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Urea Denaturation of Horse Heart Ferricytochrome *c*. Equilibrium Studies and Characterization of Intermediate Forms†

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ABSTRACT: Equilibrium studies of the urea denaturation of horse heart ferricytochrome *c*, pH 7.0, through alteration of the absolute extinction of the 695-nm band and of the fluorescence efficiency of the tryptophan side chain, have been reported. The denaturation profiles using the two probes have been analyzed in terms of similarities and differences as well as to determine the nature of the intermediate forms. The intermediate forms in the 4–4.5 M urea concentration range have been further characterized by circular dichroism spectroscopy in the Soret and the intrinsic absorption regions, stability to temperature and pH, and reactivity of the methionine and histidine side chains to bromoacetic acid. The scheme $N \rightleftharpoons X_1 \rightleftharpoons X_2 \rightleftharpoons D$, in which N and D are the 0 and 9 M urea forms and X_1 and X_2 are the two intermediate forms, with midconcentrations of urea of 2–2.5, 6.2, and 6.9 M, respectively, for the three transitions, is proposed as an explanation of the observed denaturation profiles of the protein. The N to X_1 transition is seen in the enhanced absorptivity of the 695-nm band and the further quenching of tryptophan fluorescence. Form X_1 exhibits lowered temperature and pH stability, a nativelike intrinsic CD spectrum, and reactivity of both His-18 and Met-80 to bromoacetic acid. The X_1 to X_2 transition is apparent only in the 695-nm absorptivity–urea profile, while the X_2 to D transition is discerned from both the enhancement of tryptophan fluorescence and the final quenching of the 695-nm absorptivity. The extent of alteration of $|\epsilon|_{695}$ during the X_1 to X_2 transition is about two-thirds the total extinction. The two high molar urea transitions yield reference free-energy changes of 25.1 and 9.1 kcal/mol, and the corresponding parameter determining the extent of solvent exposure, the parameter *m*, amounts to about 4000 and 1300 cal/M^2, respectively. The course of urea denaturation of horse heart ferricytochrome *c*, in terms of the alteration of protein structures, is thus described as follows. Step 1, N to X_1, reflects in the main the loosening of the frontal section of the heme crevice, without alteration of either the coordination configuration of the native protein or its polypeptide structures. However, definite destabilization of the molecular conformation, as well as of the heme coordination configuration, occurs, which is detailed with reference to the reciprocity of the heme crevice to molecular stability. Step 2, the X_1 to X_2 transition, is characterized simply as a reflection of the solvent exposure of the protein backbone, again without alteration of either the coordination configuration of heme iron or the tryptophan–heme domain of the molecule. The reduction of the 695-nm extinction during this step is considered to be linked to the molecular conformation. Step 3, the X_2 to D transition, is regarded as a composite of both the disruption of the Met-80–S–iron linkage and the loosening of the crevice in the tryptophan–heme domain of the molecule; it may reflect the reorganization of the polypeptide chain.

The structural, conformational, and possibly functional aspects of enzymes can be better understood from a detailing of the folding and unfolding processes. In the case of the constituents of the electron-transport chain, cytochrome *c* is the only component susceptible to such investigation, primarily because of its availability in a pure, well-defined form and the wealth of information regarding its structure, conformation, and function (Margoliash & Schejter, 1966; Harbury & Marks, 1973; Dickerson & Timkovich, 1975; Myer, 1978; Myer & Pande, 1978; Ferguson-Miller et al., 1979). The availability of the three-dimensional structures of a number of preparations, on the one hand, and details of structural–conformational–functional relationships, on the other, provide the necessary bases for the interpretation of not only the course of the folding and unfolding processes but also of the state of the intermediate forms.

Horse heart ferricytochrome *c* has been denatured by a number of agents and studied by both kinetic and equilibrium approaches, yet there is no consistent view regarding either the mechanism for denaturation and folding of the protein or an understanding of the processes in terms of various structural aspects of the molecule. From earlier studies with urea using circular dichroism and absorption spectroscopy, it was proposed that the course of urea denaturation can be described by a two-step process, i.e., a three-state model (Myer, 1968a; Stellwagen, 1968). Similar inferences were made when the denaturation was induced with a variety of alcohols (Kaminsky et al., 1973). Using fluorescence spectroscopy as the probe,

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others suggested that urea denaturation is a simple single-step process (McLendon & Smith, 1978; Aviram & Weissmann, 1978). Studies of the kinetics of the folding and unfolding with urea have been limited, but the possibility of a sequential mechanism has been proposed (Tsong, 1976). A single-step model has also been proposed for the denaturation with guanidinium hydrochloride from equilibrium investigations using optical activity (Knapp & Pace, 1974) and fluorescence spectroscopy (McLendon & Smith, 1978). From the kinetics of the folding and unfolding with guanidinium hydrochloride, the proposals range from a simple mechanism with one intermediate and one incorrectly folded form, three steps (Ikai et al., 1973; Henkens & Turner, 1973), to a mechanism involving several conformational forms in a sequential manner (Tsong, 1976). Recently, a three-step mechanism similar to that from the kinetic studies of guanidinium hydrochloride denaturation has been found applicable to the denaturation of the protein with alcohols (Drew & Dickerson, 1978). There are undoubtedly similar inconsistencies regarding the state of the intermediate forms, as the variation in possible forms in a given scheme dictates the differences in the forms themselves. In view of an earlier observation from CD studies of the urea denaturation of cytochrome *c*—that the course of the reaction proceeds through a minimum of two steps (Myer, 1968a), none of which was clearly defined in terms of protein structures—we undertook to investigate the details of this process, with particular attention to not only the course of the reaction but also to the structural characterization of the various intermediate forms. Using two well-characterized structural–conformational probes, the 695-nm absorption band, a feature directly linked to the presence or absence of the Met-80–S–iron linkage (Shechter & Saludjian, 1967; Sreenathan & Taylor, 1971), and the fluorescence efficiency of the single tryptophan side chain, a direct measure of the conformational and configurational aspects of the heme–tryptophan domain (Stryer, 1968; Tsong, 1976), i.e., the deepest part of the heme crevice (Dickerson & Timkovich, 1974), we reinvestigated in detail the course of urea denaturation of this protein. In addition, we have characterized the intermediate forms, using circular dichroism spectroscopy, thermal denaturation, pH–spectroscopic behavior, and the reactivity of the protein functional groups to group-specific reagent as conformational probes (Myer, 1978; Myer & Pande, 1978), on the one hand, and of the microscopic environment of a number of protein functional groups, on the other hand (MacDonald, 1974). This report is the result of these investigations.

