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Glycation of Calmodulin: Chemistry and Structural and Functional Consequences[†]

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ABSTRACT: In the presence of Ca^{2+} and glucose, calmodulin incorporates 2.5 mol of glucose/mol of protein. In the absence of Ca^{2+}, only 1.5 mol of glucose is incorporated per mole of calmodulin. Glycation of calmodulin is associated with variable reductions in its capacity to activate three Ca^{2+}/calmodulin-dependent brain target enzyme systems, including adenylyl cyclase, phosphodiesterase, and protein kinase. In addition, glycated calmodulin exhibits a 54% reduction in its Ca^{2+} binding capacity. Isolated CNBr cleavage fragments of glycated calmodulin suggest that glycation follows a nonspecific pattern in that each of seven available lysines is susceptible to modification. A limit observed on the extent of glycation appears related to the accompanying increase in negative charge on the protein. Glycation results in minimal structural rearrangements in calmodulin, and the Ca^{2+}-induced increase in α-helix content and radius of gyration is the same for glycated and unmodified calmodulin. Since glycated calmodulin's Ca^{2+} binding capacity is reduced, this implies that the Ca^{2+}-induced conformational changes in calmodulin do not require all four Ca^{2+} binding sites to be occupied. Examination of the lysine positions in calmodulin suggests that Ca^{2+} binding to domains II and IV is sufficient to induce these changes. The functional consequences of calmodulin glycation therefore cannot be attributed to inhibition of these conformational changes. An alternative explanation is that the inhibition arises from interference at the target enzyme binding site by bound glucose. While glycation shows minimal structural effects, a large pH dependence is observed for the α-helix content of unmodified calmodulin. It is suggested that pH, as well as ionic strength and Ca^{2+} concentration, may be important in stabilizing the crystal form of the protein.

Since the earliest descriptions of nonenzymatic glycation, there has been interest in the possibility that this covalent modification of protein lysine residues and N-terminal amino moieties might alter the function, regulation, or recognition of the affected proteins (Brownlee & Cerami, 1981; Bunn, 1981; Brownlee et al., 1984). The glycation reaction proceeds through a Schiff base intermediate which undergoes Amadori rearrangement to a stable ketoamine. There is an accompanying increase in the net negative charge of the recipient protein (Bitensky et al., 1988). Because the rate of protein glycation is a function of glucose concentration, the reaction occurs more rapidly in the diabetic milieu and might contribute to the long-term histopathological complications of diabetes mellitus (Vlassara et al., 1986). Several proteins have been

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studied for changes in functional properties that might arise as a result of glycation. Proteins in which functional changes have been detected include hemoglobin, albumin, tubulin, lens crystalins, low-density lipoprotein, high-density lipoprotein, fibrinogen, and myelin basic protein (Brownlee & Cerami, 1981; Brownlee et al., 1984; Bunn, 1981; Williams et al., 1981).

Calmodulin is a ubiquitous, Ca^{2+}-dependent regulator of intracellular processes that was first described by Kakiuchi and co-workers (Kakiuchi et al., 1969; Kakiuchi & Yamazaki, 1970). It has been found in every cell type in which it has been studied, including erythrocytes (Gopinath & Vincenzi, 1977) and photoreceptors (Nagao et al., 1987), and is especially prevalent in neuronal tissues where it contributes more than 1% of total protein. Calmodulin mediates the effects of Ca^{2+} on a variety of cellular, enzymatic, or cytoskeletal targets (Cheung, 1980). Kakiuchi et al. (1982) have described a series of calmodulin binding proteins and calmodulin/Ca^{2+}-dependent functions, especially in neurons and smooth muscle cells.

Calmodulin belongs to the family of structurally homologous Ca^{2+} binding proteins that includes parvalbumin, troponin C, and S-100. The crystal structure of calmodulin fully saturated with Ca^{2+} has been solved (Babu et al., 1985). The structure is unusual in that it consists of two globular domains connected by a single α-helix of approximately eight turns. The interconnecting helix is mostly exposed to solvent and forms few contacts with the rest of the molecule. Each globular domain contains two Ca^{2+} binding sites. Small-angle X-ray scattering experiments (Heidorn & Trewhella, 1988) have shown, however, that the globular domains are closer to each other by several angstroms in solution compared with the crystal structure. This requires some structural rearrangement of the interconnecting helix in solution, when compared with that in the crystal form.

Calmodulin undergoes a conformational change when it binds Ca^{2+}. Hennessey and co-workers (Hennessey et al., 1987) have shown that there is a 4% increase in α-helix due to Ca^{2+} binding, and small-angle X-ray scattering studies (Seaton et al., 1985; Heidorn & Trewhella, 1988) have shown that this is accompanied by an increase in the radius of gyration (R_g)[1] and maximum dimension of the molecule on Ca^{2+} binding. These structural effects have been postulated to be important in facilitating binding to the target enzyme. In particular, it has been suggested that flexing of the interconnecting helix region is a mechanism by which the disposition of the two globular Ca^{2+} binding domains could be positioned optimally for binding to a wide variety of target enzymes.

Here we report the consequences of in vitro nonenzymatic glycation on the capacity of calmodulin to activate three brain target enzymes. In addition, we have estimated the extent to which each of the seven lysine residues in calmodulin contributes to its overall glycation pattern. A molecular mechanism for the glycation-related changes in calmodulin function is suggested on the basis of small-angle X-ray scattering and circular dichroism (CD) studies. The CD studies also give some insights into the factors that may be important in stabilizing the crystal structure form of calmodulin. A possible mechanism is also proposed to explain the apparent limit of 2.5 mol of glucose/mol of calmodulin by which calmodulin glycation is constrained.

