeletal troponin-C is occupied by a phenylalanine and in neither carp MCBP nor skeletal troponin-C is there an amino acid residue proposed to participate in hydrophobic interactions whose proton resonances can easily be assigned. Tyrosine-145 of cardiac troponin-C occupies a position homologous to tyrosine-138 of calmodulin and should exhibit similar spectral behavior reflecting the structure of the hydrophobic interior of the protein.

Structural similarities between skeletal troponin-C and calmodulin are indicated by the qualitative features of their respective NMR spectrum. In the absence of any metals the spectra of both proteins exhibit the majority of phenylalanine ring proton intensity at a position characteristic of solvent-exposed phenylalanine rings. The binding of metals by both proteins results in a net shift of phenylalanine intensity upfield of this position, indicating that the metal ion induced conformational transitions are affecting a number of phenylalanine residues. This could be explained by a further structuring of the hydrophobic core of the protein and is also consistent with the increase in helicity observed to occur for both proteins upon binding calcium. The calcium-induced conformational changes may also result in closer interactions between various regions or chains of the protein. Both proteins exhibit unique high-field phenylalanine resonances in the metal-free conformation. The two resonances in skeletal troponin-C are affected differently by the binding of calcium at the high- and low-affinity sites of the protein (Seamon et al., 1977; Levine et al., 1977a). The corresponding resonance of calmodulin shows similar behavior, that is, a downfield shift which is attributed to both calcium-induced conformational transitions. Further similarities in the proteins' spectra are evident in the high-field aliphatic region corresponding to ring current shifted aliphatic resonances. Numerous ring current shifted resonances are seen in the spectra of the metal-free proteins which are not observed in the spectra of the calcium-saturated proteins. These results suggest that the conformations of the metal-free proteins have regions of tertiary structure characterized by very close interactions between aromatic and aliphatic groups. Upon binding calcium the interactions holding these groups in close proximity are weakened, which is reflected by the disappearance of these resonances.

The results reported here for calmodulin are similar to published studies on skeletal troponin-C (Seamon et al., 1977; Seamon, 1977; Levine et al., 1977a,b, 1978), a cyanogen bromide fragment of skeletal troponin-C (Birnbaum & Sykes, 1978), and carp MCBP (Parello et al., 1974; Seamon, 1977). These studies along with other investigations of the metal ion induced conformational transitions of calcium binding proteins (Levine et al., 1977b; Nagy, 1979) can be incorporated into a general description of the solution structures of the proteins. In the absence of metals the proteins exhibit a constrained

conformation where possible helical regions are prevented from being completely formed. This conformation will in general be more extended or less compact than the calcium-saturated conformation. The protein conformation, however, will not represent a random coil or denatured conformation but will have regions of local structure. The constraining forces keeping the configuration extended will most probably be due to an electrostatic interaction between free acidic groups and binding groups which will not be neutralized or coordinated in the absence of metals. Upon binding metals negative charges will be neutralized which will then release the conformational restraint on the rest of the protein's structure, allowing complete formation of the helical regions. This conformational transition will be associated with closer associations between regions of the protein, resulting in a more compact structure. It will be this conformation of the protein which is responsible for the metal ion induced activation of the associated enzyme systems.

In summary, calcium-dependent solution conformations of calmodulin have been characterized, and spectral parameters have been associated with the metal-free protein, the [Ca_2–calmodulin] complex, and the [Ca_4–calmodulin] complex. The spectral assignments are consistent with the proposed sequence homology with skeletal troponin-C, and the calcium-induced conformational transitions for calmodulin are similar to those which have been reported for skeletal troponin-C. These results have been incorporated into a general description of the calcium-induced conformational transitions in high-affinity calcium binding proteins. The results reported here also provide a basis for studying the possible interactions of calmodulin with drugs (Levin & Weiss, 1978), which are known to affect its function, and other proteins with which calmodulin interacts.

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Mechanism of Inhibition of Deoxyribonucleic Acid Synthesis by 1-β-D-Arabinofuranosyladenosine Triphosphate and Its Potentiation by 6-Mercaptopurine Ribonucleoside 5′-Monophosphate†

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ABSTRACT: The mechanism of inhibition of DNA synthesis by 1-β-D-arabinofuranosyl-ATP (*ara*-ATP) and the potentiation of this inhibition by 6-mercaptopurine ribonucleoside 5′-monophosphate (6-MPR-P) have been investigated with mammalian DNA polymerase δ by using poly(dA-dT) as the template. The inhibition of DNA synthesis by *ara*-ATP correlates with incorporation of *ara*-AMP into poly(dA-dT). Nearest-neighbor analysis indicates that *ara*-AMP does not act as an absolute chain terminator but rather that chains with 3′-terminal arabinosyl nucleotides are extended slowly. The inhibition of DNA synthesis by *ara*-ATP is markedly enhanced by the addition of the nucleotide derivative of 6-mercaptopurine, 6-mercaptopurine ribonucleoside 5′-monophosphate. The increased inhibition of DNA synthesis in the presence of 6-MPR-P is due to increased incorporation of *ara*-AMP. The mechanism by which 6-MPR-P increases the incorporation of *ara*-AMP is by selective inhibition of the 3′ to 5′ exonuclease activity of DNA polymerase, thereby preventing the removal of newly incorporated *ara*-AMP at 3′ termini of DNA chains.

The purine nucleoside analogue 1-β-D-arabinosyladenine (*ara*-A) and the pyrimidine nucleoside analogue 1-β-D-arabinosylcytosine (*ara*-C) inhibit the growth of a variety of mammalian cells and viruses. The active metabolites of these nucleoside analogues are their corresponding 5′-triphosphates, *ara*-ATP and *ara*-CTP, which are potent inhibitors of DNA synthesis in vitro. Kinetic studies have shown that *ara*-ATP and *ara*-CTP are competitive inhibitors of DNA synthesis when the corresponding deoxynucleoside triphosphate, dATP or dCTP, is the variable substrate (Furth & Cohen, 1968; Müller et al., 1975). However, the exact mechanism by which *ara*-CTP inhibits DNA synthesis remains uncertain. Studies with partially purified DNA polymerases have shown that nearly all of the incorporated *ara*-CMP is present at the 3′-hydroxyl termini of DNA chains (Momparler, 1969, 1972; Waqar et al., 1971). These findings led to the proposal that *ara*-C inhibits DNA synthesis by acting as a chain terminator, either preventing or markedly slowing the addition of deoxynucleotides (Momparler, 1969, 1972; Waqar et al., 1971; Magnusson et al., 1974; Burgoyne, 1974). However, studies with intact cells and cell lysates have indicated that *ara*-CMP and *ara*-AMP are incorporated primarily in internucleotide linkage of DNA. It was also found that inhibition of DNA synthesis by low concentrations of *ara*-C is readily reversible in vivo; i.e., removal of the inhibitor leads to the rapid re-

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