Experimental Procedure

Materials. Horse heart cytochrome *c*, type III, was purchased from Sigma Chemical Co. and used without further purification. Ultrapure urea, a Schwarz/Mann product, was used after evacuation for a period of at least 12 h and always as fresh solutions, i.e., within 1 to 2 h of preparation. Bromoacetic acid was twice crystallized, and all other chemicals were of analytical grade.

Procedures. Carboxymethylation. The carboxymethylation of the protein in the absence and/or presence of urea was performed according to the procedure described by Stellwagen (1968) and detailed by MacDonald (1974). The procedure in general consists of the treatment of cytochrome *c* solution in 0.1 M phosphate, pH 7.5, with or without urea, with bromoacetic acid, final concentration 0.2 M, at room temperature for a period of 72–75 h. The reaction was terminated by gel filtration with Sephadex G-25, equilibrated with the same buffer but without the additives. The resulting products were further purified by using Sephadex G-75 and 0.1 M phosphate, pH 7.5, and the major heme-containing fractions were pooled, desalted, and lyophilized.

Amino Acid Analysis. The amino acid analysis of the products was performed after acid hydrolysis with the aid of the self-assembled amino acid analyzer. The details of the procedure and of the analyzer are reported elsewhere (Myer, 1972).

Heme Undecapeptide. The heme-containing protein fragment of the native and modified preparations from residue 11–21, both inclusive, was prepared according to the procedure outlined by Harbury & Loach (1960).

Absorption Spectra. Spectroscopic measurements were performed with a Cary 15 spectrophotometer using the specially designed apparatus for pH studies (Myer & Harbury, 1973). The absolute extinction of the 695-nm band was determined according to the procedure outlined by Kaminsky et al. (1973). The concentrations of the solutions were determined by using an extinction of 1.04×10^4 at 528 nm, pH 7.0.

Circular Dichroism Spectra. The circular dichroism spectra were recorded with a JASCO-J-15 spectrodichrograph. Details of the measurement of the CD spectrum in the intrinsic and the Soret regions, as well as the measurement of effects of temperature, have been given in an earlier publication (Myer, 1968a,b), or they can be found in one of the recent reviews on this subject (Myer, 1978; Myer & Pande, 1978).

Fluorescence Measurements. Fluorescence measurements were performed with a setup consisting of the following: two double monochromators, Spex minimates; a light source, a xenon 450-W lamp; an IP-28 photomultiplier; an ISCO linear log amplifier; a recorder; the necessary optics for proper matching of the monochromator characteristics, focusing of the excitation beam, and collection of the emission beam. L-Tryptophan, a Sigma product, was used to standardize the instruments. Solutions containing 2 μM protein were excited at 280 nm and the spectra recorded between 300 and 420 nm. A constant slit width of 1.25 mm was maintained, both for the exit of the illuminator and for the analyzer. The results are normalized with respect to the fluorescence intensity of L-tryptophan as 100%. All measurements are in 0.05 M phosphate buffer with 0.2 M KCl, pH 7.0, unless otherwise stated.

Analysis of Equilibrium Data. The equilibrium parameters for the denaturation were determined according to the procedure described by Pace (1975). The limiting values of the transition were determined either through extrapolation of the curves or through least-squares fitting. The conditional equilibrium constant, K_D, was determined by using the expression $(P_i - P_{obsd})/(P_{obsd} - P_f)$, where P_i, P_f, and P_{obsd} are the initial, final, and observed values of the parameter, respectively. The apparent free energy for denaturation, and reference free-energy change, ΔG^*, and the parameter m were determined by linearization of the data through the empirical equation $\Delta G_D = -RT \ln K_D = \Delta G^* - m[U]$, where [U] is the effective concentration of urea.

The pH–spectroscopic data were analyzed by using the Henderson–Hasselbach equation, $pH = pK_a + n \log (A/AH)$, where A and AH are the proportions of the deprotonated and protonated forms. The pH–spectroscopic titrations were linearized by using an extended version of the Reed & Berkson method (1929) from data points between 20 and 80% of the titration as the reference points, as described by Clark (1960). The midpoint of the titration and the initial and final values of the variable parameters were then floated for best fit by using unweighted least-squares programming, and the reverse

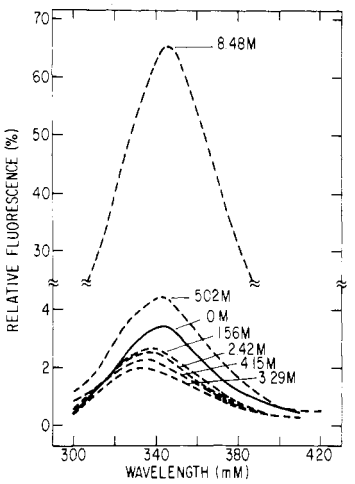

FIGURE 1: Fluorescent spectra of horse heart ferricytochrome *c* in the presence of different concentrations of urea. Conditions: 0.05 M phosphate + 0.2 M KCl + varying concentrations of urea, pH 7.0; temperature 22 °C; excitation wavelength 280 nm; protein concentration 2 μM. Relative fluorescence with respect to free tryptophan, 2 μM, in the same buffer, but without urea.