Materials and Methods

Glycation of Brain Calmodulin. Affinity-purified bovine brain calmodulin was purchased from Calbiochem and consistently gave a single band (at 17-kDa) when 20 μg was analyzed on an SDS–polyacrylamide gradient gel. For glycation, purified calmodulin (60 μM) was incubated at 30 °C or room temperature in phosphate buffer (pH 7.4) containing 1 mM $CaCl_2$ with 28 mM [6-^{3}H]glucose for 9 days. The reaction mixture was sterilized by filtration through a 0.2-μm Millipore filter, and 0.02% sodium azide was added to prevent bacterial growth. Aliquots of calmodulin were taken periodically and precipitated with TCA to determine the rate and extent of incorporation of [6-^{3}H]glucose into the protein fraction. Glycated calmodulin was separated from nonglycated calmodulin by Glycogel B (Pierce) affinity chromatography (Kowluru et al., 1987b). While the rate for calmodulin glycation was slightly faster at higher glucose concentrations, the extent of glycation (2.5 mol of glucose/mol of calmodulin) was not different for glucose concentrations of 28 or 100 mM.

Enzyme Assays. Phosphodiesterase activity was measured in a calmodulin-depleted, purified, brain phosphodiesterase preparation (Boehringer) by using the procedure of Thompson and Appelman (1971). Adenylyl cyclase was measured in a rat brain membrane fraction by the method of Valverde et al. (1979). The heavy membrane fraction was washed 3 times with buffer containing EGTA (2 mM) and EDTA (1 mM) followed by three washes with buffer containing only $MgCl_2$ and DTT. For each 100-μL assay volume, 5–7 μg of membrane protein was used. [^{3}H]cAMP was separated from ATP with Dowex 50 W-X4 and neutral alumina (Krishna & Krishnan, 1975). Ca^{2+}/calmodulin-dependent brain protein kinase was prepared from rat brain cytosol and assayed as previously reported with myelin basic protein as substrate (Fukunaga et al., 1982). On the basis of the vigorous activity shown with myelin basic protein as substrate, type II protein kinase is abundant in this preparation, which may also contain lesser quantities of types I and III (Kennedy et al., 1987). EGTA (10 mM) was used to establish a Ca^{2+}-free system.

Quantitation of Calcium Binding. The binding of ^{45}Ca to unmodified and glycated calmodulin was measured by equilibrium dialysis (Teraoka & Nierhaus, 1979), which was carried out for 36 h. Free ^{45}Ca and bound ^{45}Ca were measured by scintillation spectroscopy. At saturating Ca^{2+} levels, the mole ratio of Ca^{2+} to calmodulin was 3.9.

CNBr Cleavage. Calmodulin was glycated in the presence of [6-^{3}H]glucose for 7 days at 30 °C (as above). Glycated calmodulin was cleaved with CNBr as described by Watterson et al. (1980). Fractions were separated by FPLC using a reverse-phase column (High Pore RP318, Bio-Rad) and a trifluoroacetic acid (TFA)/acetonitrile gradient (0.1% TFA and 0.1% TFA in acetonitrile). The amount of protein in each fraction was determined fluorometrically according to the method of Bohlen et al. (1973). An aliquot of each fraction was also counted to measure incorporation of [6-^{3}H]glucose. The CNBr cleavage produces eight recoverable fragments which are numbered I–VIII on the basis of their sequence in the parent peptide, and A–H on the basis of their elution sequence from the reverse-phase column (Figure 3). CNBr peptide fragments I (three lysines) and V (two lysines) were distinguished by using intrinsic fluorescence measured in an Aminco SPF-500 spectrophotofluorometer. CNBr fragments IV and VIII were distinguished by susceptibility to trypsin. An aliquot of the pooled peaks was treated with a trypsin–

[1] Abbreviations: AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; FPLC, fast protein liquid chromatography (Pharmacia); HSL, homoserine lactone; MOPS, 3-(*N*-morpholino)propanesulfonic acid; R_g, radius of gyration; TFA, trifluoroacetic acid; UV, ultraviolet.

agarose suspension (Sigma) for 2 h on ice. The suspension was centrifuged, and the supernatant was chromatographed on silica gel thin layers using butanol/H_2O/acetic acid (volume ratios 250:250:60) as solvent. Spots were identified with ninhydrin.

Samples for Structural Studies. All structural studies were done using calmodulin that was glycated in the presence of Ca^{2+} and had, therefore, on average 2.5 glucose molecules per calmodulin molecule. Protein concentrations for the X-ray scattering experiments were determined by measuring the UV absorption and using an extinction coefficient $\epsilon^{0.1\%} = 0.19$ at 280 nm. For the CD measurements, protein concentration was determined from the amide absorption band at 190 nm by using an extinction coefficient of 10600 L $(mol{\cdot}cm)^{-1}$ (Hennessey et al., 1987).

Samples for X-ray scattering were in 50 mM MOPS at pH 7.4 with 100 mM KCl and 0.02% sodium azide. Ca^{2+}-free samples contained 5 mM EGTA, while the Ca^{2+}-saturated samples contained 50 mM $CaCl_2$. For each series, concentrations were measured for a stock solution by using UV absorption, and dilutions were determined gravimetrically.

Samples for CD measurements were dialyzed several times against 5 mM MOPS, pH 7.4, with 10 mM EGTA to remove any Ca^{2+} ions, followed by dialysis against 5 mM MOPS buffer, pH 7.4. The Ca^{2+} content of the final buffer solutions used to prepare the Ca^{2+}-free calmodulin samples was checked by inductively coupled atomic emission spectrometry (courtesy of the Colorado State University Soil Testing Laboratory). The protein concentration of the final sample was approximately 0.05 mM in a 5 mM MOPS buffer, pH 7.4. The high ionic strength and Ca^{2+}-containing samples were prepared by adding (gravimetrically) concentrated solutions of $NaClO_4$ and $CaCl_2$ to final concentrations of 150 and 5 mM, respectively. The $NaClO_4$ was chosen for its transparency in the vacuum UV spectral region.

X-ray Scattering. X-ray scattering data collection and analysis procedures have been described previously (Heidorn & Trewhella, 1988). R_g values were calculated by using a Guinier analysis with data that satisfied the condition $R_g q_{max} < 1.3$, where $q = (4\pi \sin \theta)/\lambda$. Length distributions, $P(r)$, were calculated by Fourier inversion of the scattering data to $q^2 = 0.07$ Å^{-2}. The length distribution is the frequency of vectors connecting small-volume elements within the volume of the scattering particle, weighted by the product of the X-ray scattering power in each volume element. R_g values were also computed from the second moments of the $P(r)$ functions.

