Chemical Modification Studies on the Ca^{2+}-Dependent Protein Modulator of Cyclic Nucleotide Phosphodiesterase†

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ABSTRACT: Cyclic nucleotide phosphodiesterase can be activated by a Ca^{2+}-dependent protein modulator. It is generally believed that upon binding Ca^{2+} the modulator undergoes a conformational change and that the Ca^{2+}-modulator complex binds to the phosphodiesterase to give an activated enzyme-modulator complex (Wang, J. H., Teo, T. S., Ho, H. C., and Stevens, F. C. (1975), *Adv. Cyclic Nucleotide Res. 5*, 179-194). Chemical modification studies have provided an overview of the amino acid residues which may be involved in the binding of the Ca^{2+}-modulator complex to the enzyme and in the conformational changes occurring in the modulator upon binding of Ca^{2+}. The single histidine residue can be carbethoxylated, the two tyrosine residues can be nitrated and 4 out of the 6 arginine residues can be modified with 1,2-cyclohexanedione without affecting the ability of the modulator to stimulate phosphodiesterase; the histidine residue and the two tyrosine residues are more reactive in the Ca^{2+}-modulator complex than in free modulator. Carboxymethylation of one (or more) methionine residues showed that these may be of importance for the binding of the Ca^{2+}-modulator complex to phosphodiesterase. Modification of methionine residues by mild oxidation with *N*-chlorosuccinimide or Chloramine-T, carbamoylation of one or more lysine residues, and blockage of several carboxyl groups with glycine methyl ester all resulted in decrease or complete loss of activity and in each of these cases inactivation of the modulator is faster in the absence of Ca^{2+} than in the presence of Ca^{2+}.

The enzyme cyclic nucleotide phosphodiesterase occurs in multiple forms in most mammalian tissues (Appleman et al., 1973). We have been interested for some time in the mechanism of regulation of a bovine heart cyclic nucleotide phosphodiesterase which is activated by a Ca^{2+}-dependent protein modulator. This protein modulator was independently discovered by Cheung (1970, 1971) and Kakiuchi et al. (1970) in rat brain, and by Goren and Rosen (1971) in bovine heart. Subsequently, the modulators from bovine heart (Teo et al., 1973) and bovine brain (Lin et al., 1974) were extensively purified. Recently, it has been shown that the bovine heart and bovine brain modulators are probably identical (Stevens et al., 1976; Watterson et al., 1976).

The mechanism of activation of cyclic nucleotide phosphodiesterase by the Ca^{2+}-dependent protein modulator has been studied by a number of workers (Teo and Wang, 1973; Teshima and Kakiuchi, 1974; Lin et al., 1974, 1975; Wang et al., 1975). It is generally agreed that activation of the enzyme (which exhibits a basal level of activity in the absence of Ca^{2+} and modulator) is initiated by binding of Ca^{2+} to the protein modulator, whereupon the modulator assumes an active conformation; this active form of the modulator (i.e., the modulator-Ca^{2+} complex) then binds to the phosphodiesterase to form an enzyme-modulator-Ca^{2+} complex which is six- to tenfold more active than the free enzyme. It has recently been discovered by Brostrom et al. (1975) and confirmed by Cheung et al. (1975) that the protein modulator also activates brain adenylate cyclase. It has been demonstrated (Lynch et al., 1976) that the activation of the adenylate cyclase also involves the Ca^{2+}-dependent formation of an enzyme-modulator complex.

The mechanism of Ca^{2+} activation of cyclic nucleotide phosphodiesterase closely resembles the mechanism of Ca^{2+} regulation of muscle actomyosin ATPase which is also modulated by a Ca^{2+} binding protein, troponin (Wang et al., 1975). Recently Stevens et al. (1976) and Watterson et al. (1976) have provided convincing evidence in support of the postulate that the protein modulator and troponin C, the Ca^{2+} binding subunit of troponin, are homologous proteins which have evolved from a common ancestral protein. Moreover, Amphlett et al. (1976) were able to demonstrate that the protein modulator could substitute for troponin C in a reconstituted Ca^{2+}-sensitive actomyosin ATPase system. Earlier studies (Waisman et al., 1975) on the distribution of the protein modulator have shown it to be ubiquitous in the animal kingdom and it appears that the protein modulator may be one of the earliest Ca^{2+}-binding proteins to emerge with a defined function and be a precursor of troponin C.