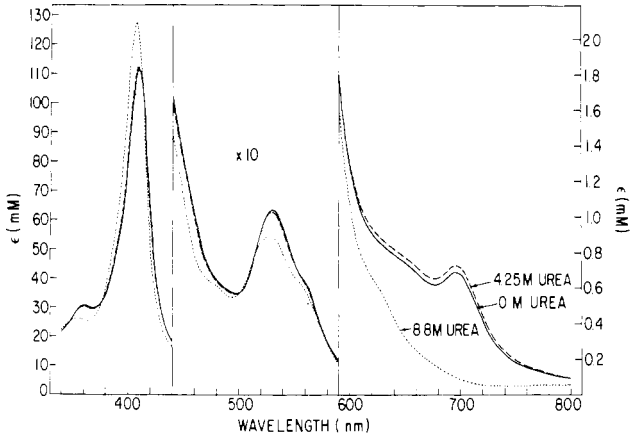

FIGURE 2: Absorption spectra of native horse heart ferricytochrome *c* and in the presence of 4.5 and 9 M urea. The buffer was the same as that used in Figure 1. "×10" in the central section applies to the ordinate on the right.

calculations were performed to obtain the calculated curves.

The resolution of an asymmetric titration into its components was performed by assuming that one of the extreme limbs of the titration is primarily the contribution of one component. The curve for the limiting component was generated and subtracted from the observed titration curve, followed by similar analysis of the residual information until a best fit was obtained after floating the information of the first component and subsequent components for a nonlinear least-squares fit. The final resolution was found to be acceptable when the root mean square reached a minimal value of 0.001.

The thermodynamic parameters from the temperature–denaturation curves were obtained through analysis of data by using the well-known van't Hoff relationship $\Delta G° = RT \ln K_{eq} = \Delta H° - T\Delta S°$.

Results

(A) Urea Denaturation, 695-nm Extinction and Tryptophan Fluorescence. The effect of increasing urea concentrations on the fluorescence spectra of ferricytochrome *c* at pH 7.0 is shown in Figure 1, and in Figure 2 are compared the absorption spectra at certain critical urea concentrations. The

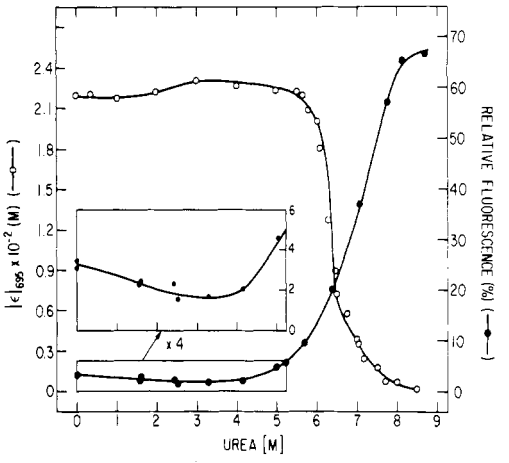

FIGURE 3: Denaturation profiles of horse heart ferricytochrome *c* with urea from changes of absolute extinction of the 695-nm band (○) and the fluorescence efficiency (●) of the tryptophan residue. Fluorescence data were taken from Figure 1; values were at the maxima of the emission spectra. Absolute extinction of the 695-nm band was determined according to the procedure of Kaminsky et al. (1973). Conditions of measurement were identical with those listed in Figure 1.

denaturation profiles resulting from increasing concentrations of urea, as exhibited by alteration of the fluorescence efficiency of the tryptophan side chain and of the absolute extinction of the 695-nm band, are compared in Figure 3. The effect of increasing concentrations of urea on the tryptophan fluorescence is, first, the lowering of fluorescence efficiency with a concurrent blue shift in the position, followed by enhancement and a return to the normal position (Figures 1 and 3). The absolute extinction of the 695-nm band, on the other hand, first increases, reaching a maximum value at 3.5–4.5 M urea, and subsequently decreases to zero value at ~9 M urea (Figure 3). The absorption spectrum in the Soret and visible regions for the protein in solution with 3.5–4.5 M urea is barely distinguishable from that of the native protein, while in 9 M urea the Soret absorptivity is enhanced with a blue shift in position (Figure 2). In the visible region, the absorptivity at 530 nm is slightly lowered, without any indication of a band in the 620-nm region. There is a definite enhancement of the 695-nm band for solutions containing 3.5–4.5 M urea, which is extinguished at ~9 M urea.

The denaturation profiles in Figure 3 can be described as a composite of at least two distinct transitions, one centered below 4–5 M urea and the other major transition localized at higher urea concentrations. The presence of a denaturation transition in the region above 5 M urea is a well documented observation for this protein (Myer, 1968a; Tsong, 1976; Aviram & Weissmann, 1978), as well as finding a transition below 4.5 M urea (Myer, 1968a). A denaturation step parallel to the low molar urea step was also observed in studies of the effect of temperature on the CD spectrum of the protein (Myer, 1968a). The small enhancement of the absolute extinction of the 695-nm band, 219 to 229 M^{-1} (Figure 2; Table I), and the small, but definite, lowering of the fluorescence efficiency of the tryptophan side chain, 3 to 1.9% (Figure 1; Table I), thus reflect the transition centered in this region of the urea scale. The transition below 4 M urea, whether observed through alteration of 695-nm extinction or fluorescence of the tryptophan side chain, behaves the same; i.e., both exhibit midtransition urea concentrations of ~2–2.5 M (Figure 3). However, above 5 M urea the situation with regard to the major denaturation profiles is quite different. The absorptivity–urea profile is definitely asymmetric, while the

Table I: Thermodynamic Parameters of Various Transitions and Properties of Limiting States from Trp Fluorescence and 695-nm Extinction Studies of Urea Denaturation of Horse Heart Ferricytochrome c[a]

transition	thermodynamic parameters[b] midtrans [urea] (M)	ΔG^* (kcal/mol)	m (cal/M^2)	properties of limiting states initial	final
	Tryptophan Fluorescence				
first	2	ND[c]	ND	3.0%[d]	1.9%
second	6.9	7.6	1116	1.9%	70.0%
	Absolute Extinction of 695-nm Band[e]				
first	2.5		ND	219 M^{-1}	229 M^{-1}
second	6.5	biphasic		229 M^{-1}	0 M^{-1}
after resoln[f]					
first component	6.2	25.1	4048	229 M^{-1}	82 M^{-1}
second component	7.0	9.1	1318	82 M^{-1}	0 M^{-1}

[a] Conditions: 0.05 M phosphate + 0.2 M KCl, pH 7.0. [b] From analysis using the equation $\Delta G_D = \Delta G^* - m[U] = RT \ln K_D$, where ΔG_D, ΔG^*, [U], and K_D are the denaturation free-energy change, the reference denaturation free-energy change, the urea concentration (Pace, 1975), and the apparent equilibrium constant. K_D is given by the expression $(P_i - P_{obsd})/(P_{obsd} - P_f)$, where P_i, P_f, and P_{obsd} are the initial, final, and observed values of the probe used. [c] ND means not determined. [d] The percent fluorescence efficiency is with respect to free tryptophan. [e] Absolute extinction determined according to the procedure of Kaminsky et al. (1973). [f] From resolution shown in Figure 5 and analysis shown in Figure 4.