CD Measurements. The far-UV absorption and CD spectra were measured at room temperature in 0.1-cm path-length cells (Hellma) with a modified Jasco 40CS spectropolarimeter, as previously described (Edmondson & Salzman, 1987). The spectral bandwidth was 1.0 nm, and time constants of 3 and 10 s were used for the CD measurements. The total optical density was less than 1.0 over the measured spectral range (260–177 nm). The CD was calibrated at 290.5 nm with *d*-camphor-10-sulfonic acid using $\Delta\epsilon^{0.1\%} = 2.36$ (Chen & Yang, 1977). The ratio of $\Delta\epsilon_{192.5}/\Delta\epsilon_{290.5}$ for *d*-camphor-10-sulfonic acid was −2.05. CD spectra were smoothed by the method of Savitsky and Golay (1964).

The fractions of protein secondary structure were determined by fitting the average CD spectra from 260 to 178 nm in 2-nm intervals with the five most significant basis spectra generated by singular value decomposition of the CD spectra of proteins with known secondary structures (Hennessey & Johnson, 1981). The "variable selection" method developed by W. C. Johnson, Jr. (personal communication), was used

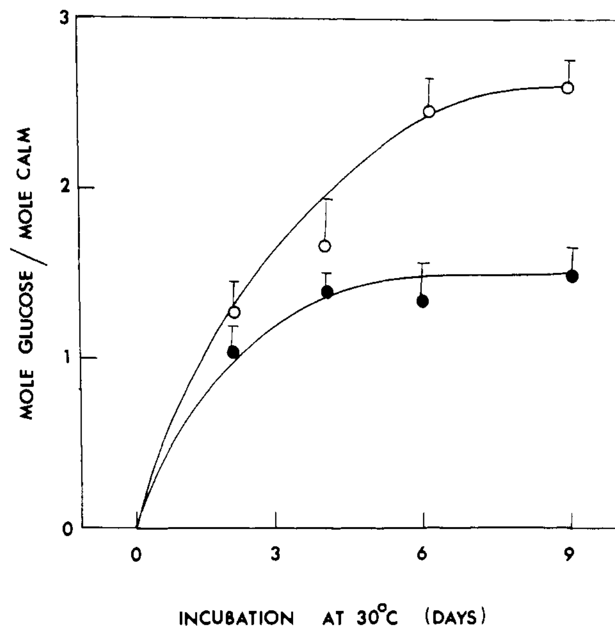

FIGURE 1: In vitro glycation of bovine brain calmodulin. Purified calmodulin was incubated with 28 mM [6-^{3}H]glucose in the presence (○) or absence (●) of Ca^{2+} as described in the text. Aliquots were removed from the incubation medium, and the incorporation of radioactivity into the TCA-precipitable fraction was quantitated by scintillation spectrometry. Incorporation of radioactivity into calmodulin was calculated at different time intervals and the extent of reaction between glucose and calmodulin determined as a function of incubation time.

to eliminate from the reference set those proteins that contain CD contributions not found in the CD spectrum of calmodulin. The results reported here are the average ± 1 standard deviation from all possible combinations containing 19 reference proteins (out of an initial set of 22) that fit the selection criteria. The self-consistency of the method was checked by repeating the procedure after excluding the reference protein(s) that failed to meet the selection criteria, and similar results were obtained although the number of solutions increased from between 20 and 100 to between 100 and 250. Similar results were also found when the data were truncated at 182 nm. The selection criteria were (a) the fraction of each secondary structure must be greater than −0.05, (b) the sum of the secondary structures must be between 0.9 and 1.1, and (c) the root mean square error between the measured and calculated spectra is less than 0.21 $\Delta\epsilon$ unit. A description of this technique applied to calmodulin at different ionic strengths has been published (Hennessey et al., 1987).

RESULTS

Glycation of Calmodulin. The incorporation of [6-^{3}H]-glucose into calmodulin at 30 °C over a 9-day period is shown in Figure 1. These experiments were also carried out at 22 °C (not shown) at a glucose concentration of 28 mM. No difference either in the shape of the time course or in the extent of glycation was observed between 22 and 30 °C, although the rate of glycation was slower at 22 °C. After 6 days, approximately 2.5 mol of glucose was incorporated per mole of calmodulin. After 9 days, the extent of glycation had not measurably increased. When glycation was carried out in the absence of Ca^{2+} (in the presence of 10 mM EGTA), it saturated at 1.5 mol of glucose/mol of calmodulin (Figure 1). Fully glycated calmodulin (prepared in the presence of Ca^{2+}) was used unless otherwise noted.

Effects of Glycation on ^{45}Ca Binding and Activation of Ca^{2+}/Calmodulin-Sensitive Enzymes. Figure 2 and Table I

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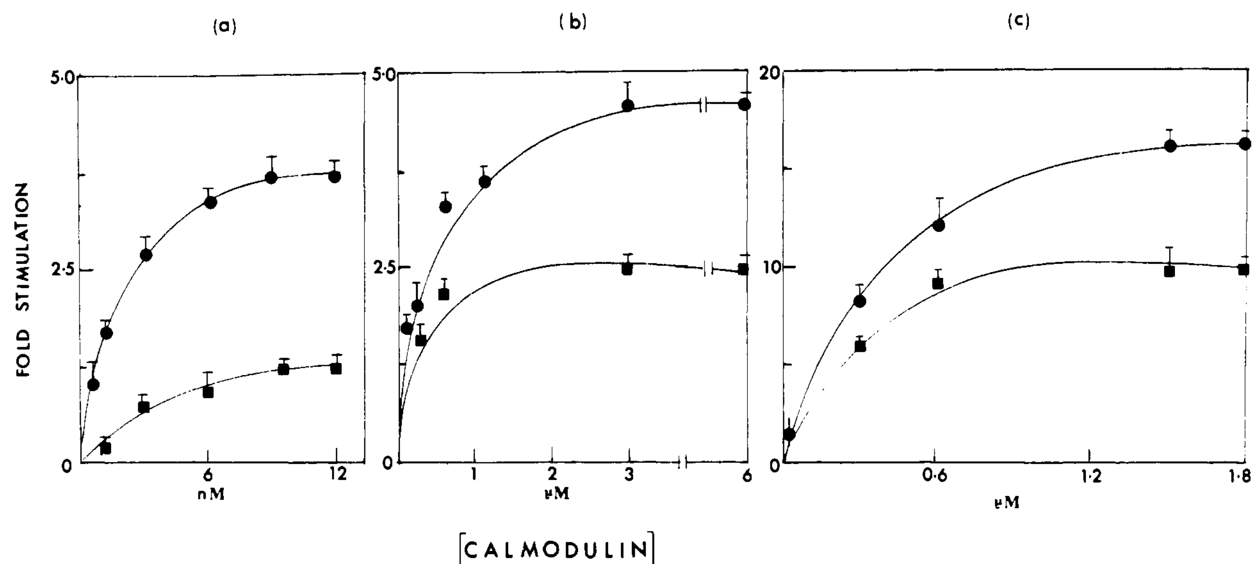