Because of the importance of Ca^{2+} binding proteins in the regulation of several important biological processes, we believe it is of interest to gain a better understanding of their mode of action using the protein modulator as a model. We are currently concerned, firstly, with identifying the specific amino acid residues required by the protein modulator for its interaction with cyclic nucleotide phosphodiesterase, and secondly, with gaining insight into the conformational changes occurring in the modulator as it binds Ca^{2+} (Ho et al., 1975). The strategy we are employing to tackle these problems is that of chemical modification of specific amino acid side chains. This paper describes a survey study of the effects of various chemical modifications on the ability of the modulator to activate cyclic nucleotide phosphodiesterase, and observed alterations in the accessibility of amino acid side chains to modifying agents as the protein modulator binds Ca^{2+}. The protein modulator is ideally suited for such a chemical modification study because the absence of cysteine and tryptophan facilitates the interpretation of many of the modification results. The amino acid composition of the protein modulator is shown in Table I.

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TABLE I: Amino Acid Composition of Bovine Heart Protein Modulator.[a]

Lys	9	Glu	30	Met	9
His	1	Pro	2	Ile	8
Arg	6	Gly	12	Leu	10
Asp	25	Ala	12	Tyr	2
Thr	12	Cys	0	Phe	10
Ser	5	Val	8	Trp	0

[a] Data taken from Stevens et al. (1976).

Materials and Methods

Materials. The bovine heart protein modulator of cyclic nucleotide phosphodiesterase was purified and checked for homogeneity as outlined by Teo et al. (1973). Modulator deficient enzyme was prepared from bovine heart as previously described (Teo et al., 1973). *N*-Chlorosuccinimide, diethylpyrocarbonate, 1-ethyl-3-dimethylaminopropylcarbodiimide, glycine methyl ester, iodoacetic acid, iodoacetamide, mercaptoacetic acid, and 3-nitrotyrosine were all obtained from Sigma Chemical Co. (St. Louis, Mo.). Cyanogen bromide, 1,2-cyclohexanedione, and dithioerythritol were products of Pierce Chemical Co. (Rockford, Ill.). Homocitrulline was purchased from the Cyclo Chemical Corp. (Los Angeles, Calif.). Tetranitromethane was obtained from Schwarz/Mann (Orangeburg, N.Y.), *O*-methylisourea from National Biochemicals (Cleveland, Ohio), and potassium isocyanate from Eastman Organic Chemicals (Rochester, N.Y.). All other chemicals were reagent grade or better.

Assay Procedure. Cyclic nucleotide phosphodiesterase activity was measured by the procedure of Butcher and Sutherland (1962) as described in detail by Teo et al. (1973). The protein modulator was assayed for its ability to activate a standard amount (0.4–0.5 unit) of modulator deficient phosphodiesterase; one unit of modulator is defined as the amount giving 50% maximal activation of the enzyme (Teo et al., 1973).

Acid Hydrolysis. Samples containing 0.02 to 0.1 μmol of protein were hydrolyzed with 6 N HCl, containing 50 μL of 5% aqueous phenol per mL to protect tyrosine against destruction (Howard and Pierce, 1969), at 110 °C in sealed, evacuated tubes for 22 h.

Performic Acid Oxidation. Performic acid oxidation was performed according to Stegemann (1958). The protein (1 mg) was dissolved in 220 μL of performic acid (1 mL of formic acid mixed with 0.1 mL of 30% hydrogen peroxide and kept in a stoppered tube for 15 min at room temperature) and after 15 min at room temperature 1 mL of deionized water was added and the mixture lyophilized. The residue was redissolved in 0.5 mL of deionized water and again lyophilized prior to acid hydrolysis and amino acid analysis.

Amino Acid Analyses. Analyses were carried out in a Spinco 120/139 amino acid analyzer as outlined in the Spinco manual.