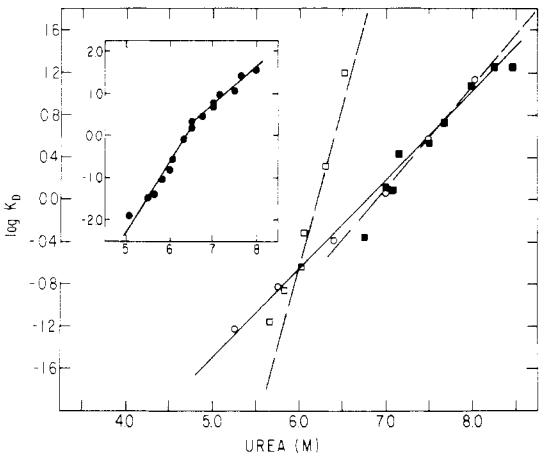

FIGURE 4: Thermodynamic representation, log K_D vs. [urea], of denaturation profiles at high urea concentrations and of the two components of the 695-nm absorption–urea transition shown in Figure 5, according to the expression $\Delta G_D = -RT \ln K_D = \Delta G^* - m[U]$ (Pace, 1975), where the apparent denaturation constant, K_D, is given by $(P_i - P_{int})/(P_{int} - P_f)$ with P_i, P_f and P_{int} as the initial, the final, and the intermediate values of the probe used. The limiting values, P_i and P_f, for each denaturation transition or its components are listed in the last two columns of Table I. Inset: log K_D vs. [urea] plots of the 695-nm absolute extinction–urea transition above 4 M urea, shown in Figure 3. (●) Actual data. Main body: (○) log K_D vs. [urea] plot of the high molar urea transition from fluorescence changes of tryptophan (Figure 3); (--□-- and --■--) log K_D vs. [urea] representation of the two resolved components of the 695-nm absorption–urea transition (Figure 5). The solid line is the least-squares fit to the calculated values. The dotted lines are the linear transformations of the resolved components, and the values denoted by □ and ■ are those calculated from the observed data points by superimposing the two components of the 695-nm absorption–urea transition (Figure 5), respectively.

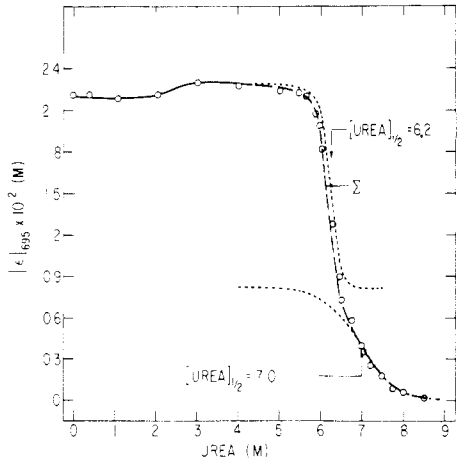

FIGURE 5: Resolution of the high molar urea 695-nm absorption–urea denaturation transition. (---) Resolved components; (–·–) summation of the two resolved components; (—) unresolved denaturation profile. (○) Observed absolute extinction of the 695-nm band at various urea concentrations (data in Figure 3). Details for the resolution are outlined in the text.

fluorescence–denaturation profile is symmetric. The mid-transition urea concentration of the former is ~6.5 M, whereas the latter is 7.0 M. The lack of coincidence between the denaturation profiles from different physical probes is a clear indication of the inapplicability of the simple two-state model for the course of perturbation (Lumry et al., 1966). The inapplicability of a simple two-state model to the major urea denaturation step is also apparent from the asymmetry of the denaturation profile. The interpretation that the urea denaturation of cytochrome c above 5 M urea may reflect a combination of events, the unfolding of the polypeptide chain and the disruption of Met-80–S from heme iron with replacement by another strong ligand field group of the protein (Myer, 1968a), further attests to the complexity of this transition of the system.

The analysis of the denaturation profiles according to the procedure of Pace (1975) shown in Figure 4 not only supports the above inference but also indicates that the absorption–urea denaturation profile encompasses a minimum of two steps. The log K_D vs. [urea] plot from the fluorescence–denaturation profile is a straight line (Figure 4, main body), a feature typical of a system conforming to a two-state, single-step process, whereas a similar plot from the absorption–denaturation profile exhibits a distinct break (Figure 4, inset), behavior expected for a process conforming to a mechanism with a minimum of two steps, i.e., a mechanism with one intermediate form.

The asymmetry of the absorption–urea transition provides the necessary ingredient for resolving the contributions from each step of the denaturation profile, since the asymmetry can only mean that the two transitions are sufficiently separated so that the contributions at the two extremes of the denaturation profile are mainly contributions from each process. Using the transition profile at high urea concentrations, i.e., above 7 M, as contributions from the second step of the denaturation process, adopting the simple procedure of generation of a transition profile, and peeling off the contributions of a given transition, we can effectively resolve the absorptivity–

denaturation profile into its constituents. This is shown in Figure 5, and the resulting log K_D vs. [urea] plots of the resolved components, along with the observed data points, are shown in Figure 4 for ease of comparison. It is important to note that the log K_D vs. [urea] plot resulting from the high molar urea component of the absorption–urea denaturation profile, referred to as component 2, is very similar, if not identical, to the plot from the fluorescence profile. The thermodynamic characteristics associated with the two transitions, such as the midtransition urea concentrations of 7.0 and 6.9 mol/L, the reference free-energy changes of 9.1 and 7.6 kcal/mol, and the values of parameter m of about 1300 and 1100 cal/M^2, respectively (Table I), are indeed very similar, if not identical. The magnitude of parameter m, when compared to the values of 50–80 cal/M^2 for solvent exposure of a single peptide unit (Nozaki & Tanford, 1970), reflects the exposure of 15–24 peptide units.