FIGURE 2: Effect of glycated and unmodified calmodulin on brain phosphodiesterase, adenylyl cyclase, and protein kinase activities. Enzyme activites were assayed in the presence of varying concentrations of glycated (■) and unmodified (●) calmodulin. Unstimulated activities were 1.5 nmol of cyclic AMP hydrolyzed (mg of protein)$^{-1}$ min^{-1} (phosphodiesterase), 85 pmol of cyclic AMP formed (mg of protein)$^{-1}$ min^{-1} (adenylyl cyclase), and 223 pmol of $^{32}PO_4^{3-}$ incorporated (mg of protein)$^{-1}$ min^{-1} (protein kinase).

Table I: Effects of Glycation on Calmodulin Function and Ca^{2+} Binding

act.	concn (nM) of calmodulin required for half-max activation: unmodified	glycated	% inhibn of calmodulin act. by glycation
phosphodiesterase	1.5	3	70
adenylyl cyclase	247	94	40
protein kinase	300	240	39
Ca^{2+} binding			54

summarize the effects of unmodified and glycated calmodulin on the activity of three brain enzymes. Maximal activation of phosphodiesterase was achieved with 12 nM calmodulin (Figure 2a). At a half-saturating concentration (3 nM) of glycated calmodulin, there was a 70% reduction in its ability to activate phosphodiesterase compared with the activation produced by a half-saturating concentration (1.5 nM) of unmodified calmodulin. For adenylyl cyclase, half-maximal activation required 247 nM calmodulin (Figure 2b) in contrast with the 1.5 nM calmodulin required for half-maximal activation of phosphodiesterase. This could reflect the fact that the adenylyl cyclase system was assayed in a membrane preparation which also contained other potential calmodulin binding sites. At a half-saturating glycated calmodulin concentration of 94 nM, there was a 40% reduction in the activation of adenylyl cyclase, as compared with the effects of half-saturating unmodified calmodulin. In the case of protein kinase (Figure 2c), a 39% decrease was observed in the capacity of half-saturating concentrations of glycated calmodulin (240 nM) to activate that protein compared to the activation produced by a half-saturating concentration of unmodified calmodulin (300 nM). Finally, a 54% reduction in the binding

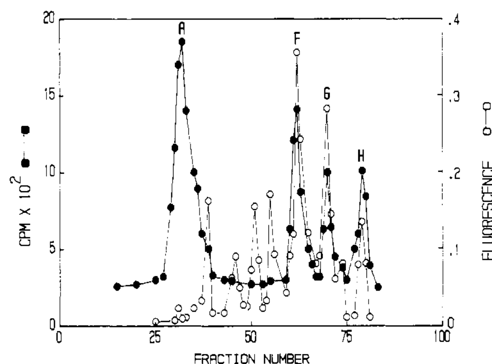

FIGURE 3: Reverse-phase FPLC of CNBr fragments generated from calmodulin showing incorporation of radioactivity in four of the eight peaks. Each of the labeled peaks (A, F, G, and H) is assigned to a numbered CNBr fragment.

of Ca^{2+} to the maximally glycated form of calmodulin was observed (Table I).

For calmodulin glycated in the presence of EGTA (i.e., to 1.5 mol of glucose/mol of calmodulin), the inhibition of calmodulin function is reduced compared with the values given above. For example, the activation of phosphodiesterase (data not shown) is only reduced by 23% compared with 70% for 2.5 mol of glucose/mol of calmodulin.

Identification of Glycation Sites. There are seven lysine residues in calmodulin available for glycation (Table II). Lysine residue 115 is trimethylated, and the N-terminus (alanine) is acetylated, and thus neither functions as a site for glycation. Eight CNBr fragments were obtained from calmodulin glycated in the presence of Ca^{2+}. The CNBr fragments are numbered in sequence starting at the N-terminus. Following exhaustive dialysis of glycated calmodulin (prepared

Table II: Glycation of Calmodulin CNBr Fragments

CNBr fragment	amino acids	chromatography peaks	Lys residue	glycation of CNBr fragments (mol of glucose/ mol of calmodulin)	no. of Phe/no. of Tyr
I	1–36	A	13, 21, 30	1.40	3/0
IV	73–69	G	75	0.30	
V	77–109	F	77, 94	0.48	2/1
VIII	146–148	H	148	0.30	

Table III: R_g and d_{max} Values Calculated for Infinite Dilution

		R_g (Å)	d_{max} (Å)
with calcium			
calmodulin	$P(r)$	21.1 ± 0.4	62 ± 2
	Guinier	21.0 ± 0.3	
glycated calmodulin	$P(r)$	21.4 ± 0.2	66 ± 2
	Guinier	20.9 ± 0.2	
without calcium			
calmodulin	$P(r)$	19.6 ± 0.3	58 ± 2
	Guinier	19.9 ± 0.6	
glycated calmodulin	$P(r)$	19.8 ± 0.3	63 ± 2
	Guinier	19.7 ± 0.1	

by using [6-^{3}H]glucose), the protein was subjected to complete CNBr fragmentation, and the fragments were separated on reverse-phase FPLC (Figure 3).