With regard to the first component of the absorption–urea transition, component 1, there is no counterpart in the denaturation profile seen through enhancement of tryptophan fluorescence of the protein (Figure 4). This step of the transition is discerned primarily through a decrease in the absolute extinction of the 695-nm band from a value of 229 to 82 M^{-1} and is centered at a urea concentration of ~6.2 mol/L (Figures 4 and 5). The thermodynamic parameters associated with this step are a reference free energy of ~25 kcal/mol and a parameter m of ~4000 cal/M^2 (Table I). On the basis of the magnitude of parameter m and by use of the same procedure as that used for the second component (see preceding paragraph), solvent exposure of a minimum of 50–80 peptide units can be estimated during this step of the denaturation transition. X-ray diffraction, however, indicates that only ~50–60 of the peptide bonds of the protein are inaccessible to the solvent (Dickerson & Timkovich, 1975). Since the last step reflects in part the solvent exposure of at least some peptide units and since the above estimates are based on an estimation for exposure of peptide bonds in guanidinium hydrochloride (Nozaki & Tanford, 1970; Tanford, 1970), the overestimate of exposure could simply be due to an inappropriate value of m, as its magnitude in urea could be significantly different from that in guanidinium hydrochloride. In any event, this step of the transition seems to be predominantly the exposure of the peptide backbone.

In summary, the above considerations show that the urea denaturation of ferricytochrome c involves a minimum of two intermediate forms, the first forming with a midtransition urea concentration of 2–2.5 M and the second with a concentration of ~6.2 M. The second intermediate form converts to the so-called denatured form, the 9 M urea form, with a midtransition urea concentration of ~7.0 M. The formation of the first intermediate form is discerned through the enhancement of the absolute extinction of the 695-nm band and the concurrent small, but definite, decrease of the fluorescence efficiency of the single tryptophan side chain; the conversion of the first intermediate form to the second involves a decrease of absolute extinction of the 695-nm band from 229 to ~82 M^{-1} with no counterpart in the fluorescence–urea denaturation profile. This step involves a reference free-energy change of ~25 kcal/mol and a parameter m of ~4000 cal/M^2. The transformation of the second intermediate form to the 9 M urea form is seen through the quenching of the residual absolute extinction of the 695-nm band as well as the enhancement of the fluorescence efficiency of the tryptophan side chain. The thermodynamic quantities associated with this step are reference free-energy changes of about 9.1 and 7.6 kcal/mol and parameter m values of about 1300 and 1100 cal/M^2, respectively.

(B) Characterization of Various Urea-Perturbed Forms. *(1) Absorption Spectra.* The absorption spectra of molecular form X_1 and of form D are typical of hemoproteins in the low-spin state of heme iron: a Soret band above 400 nm, a well-defined 530-nm band, and the absence of the characteristic high-spin band at ~620 nm (Figure 2) (Smith & Williams, 1970; Day et al., 1967). The absence of the 695-nm band in the spectrum of form D and its presence, as in the native protein, in the spectrum of form X_1 (Figure 2) leave little doubt about the state of the heme coordination configuration in the two cases: an altered configuration in form D and nativelike in form X_1 (Dickerson & Timkovich, 1975; Shechter & Saludjian, 1967; Sreenathan & Taylor, 1971; Harbury & Marks, 1973). The limiting value of the absolute extinction of the 695-nm band of 82 M^{-1} after the X_1 to X_2 transition (Figure 5; Table I) is a definite indication of the presence of the 695-nm band in form X_2 and consequently the integrity of the Met-80–S–iron linkage of form X_2 of the protein (Dickerson & Timkovich, 1975). The absolute extinction of 82 M^{-1} for form X_2 is very close to the absolute extinction generated upon addition of *N*-acetylmethionine ester to the heme undecapeptide (Shechter & Saludjian, 1967), which is devoid of the molecular conformational implications inherent to the native protein.

(2) Tryptophan Fluorescence. The fluorescence efficiency of the single tryptophan residue of cytochrome c has been shown to reflect primarily the interchromophoric distance between the indole moiety and the heme group as well as their relative orientation (Stryer, 1968; Tsong, 1976). Since cytochrome c contains a single tryptophan residue and since it is localized in the deepest part of the crevice (Dickerson & Timkovich, 1975), the alteration of the fluorescence efficiency of tryptophan provides a measure of the perturbation in the deepest part of the crevice, or the tryptophan–heme domain of the molecule. The further quenching of fluorescence efficiency during the transition centered at ~2–2.5 M urea (Figures 1 and 3) is an indication of small, but definite, perturbation in the tryptophan–heme region of the molecule. Whether closer packing of the donor and acceptor moieties, tryptophan and heme, is responsible or whether the relative orientation of the two moieties is altered (Stryer, 1968; Tsong, 1976) is difficult to discern, but one aspect is incontestable; corresponding to the N to X_1 step of the urea denaturation transition, definite, although small, alterations occur in the deepest part of the crevice which are not of the type encountered during other urea denaturation transitions, as the changes are opposite to those seen for the N to X_1 transition (Figures 1 and 3).

The lack of a counterpart to the first component of the major absorption–urea transition in the fluorescence–denaturation profile (see section A) reflects the integrity of the deepest part of the crevice during this denaturation step of the protein. In contrast, the enhancement of the tryptophan fluorescence to ~70% of free tryptophan, which seems to parallel the second component of the major urea transition, suggests the occurrence of loosening of the structures in the deepest domain of the crevice.