Only four of the eight CNBr peptides contain one or more lysines as summarized in Table II. These are fragments I, IV, V, and VIII. When the CNBr fragments were separated on reverse-phase FPLC, eight peptide peaks were identified with fluorescamine. Four of these peaks had incorporated [6-^{3}H]glucose. The four labeled chromatographic peaks (A, F, G, and H) were assigned to their CNBr fragments in the native peptide by the following reasoning. CNBr fragments IV and VIII contain neither tyrosine nor phenylalanine. In contrast, fragment V contains both phenylalanine and tyrosine, while fragment I contains tyrosine but no phenylalanine. Using native fluorescence, it was therefore possible to definitively assign peak A to fragment I and peak F to fragment V. Note that peak A shows 1.4 mol of bound glucose/mol of calmodulin, and hence its assignment to fragment I is consistent with the fact that this fragment contains more than one lysine. Peaks G and H were assigned to the remaining fragments by using their differential sensitivity to trypsin digestion. Fragment IV contains a trypsin-sensitive site, while fragment VIII does not. After exposure to trypsin, peak G gave two spots while peak H gave only one by thin-layer chromatography. Peaks G and H were therefore assigned to fragments IV and VIII, respectively. The CNBr peptide fragments as isolated on reverse-phase FPLC and the extent (in terms of the incorporation of [6-^{3}H]glucose) of their labeling are summarized in Table II.

For calmodulin glycated in the presence of EGTA, CNBr fragment V shows no detectable glycation, and the remaining fragments also exhibit some reduction in their levels of glycation compared with glycation in the presence of Ca^{2+} (data not shown).

X-ray Scattering. R_g values were determined by using both the Guinier and $P(r)$ analyses for both glycated and nonglycated calmodulin in the presence and absence of Ca^{2+} over the concentration range 10–50 mg/mL (in steps of 10 mg/mL). Table III gives the respective R_g values calculated by extrapolation to infinite dilution (to eliminate effects of interparticle interference). Glycation resulted in no measurable change in R_g for either Ca^{2+}-free or Ca^{2+}-bound calmodulin.

Figure 4 shows the $P(r)$ functions calculated for infinite dilution, for glycated and nonglycated calmodulin both with and without Ca^{2+}. There are small changes in the $P(r)$ curves on glycation that are at the detection limits of small-angle X-ray scattering. The shoulders observed near 38 Å in the $P(r)$ functions of the unmodified forms are slightly less accentuated in the glycated form. Figure 5 shows the $P(r)$ difference functions calculated between the Ca^{2+}-saturated and Ca^{2+}-free forms of both glycated and nonglycated calmodulin. There is qualitative agreement in the difference curves in that they both show fewer short vectors and more long vectors on Ca^{2+} binding, though in the region between 15 and 35 Å, there are differences that are larger than the errors propagated from the statistical errors in the data and corrections such as slit desmearing.

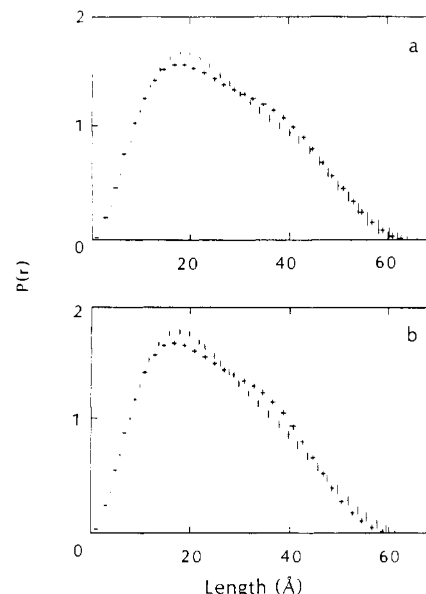

FIGURE 4: $P(r)$ functions for calmodulin (+) and glycated calmodulin (|) in the presence of Ca^{2+} (a) and in the absence of Ca^{2+} (b).

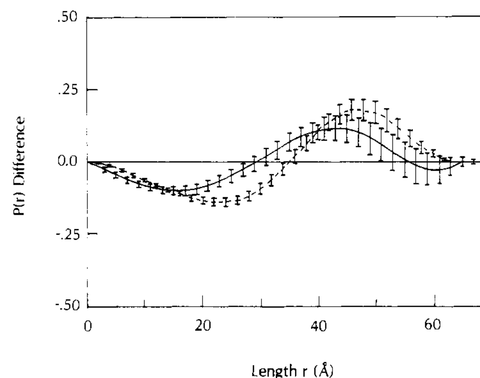

FIGURE 5: $P(r)$ difference curves calculated as the Ca^{2+}-saturated minus the Ca^{2+}-free form for calmodulin (dashed line) and glycated calmodulin (solid line).

Circular Dichroism. The CD spectra for glycated and nonglycated calmodulin at pH 7.4 in 150 mM $NaClO_4$, both with and without Ca^{2+}, are shown in Figure 6a. The spectra are in qualitative agreement with previous measurements (Hennesey et al., 1987). However, the magnitude of the 192-nm band is about 15% less than that reported by Hennessey and co-workers. This difference is reflected in a lower estimate of the total α-helix content compared with these workers' estimate. The results of the secondary structure analyses are presented in Table IV. As was found by Hennessey and co-workers, the binding of Ca^{2+} at high ionic strength (150 mM $NaClO_4$) results in a 4% increase in the α-helical content for unmodified calmodulin.

At low ionic strength (without $NaClO_4$), pH 7.4, and in the absence of Ca^{2+}, glycation has a significant effect on the CD spectrum of calmodulin (see Figure 6b and Table IV). Both the glycated and nonglycated forms show significantly less α-helix than any of the forms at physiological ionic strength (150 mM $NaClO_4$), with the unmodified calmodulin showing the least α-helix. In contrast, glycating calmodulin has little effect on the secondary structure at low ionic strength in the presence of Ca^{2+}, or at physiological ionic strength either with or without Ca^{2+}. As is observed for unmodified calmodulin,

Table IV: Percentage Secondary Structure Contents from CD Analysis

mol of glucose/mol of CaM	pH	[$NaClO_4$] (M)	[$CaCl_2$] (M)	α-helix	antiparallel β-sheet	parallel β-sheet	turns	other
0	5.5	0	0	59 ± 2	0 ± 2	1 ± 1	18 ± 1	15 ± 2
0	7.4	0	0	37 ± 2	12 ± 5	0 ± 2	22 ± 1	23 ± 2
2.5	7.4	0	0	44 ± 1	6 ± 3	0 ± 2	22 ± 1	22 ± 3
0	5.5	0	0.005	59 ± 2	2 ± 3	1 ± 2	21 ± 2	18 ± 4
0	7.4	0	0.005	53 ± 2	7 ± 3	2 ± 2	23 ± 2	18 ± 3
2.5	7.4	0	0.005	53 ± 3	4 ± 3	1 ± 3	24 ± 2	19 ± 2
0	5.5	0.15	0	57 ± 2	3 ± 3	1 ± 1	20 ± 1	17 ± 3
0	7.4	0.15	0	49 ± 2	9 ± 3	2 ± 1	22 ± 2	19 ± 3
2.5	7.4	0.15	0	50 ± 1	5 ± 3	0 ± 2	21 ± 2	17 ± 3
0	5.5	0.15	0.005	59 ± 2	3 ± 3	0 ± 2	22 ± 2	17 ± 3
0	7.4	0.15	0.005	53 ± 2	9 ± 4	1 ± 2	24 ± 2	17 ± 2
2.5	7.4	0.15	0.005	54 ± 1	5 ± 2	1 ± 1	24 ± 1	18 ± 2

there is an increase in the α-helix content of 4% on Ca^{2+} binding at physiological pH and ionic strength.

CD spectra were also recorded for unmodified calmodulin at pH 5.5 (Figure 6c). Lowering the pH results in an increase in α-helix of 6–7% for all conditions studied except for the Ca^{2+}-free, low ionic strength condition, where the increase is much larger (22%) (Table IV). At low pH and physiological ionic strength, the α-helix content increases by only 2% on Ca^{2+} binding, but, unlike the pH 7.4 case, there is no apparent change in the CD spectra when they are compared directly, suggesting that this change may not be significant.

Discussion

Glycation of calmodulin in the presence of Ca^{2+} has been shown to cause a reduction in its capacity for binding Ca^{2+}, and a variable (39–70%) decrease in its ability to activate three target enzymes. The process of glycation appears to involve all of the available lysines, but no more than two or three on any single calmodulin molecule. Moreover, while some lysines (those in fragment I) are somewhat more and others (those in fragment V) somewhat less reactive than the average (see Table II), no single lysine residue appears either to be excluded from or to dominate the glycation process.

There are a number of factors that could influence the glycation process:

(1) It has been observed in a number of cases that glycation is associated with a reduction in p*I* (Kondo et al., 1987; Kowluru et al., 1987b; Spicer et al., 1978; Williams & Siegal, 1985). In the case of calmodulin, the p*I* was reduced from 4.3 to 4.1. An increase in the negative charge of glycated proteins appears to be a constant feature of this process (Bitensky et al., 1988).

(2) Local changes in the pK_a values of a variety of charged groups have been reported (Matthew et al., 1979). Moreover, it has been suggested that two adjacent lysines (or other positively charged groups) could exhibit different pK_a values in consequence of the proximity of their positive charges. In the case of calmodulin glycation, however, the widely spaced distribution of lysines and arginines does not support the operation of this mechanism.

(3) The first step in the glycation process (i.e., the Schiff base formation) is readily reversible, while the Amadori product appears relatively stable. Thus, the amount of product observed in glycation is a function of the amount of ketoamine formed by rearrangement. In some proteins, it has been suggested that glycation appears to favor lysines adjacent to carboxylate anions or ammonium ions which can serve to catalyze the Amadori rearrangement (Garlick & Mazer, 1983; Shapiro et al., 1980).

The increase in negative charge that is observed with glycation of proteins may result from the pK_a of the resulting