(3) Circular Dichroism Spectra. The circular dichroism spectrum is well acknowledged to be an exceedingly sensitive probe for the characterization of the hemoproteins and proteins in general in terms of not only the nature and magnitude of protein organizing but also of the conformation of the heme group (Myer, 1978; Myer & Pande, 1978). The Soret and

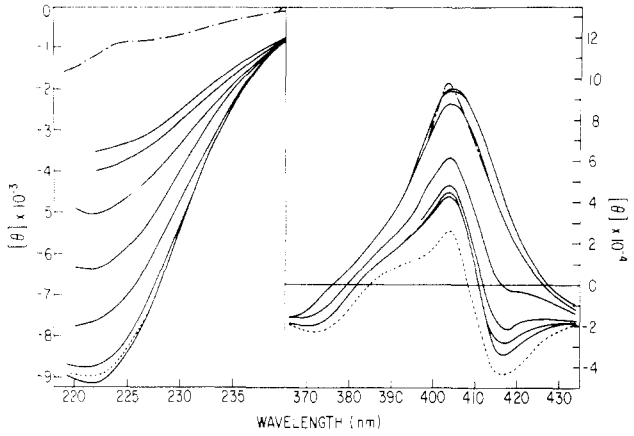

FIGURE 6: Intrinsic and Soret CD spectra of horse heart ferricytochrome *c* in the absence of urea and in the presence of 4.5 and 9 M urea and of the protein in the presence of 4.5 M urea with increasing temperatures. (---) Absence of urea; (– – –) in 9 M urea; (—) in 4.5 M urea, at different temperatures (from bottom to top: 20, 40, 50.5, 56, 61, 66, and 70 °C in the left section and 20, 30, 40, 50, 60, 70, and 80 °C in the right section, respectively). CD spectra of the protein in the absence of urea and in the presence of 9 M urea were measured at 22 °C. Buffer, in addition to the varying concentrations of urea, contained 0.05 M phosphate + 0.02 M KCl, pH 7.0.

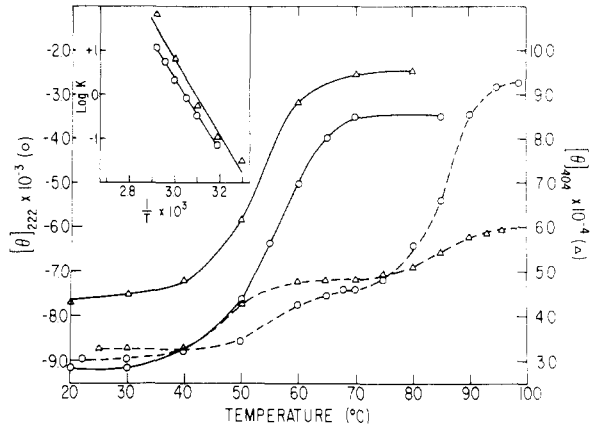

FIGURE 7: Thermal denaturation profiles of horse heart ferricytochrome *c* in the presence and absence of 4.5 M urea. (---) In the absence of urea; (—) in the presence of urea. (○) Ellipticities at 222 nm; (△) ellipticities at 404 nm. Inset: log K_{eq} vs. $1/T$ plots of the thermal denaturation profiles of the protein in the presence of 4.5 M urea. The apparent equilibrium constant, K_{eq}, was calculated by using the expression $([\theta]_i - [\theta]_{obsd})/([\theta]_f - [\theta]_{obsd})$, where $[\theta]_i$, $[\theta]_f$, and $[\theta]_{obsd}$ are the initial, final, and observed values of ellipticity for each thermal denaturation transition. Buffer conditions were the same as those in Figure 6. Data for the native protein were taken from Myer (1968a).

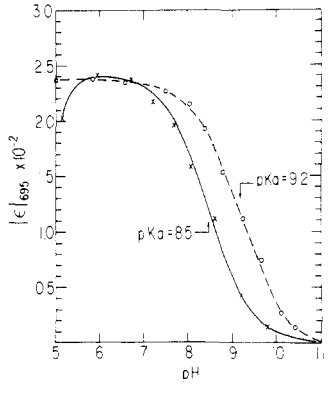

FIGURE 8: Effect of pH variation on the absolute extinction of the 695-nm band of horse heart ferricytochrome *c* in the absence and presence of 4.5 M urea. Absolute extinction of the 695-nm band was determined according to the procedure of Kaminsky et al. (1973). Conditions: 0.05 M phosphate + 0.2 M KCl, pH 7.0; temperature 22 °C. (○) In the absence of urea; (×) in the presence of urea.

intrinsic CD spectra of the native protein, the 9 M urea form, and the form stable in 4–5 urea are compared in Figure 6. Figure 6 also includes the CD spectrum resulting from increasing temperature on the 4–5 M urea form. Since the CD spectrum of this protein and those corresponding to the three forms in Figure 6 have been extensively analyzed (Myer, 1968a,b, 1978; Myer & Pande, 1978), it will suffice to state that the form in 9 M urea has been characterized as one with a disrupted coordination configuration of heme iron as well as a more or less completely disrupted protein secondary structure. The intermediate form, i.e., the form in the presence of 4.5 M urea, on the other hand, is found to have a nativelike polypeptide conformation and heme coordination configuration, but the crevice is definitely loosened.

A comparison of the thermal stability of proteins in the presence and absence of perturbants permits an evaluation of the relative conformational stabilities of these systems. In contrast to the effect of temperature on the CD spectrum of native cytochrome *c* reported earlier (Myer, 1968a), the behavior of the protein in the presence of 4.5 M urea is significantly different. The two-step denaturation profile of the native protein is rendered into a simple single-step process (Figure 7) with a midtransition temperature of only 55 °C, which is ~27 °C lower than that of the major melting temperature of the native protein. The standard changes of enthalpy and entropy for melting in the presence of 4.5 M urea, 38 kcal/mol and 115 eu, are also significantly lower than the values associated with the major thermal denaturation step of the native protein, i.e., 75 kcal/mol and 220 eu, respectively (Myer, 1968a). Since the first thermal denaturation step of the native protein has been characterized as simply the loosening of the heme crevice (Myer, 1968a), its absence in the thermal denaturation profile of the form in 4.5 M urea is consistent with the interpretation of a loosened heme crevice of the protein in the presence of 4.5 M urea. The lowering of the melting temperature further indicates that, in addition to the loosening of the heme crevice, the polypeptide structures of the molecule are significantly destabilized. The lowered values of the thermodynamic parameters associated with the thermal unfolding of the protein in the presence of 4.5 M urea are again an indication of the definite perturbation of the molecular structures so as to eliminate some of the sources contributing to the entropy and enthalpy changes during thermal unfolding of the native protein. The loosening of the heme crevice, the disruption of the hydrophobic environment, and the exposure of some peptide linkages to solvent are all possible sources of entropy and enthalpy changes.

(*4*) *Effect of pH.* The pH stability of the heme coordination configuration is another aspect permitting characterization of the molecular state of the system. In the native protein, the formation of low-spin heme coordination occurs with an apparent pK_a of ~2.5 and its disruption, possibly through deprotonation of a protein functional group, occurs with an apparent pK_a of ~9.2 (Dickerson & Timkovich, 1975). In the presence of 4.5 M urea, the alkaline pK_a is lowered to a value of 8.5 (Figure 8). Similarly, there is an indication that the acidic pK_a of the protein is also possibly shifted to higher values in the 4.5 M urea form. The lowering of the alkaline pK_a and possibly the raising of the acidic pK_a in the presence of 4.5 M urea, conditions under which the first intermediate form exists (see section A), are a clear indication that the protein in form X_1 is destabilized, not only in terms of the

Table II: Met and His Content of Horse Heart Cytochrome *c* upon Carboxymethylation[a] in the Absence and Presence of 4.5 and 9 M Urea and of Heme Undecapeptide from the 4.5 M Urea Preparation

prepn	residues/molecule Met[b]	His
native, absence of urea	0.9 (2)[c]	1.9 (3)
4.5 M urea[e]	trace[d]	0.8
9 M urea[e]	0.1	trace
undecapeptide from 4.5 M urea prepn	0.0 (0)	0.0 (1)

[a] Conditions of carboxymethylation in the presence or absence of urea: 0.1 M phosphate + 0.2 M bromoacetic acid buffer, pH 7.0; reaction time, 72 h (MacDonald, 1974; Stellwagen, 1968). [b] Methionine estimated as methionine sulfone after performic acid oxidation (Myer, 1972). [c] Values in parentheses are for the unmodified protein and its undecapeptide. [d] Trace implies a finite amount, but less than 0.1 residue/molecule. [e] Data from MacDonald (1974).

polypeptide conformation but also with respect to the coordination configuration of heme iron.

(*5*) *Carboxymethylation.* The reactivity of protein functional groups to group-specific reagents, particularly those intrinsically protected in the native protein, provides an added approach for localizing structural perturbations. Carboxymethylation with bromoacetic acid of ferricytochrome *c* without any perturbation is shown to result in modification of the Met-65 and His-33 side chains, while Met-80 and His-18, the two amino acids providing the axial ligands to heme iron (Dickerson & Timkovich, 1975), and His-26 are protected (Stellwagen, 1968; Harbury & Marks, 1973; MacDonald, 1974). If the molecule is perturbed through addition of cyanide, which replaces Met-80 from heme coordination, there is additional modification of this functional group of the protein. When the reaction is carried out in the presence of 4.5 M urea, the amino acid analysis shows that both of the methionyl side chains and at least two of the three histidine residues of the protein are modified (Table II). Since the protein contains only two methionine side chains, the apparent effect of the presence of 4.5 M urea is the generation of reactivity of the heme iron-coordinated Met-80 side chain.

Whether the modification of two of the three histidyl side chains is a reflection of the overall modification of all three residues of the protein, His-33, -26, and -18, or of two of the three, and which ones, can be determined through consideration of the data shown in the last column of Table II and the already exhibited reactivity of the molecule. Since His-33 is the normally susceptible group of the protein, whether perturbed or not (Stellwagen, 1968), one of the two modified histidines must be His-33. As to whether the modification of the second histidine side chain is an average of the modification of His-18 and -26 or specifically of His-18 or -26 is discerned from an analysis of the heme undecapeptide from the 4.5 M urea modified preparation in Table II. Since the heme undecapeptide contains only His-18 (Harbury & Loach, 1960; Harbury & Marks, 1973) and if the modification is an average of His-18 and -26, one can expect a proportional recovery of unmodified His-18. On the other hand, if the modification is specifically of His-26 or -18, the heme undecapeptide from the modified preparation should exhibit the presence or absence of histidine. As shown in Table II, the heme undecapeptide isolated from the modified preparation is completely devoid of histidine, thus establishing that the second histidine residue reactive to bromoacetic acid in the presence of urea is His-18.

The carboxymethylation of ferricytochrome *c* with bromoacetic acid thus shows that, in addition to the normal modification of the His-33 and Met-65 side chains, both Met-80 and His-18, the two centrally coordinated functional groups of the protein, are also modified. This means that the apparent effect of 4.5 M urea is the generation of a molecular form with both axial ligates accessible to anionic reagent. Since the heme group in the native protein is buried in a crevice with the exception of an edge (Dickerson & Timkovich, 1975) and since the axial ligands are intrinsically protected from anionic reagent by a hydrophobic envelope surrounding the heme group, the generation of reactivity of both axial ligands can only occur if the crevice is loosened on both sides of the heme plane so as to permit the penetration of the anionic reagent.

Discussion

The unfolding of horse heart ferricytochrome *c* at neutral pH with urea is best described by a scheme involving a minimum of two intermediates:

$$N \rightleftharpoons X_1 \rightleftharpoons X_2 \rightleftharpoons D$$

with midtransition urea concentrations of 2–2.5, 6.2, and ~7 mol/L, respectively. In terms of the various structures of the molecule, the N to X_1 transition is found to be simply the loosening of the frontal section of the heme crevice, resulting in exposure of both axial ligands to small anions but without alteration of either the coordination configuration of heme iron or the polypeptide conformation of the protein. Concurrent to the loosening of the frontal section of the heme crevice, there is a definite destabilization of the polypeptide conformation as well as of the heme coordination sphere. In addition, a small, but definite, alteration in the deepest part of the crevice occurs, which on the basis of fluorescence measurements seems to be directionally opposite to those during the latter part of the denaturation process. The transition X_1 to X_2 is a conformational perturbation step and like the first step of the transition does not involve the alteration of the coordination configuration of heme iron. The state of the tryptophan–heme domain, i.e., the deepest part of the crevice, is maintained during this transition of the denaturation scheme. The final step of the mechanism, the X_2 to D transition, involves the disruption of the Met-80–S–iron linkage, the loosening of the tryptophan–heme domain, and the almost complete derangement of the polypeptide conformation of the protein. Since form D, in spite of the displacement of Met-80–S from heme iron, is a typical low-spin form of the protein, the possibility of the reorganization of the polypeptide conformation during this step in order to provide strong ligand groups to heme iron must be considered.