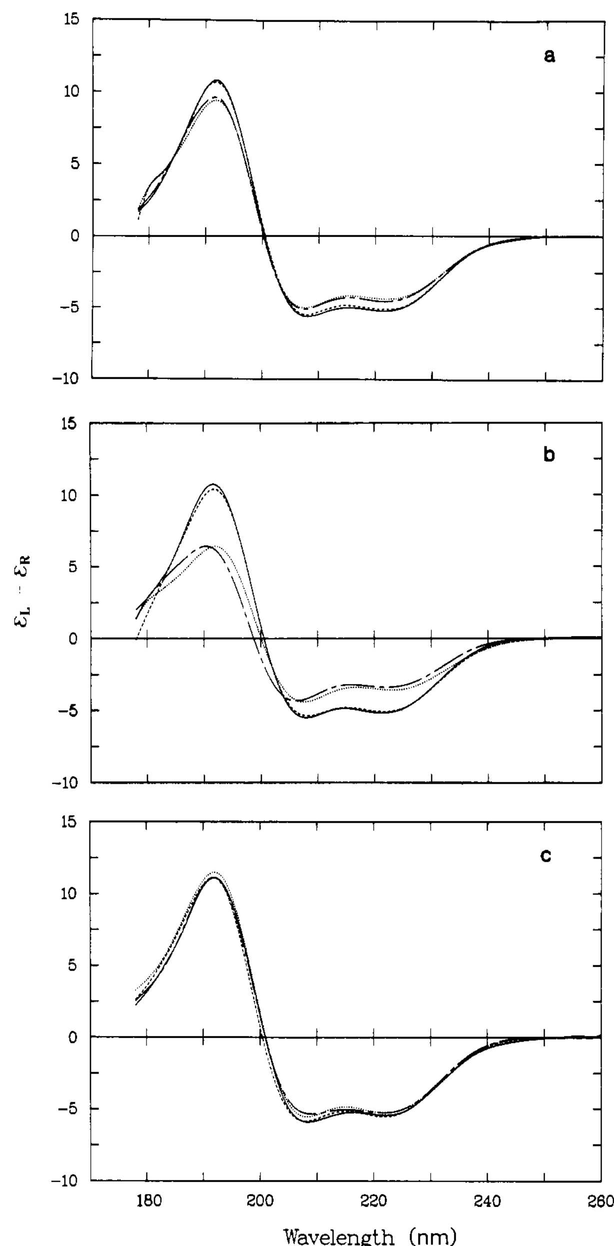

FIGURE 6: CD spectra measured for (a) unmodified calmodulin in the Ca^{2+}-saturated (—) and Ca^{2+}-free (--) form and for glycated calmodulin in the Ca^{2+}-saturated (---) and Ca^{2+}-free (···) form, at pH 7.4, 150 mM $NaClO_4$; (b) unmodified calmodulin Ca^{2+} saturated (—) and Ca^{2+} free (--) and for glycated calmodulin both Ca^{2+} saturated (---) and Ca^{2+} free (···), at pH 7.4, 0 mM $NaClO_4$; and (c) unmodified calmodulin at pH 5.5 with 150 mM $NaClO_4$ Ca^{2+} saturated (—) and Ca^{2+}free (--) and with 0 mM $NaClO_4$ both Ca^{2+} saturated (---) and Ca^{2+} free (···).

ketoamine or advanced glycation end products being lower than the pK_a of the lysine group from which it was derived. In addition, the increased negative charge of the glycated protein may elevate the pK_a values of unmodified lysine groups relative to the pK_a values prior to glycation. Such an effect might account for the observed limit on glycation observed for calmodulin, since the remaining unmodified lysines would be less susceptible to modification. In this model, the presence of adjacent acidic or basic moieties is seen a facilitating factor which would enhance glycation (Bitensky et al., 1988).

Glycation at the 2.5 mol of glucose/mol of calmodulin limit has only a very small effect on the overall structure of calmodulin at physiological pH (7.4) and ionic strength (150 mM). The redistribution in the $P(r)$ functions both in the Ca^{2+}-free and in the Ca^{2+}-bound states of glycated calmodulin suggests the structure of the protein may become slightly less dumbell-shaped and more rodlike. Model calculations (not shown) were done [according to the procedures described in Heidorn and Trewhella (1988)] in which glucose molecules were attached to the calmodulin structure to evaluate the potential impact of the extra scattering density on the $P(r)$ function, and the observed changes in the experimental $P(r)$ functions cannot be attributed to this. Hence, the data suggest the possibility of a small redistribution in scattering density in the structure on glycation, perhaps due to changes in electrostatic interactions, but with no accompanying change in R_g. Furthermore, the same increase in R_g is observed for the glycated protein on Ca^{2+} binding as for the unmodified protein, and the redistribution in the $P(r)$ functions observed on Ca^{2+} binding is at least qualitatively similar for the glycated and unmodified proteins.

The CD data also show glycated and unmodified calmodulin to be very similar in their overall secondary structure content, except in the absence of Ca^{2+} at low ionic strength. Under all conditions studied, glycation appears to inhibit the formation of the CD component attributed to antiparallel β-sheet, and this effect is strongest in the Ca^{2+}-free, low ionic strength condition. The apparent inhibition of this structure may be related to the small changes observed in the $P(r)$ functions. The small increase in α-helix (4%) observed on Ca^{2+} binding to calmodulin at physiological pH and ionic strength is, however, unaffected by glycation.

Since it has been demonstrated that the glycated calmodulin has a reduced capacity for Ca^{2+} binding, the above data demonstrate that those Ca^{2+}-dependent conformational rearrangements characterized by an increase in α-helix content and an elongation of the molecule do not require all four Ca^{2+} binding sites to be occupied by Ca^{2+}. The magnitude of the increases in R_g and α-helix content on Ca^{2+} binding is the same for the unmodified and glycated proteins, indicating that even though there is likely to be a mixture of calmodulin molecules with different combinations of Ca^{2+} binding sites occupied, every molecule must undergo the maximal Ca^{2+} binding effect on these parameters. The structural data reflect the average conformation, so if there were a significant population of molecules that did not show the changes, this would be evidenced as a reduction in the measured increases in helix content and R_g. It seems, therefore, that the functional inhibition of calmodulin on glycation is not due to inhibition of these Ca^{2+}-dependent conformational changes. A possible alternative mechanism would be interference with target enzyme binding due to glucose attachement to lysine residues in the target enzyme binding site of calmodulin. Furthermore, the absence of evidence for large conformational rearrangements on glycation suggests that the reduced Ca^{2+} binding capacity is most likely due to the Ca^{2+} binding pockets being blocked by local steric effects of bound glucose.

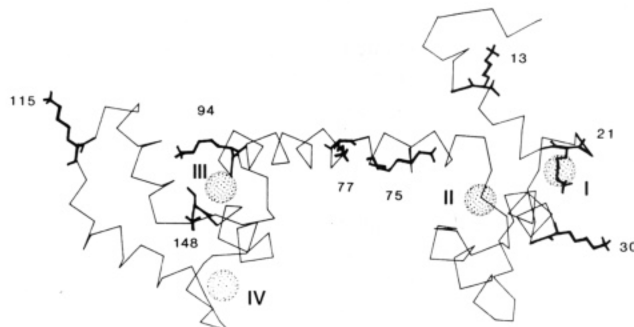

FIGURE 7: Crystallographic α-carbon backbone for calmodulin with the lysine side chains added.