The above scheme for the urea denaturation of ferricytochrome *c*, in particular the structural description of the course of events, provides a unified mechanism for all the suggestions thus far made. The single-step mechanism for urea denaturation through fluorescence investigations suggested by Aviram & Weissmann (1978) and McLendon & Smith (1978) is a consequence of the dominance of the X_2 to D transition in determining the denaturation profiles. The two-step mechanism proposed from CD studies (Myer, 1968a) is clearly a reflection of the N to X_1 transition and a composite of the last two steps in one. The above scheme is also identical with the one derived from kinetic investigations of the folding and unfolding in guanidinium hydrochloride (Ikai et al., 1973). In this regard, the inference that the last step possibly involves reorganization of the polypeptide backbone is the same as the suggestion that the last step of the guanidinium hydrochloride denaturation may reflect the refolding of the molecule in a form different from that of the native. It should be added that the above scheme is also consistent with the scheme derived

from the unfolding of the protein with methanol (Drew & Dickerson, 1978). It thus seems that the course of denaturation of cytochrome *c*, whether induced by urea, guanidinium hydrochloride, or alcohols, is almost the same. It would be of interest to determine whether the kinetics of urea denaturation would lead to a similar mechanism, which, in view of the above considerations, seems a distinct possibility.

The 695-nm Extinction and Protein Conformation. Another aspect of cytochrome *c* structure and function, the controlling factor for the extinction of the 695-nm band, is elucidated by the studies reported here. The direct link between this band and the Met-80–S–iron bond of heme systems is a well established correlation (Harbury & Marks, 1973; Dickerson & Timkovich, 1975; and references cited therein). Studies of model systems with the Met-80–S–iron linkage, however, have shown that the maximum absolute extinction is $\sim 70\ M^{-1}$, which is only above one-third that of the native protein (Shechter & Saludjian, 1967). The suggestion that the absorptivity of this band may be related in some way to the conformational aspects of the molecule was proposed as early as 1964 (Schejter & George, 1964) and further exploited by Kaminsky et al. (1973) to extract information regarding the loosening of the heme crevice of the protein, but this facet of cytochrome *c* has been more or less ignored (Dickerson & Timkovich, 1975), primarily because of the studies of Sreenathan & Taylor (1971), who concluded that the 695-nm band is sensitive to the presence or absence of the methionine ligand rather than the conformation of the molecule. It has been shown in this work that two-thirds of the absolute extinction of the 695-nm band is eliminated during the X_1 to X_2 step, which is characterized as the unfolding of the protein moiety, and only one-third of the extinction is eliminated during the step involving the disruption of the methionine–S–iron linkage, which clearly establishes the conformational link of the band, and that too to a rather large extent. It is also worth noting that the absolute extinction of this band in form X_2, possibly the limiting case of the protein without the complexity of the polypeptide conformation while still maintaining the methionine–S–iron linkage, is estimated to be $82\ M^{-1}$. This is in excellent agreement with the absolute extinction generated upon formation of the methionine complex with the heme undecapeptide (Shechter & Saludjian, 1967), which is also devoid of the conformational complexity associated with the protein. Thus, the usually accepted view that the 695-nm band measures directly the presence of an intact ligand bond between methionine–S and heme should be revised to state that, although the presence or the absence of the 695-nm band in heme systems reflects the presence or absence of the methionine–S–iron linkage, a larger proportion of its absorptivity comes from the conformational aspects of the molecule.

The Tryptophan–Heme Domain and Heme Coordination Configuration. The last step of the denaturation mechanism reflects the disruption of the methionine-80–S–iron linkage and the tryptophan–heme domain of the molecule, giving rise to the question, are the two structures of the molecule linked directly or indirectly? On the basis of the data presented here (Figure 4; Table I), the above question is difficult to resolve. Tsong (1976) has shown that the disruption of the Met-80–S–iron linkage upon addition of cyanide has little effect on the fluorescence efficiency of the tryptophan side chain, thus suggesting that the tryptophan–heme domain is not dependent on the integrity of the heme coordination sphere. We have also recently observed that the modification of Met-80 to sulfoxide, which displaces sulfur from iron coordination, does not enhance the fluorescence efficiency but further decreases it (Y. P. Myer and A. J. Pande, unpublished experiments), which supports the above inference regarding the interdependence of these two structures of the molecule. In contrast, there is a substantial line of evidence indicating that the integrity of the tryptophan–heme domain is indeed critical for maintaining the methionine coordination to heme iron. The formylation of the tryptophan residue completely prohibits the formation of the Met-80–S–iron linkage (O'Hern et al., 1975; Aviram & Schejter, 1971); the N-bromosuccinylation of tryptophan not only reduces the absorptivity of the 695-nm band but also renders it exceedingly unstable, such that the apparent pK_a for the disruption of this structure is lowered by ~ 2.7 pH units (O'Hern et al., 1975). The nitration of Tyr-67, which is also in close proximity to the tryptophan–heme domain (Dickerson & Timkovich, 1975), causes destabilization of the Met-80–S–iron linkage as in the N-bromosuccinylated derivative of the protein (Pal et al., 1975). In view of the foregoing, for the present we support the idea that the integrity of the tryptophan–heme domain determines the stability of the Met-80–S–iron linkage and not vice versa. The above proposition could be tested by investigating the unfolding of the molecule through an additional probe whose sensitivity is to one structure or the other of the molecule. This is the subject of a future publication.

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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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