Figure 7 shows the crystallographic α-carbon backbone for calmodulin with the lysine side chains. Previous small-angle scattering studies (Heidorn & Trewhella, 1988) have demonstrated that while the size and shape of the globular domains in calmodulin appear to be preserved between the solution and crystal forms, they are closer together in solution by several angstroms. There is, however, no data to indicate the precise way in which the domains approach each other in solution, and so for this discussion, we will use the crystal structure and focus on the local structure of the Ca^{2+} binding sites. The seven lysine side chains that can be glycated in the presence of Ca^{2+} are spread out in the calmodulin sequence (residues 13, 21, 30, 75, 77, 94, and 148) and are shown in Figure 7. Since the crystallographic side-chain coordinates were not available, the orientations shown in Figure 7 were derived simply by adding all the side chains to the structure in idealized configurations and then minimizing the total potential energy with the molecular mechanical program AMBER (Weiner & Kollman, 1981; Weiner et al., 1984). The resulting side-chain positions are therefore subject to uncertainty. They do, however, provide a useful guide for evaluating the potential impact of glycation on the Ca^{2+} binding domains. The four Ca^{2+} binding domains of calmodulin are normally identified as I (residues 12–39), II (48–75), III (85–112), and IV (121–148) (Klee & Vanaman, 1982). Inspection of the three-dimensional structure of calmodulin shows that Ca^{2+} binding domains II and IV are relatively free of lysine residues, each having only one lysine residue at their extreme C-terminal ends. Steric hindrance to Ca^{2+} binding in these domains resulting from the attachment of glucose molecules in the absence of a conformational rearrangement is not likely, even after allowing for possible reorientation of the lysine residues. The Ca^{2+} binding domain III also contains only one lysine residue, but it is centrally located in the Ca^{2+} binding site. The Ca^{2+} binding domain I contains three lysine residues, with lysine-21 located central to that binding domain. Given the proximity of targets for glycation, Ca^{2+} binding to domains I and III seems most likely to be affected by glycation.

NMR data on the interaction of myosin light chain kinase with calmodulin (Klevit et al., 1985; Klevit & Blumenthal, 1987) suggest that a large region of the calmodulin molecule is involved in substrate binding, including the interconnecting helix and parts of both globular domains. Further, it has been proposed that Ca^{2+}-dependent, substrate-specific conformational changes in calmodulin are important in the Ca^{2+} regulation. In addition to the changes in helix content and R_g, Ca^{2+} induces changes that result in the exposure of hydrophobic domains in the calmodulin structure (LaPorte et al., 1980), and new regions of the protein become accessible to

soluble agents (Walsh et al., 1977). In this light, it is interesting to note that the lysine residues that are protected from glycation in the absence of Ca^{2+} (residues 77 and 94) lie in the interconnecting helix region, suggesting that the Ca^{2+}-induced conformational change does involve increased solvent exposure of the interconnecting helix region of the structure. Furthermore, in the absence of Ca^{2+}, the α-helical polypeptide poly(glutamic acid) was invariably excluded from the set of reference proteins selected by the "variable selection" CD analysis. This indicates that Ca^{2+}-free calmodulin does not contain the secondary structural elements characteristic of α-helical poly(glutamic acid). However, poly(glutamic acid) did give good analyses for Ca^{2+}-bound calmodulin. It might be speculated, therefore, that the small increase in α-helix observed on binding Ca^{2+} reflects a conformational change in the four glutamic acid residues (82–87). An increase in helix stability involving these residues, which are in the interconnecting helix, could explain the small increase in R_g and increased exposure of other residues in the interconnecting helix on Ca^{2+} binding.

The pH dependence of the α-helix content in calmodulin is interesting from the perspective of the crystal structure, which was derived from crystals grown at low pH (5.5). The CD data show that the low-pH environment causes an increase in α-helix, and furthermore, the α-helix content for the low-pH form in solution is very close to that determined for the crystal structure (63% helix in terms of residues, 59% in terms of amides, Babu et al., 1985). It appears, therefore, that low pH may be a critical factor in stabilizing the interconnecting helix in the crystal environment. The CD data on calmodulin without Ca^{2+} at low ionic strength, and at neutral pH (7.4), show the lowest α-helix content of all conditions studied. Under these conditions, calmodulin is a highly electronegative molecule, and in the low ionic strength environment, there may be insufficient counterions to neutralize the protein and thus help stabilize the structure in solution. High ionic strength, Ca^{2+} binding, and lowering the pH all result in a reduction of the net charge on the molecule and an apparent increase in the α-helix content. This increase may be due to stabilization of the interconnecting helix which contains a large number of charged residues. Small-angle solution scattering from cytochrome *c* (Trewhella et al., 1988) has shown that the structure of this highly basic protein is also sensitive to ionic strength, and it has been suggested that this protein structure is destabilized at low ionic strength.

Previous studies have examined the effects of other chemical as well as genetic modifications of calmodulin. A study which produced 3 mutant calmodulins with 3, 16, or 19 amino acid substitutions also produced evidence that different domains of calmodulin interact with different target enzymes (Putkey et al., 1986). In another study, 40–100% of the lysines of calmodulin were allowed to react with one of five different chemical modifiers. The resulting chemically modified calmodulins showed variable changes in affinity for and activating efficiency of two target enzyme systems. The data also demonstrated that changes in activating efficiency were independent of concomitant changes in affinity for the target enzymes (Tetrin-Clary et al., 1987). Finally, modification of calmodulin by a fluorenyl-based spin-label showed extensive reactivity with lysines-75 and -148, forming a double adduct which showed little capacity to activate brain phosphodiesterase. The data suggest that these lysine residues are in the phenothiazine binding site of calmodulin which can recognize the tricyclic fluorene ring of the spin-label compound. Calmodulin reaction with this compound is inhibited by trifluoperazine. The fact that our data show differences in the degree to which calmodulin function is impaired by glycation for each of the target enzymes tested is consistent with the idea that different domains of calmodulin interact with different target enzymes. Alternatively, the observations may reflect differences in the class of proteins to which the target enzymes belong (i.e., integral versus soluble).

Calmodulin is a ubiquitous protein which exhibits widespread enzymatic, structural, and cytoskeletal interactions. Abnormalities in the function or recognition of calmodulin in the diabetic subject could have functional and histopathological consequences both in the nervous system and in microcirculation. It is interesting that one can recover functionally compromised calmodulin from the nervous system of animals that have been diabetic for periods of 5 weeks (Kowluru et al., 1987a). Our findings also raise the possibility that at selected subcellular domains, where calmodulin turnover might be slow (the synaptic terminus) or nonexistent (the erythrocyte), a large enough fraction of glycated calmodulin molecules could accumulate to impair localized calmodulin function.

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Registry No. Ca, 7440-70-2; L-lysine, 56-87-1; phosphodiesterase, 9025-82-5; adenylyl cyclase, 9012-42-4; protein kinase, 9026-43-1.

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