Cyanogen Bromide Cleavage. Cleavage of the protein modulator with cyanogen bromide was by the method of Gross and Witkop (1961) as modified by Steers et al. (1965). The protein (0.5 mg) was dissolved in 1 mL of 70% (v/v) aqueous formic acid containing 2 mg of cyanogen bromide. The reaction mixture was kept at room temperature in a stoppered tube in the dark for 40 h. Deionized water (8 mL) was then added and the mixture was lyophilized, redissolved in water, and again lyophilized.

Modification of Histidine Residues by Carbethoxylation. The sole imidazole function of the protein modulator was carbethoxylated with diethylpyrocarbonate using the method of Grousselle et al. (1973). Protein modulator (1 mg/mL) containing endogenous Ca^{2+} [1] was incubated at 23 °C in 0.1 M phosphate buffer, pH 6.0, with or without 2 mM EGTA in the sample and reference cuvettes of a Coleman-Hitachi Model 124 double beam spectrophotometer with a Model 165 recorder. The reaction was initiated by the addition, to the sample cell, of a fresh, concentrated solution of diethylpyrocarbonate (58.8 mM in absolute ethanol) to give a final reagent concentration of 0.588 mM; an equal volume of absolute ethanol was added to the reference cell and the formation of the carbethoxy-modulator was monitored by recording the increase in absorbance at 242 nm. Aliquots of the reaction mixture were withdrawn at selected time intervals and assayed for modulator activity; the extent of histidine modification was calculated from the absorbance using a value of 3200 M^{-1} cm^{-1} as the molar extinction coefficient, at 242 nm, of *N*-carbethoxyhistidine residues in proteins (Ovadi et al., 1967).

Modification of Tyrosine Residues by Nitration. Modification of tyrosine residues was achieved by nitration with tetranitromethane according to Sokolovsky et al. (1966). The protein modulator (final concentration 1 mg/mL) was incubated at 23 °C in 0.05 M Tris[2]-HCl buffer, pH 8.0, containing 1 M NaCl and either 2 mM $CaCl_2$ or 2 mM EGTA in the reference and sample cells of a recording spectrophotometer (see above). Nitration was initiated by the addition of a concentrated solution of tetranitromethane (60 mM in absolute ethanol) to the sample to give a final reagent concentration of 0.6 mM; an equal volume of absolute ethanol was added to the reference cell and the reaction was monitored by recording the absorbance at 428 nm. Aliquots were removed at appropriate time intervals for assay of protein modulator activity and the degree of nitration of the tyrosine residues was calculated from the absorbance at 428 nm using a value of 4100 M^{-1} cm^{-1} for the molar extinction coefficient of 3-nitrotyrosine (Riordan et al., 1967). After completion of the reaction, the reaction mixture was dialyzed extensively against water to remove excess reagent, lyophilized, and acid hydrolyzed prior to amino acid analysis; on these samples the amount of 3-nitrotyrosine was calculated from amino acid analysis using a sample of authentic 3-nitrotyrosine in a standard run.

Modification of Arginine Using 1,2-Cyclohexanedione. Selective modification of arginine residues was achieved by treatment with 1,2-cyclohexanedione according to Patthy and Smith (1975). To a solution of the protein modulator (5 mg/mL in 0.2 M sodium borate buffer, pH 9.0, containing either 2 mM $CaCl_2$ or 2 mM EGTA) was added an equal volume of a 15 mM solution of 1,2-cyclohexanedione. The reaction mixture was incubated at 37 °C and at appropriate time intervals samples were withdrawn for assay of the modulator activity. After 24 h incubation, the reaction was terminated by addition of an equal volume of cold 5% (v/v) acetic acid; the sample was lyophilized and then acid hydrolyzed in the presence of excess mercaptoacetic acid (Patthy and Smith, 1975) prior to amino acid analysis to determine the extent of arginine modification.

[1] Protein modulator isolated as described by Teo et al. (1973) is saturated with endogenous Ca^{2+} as evidenced by the fact that this modulator exhibits maximal activation of cyclic nucleotide phosphodiesterase without addition of Ca^{2+} ions. The carbethoxylation and guanidination reactions are carried out in phosphate buffer and addition of exogenous Ca^{2+} would result in the precipitation of insoluble calcium phosphate salts.

[2] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

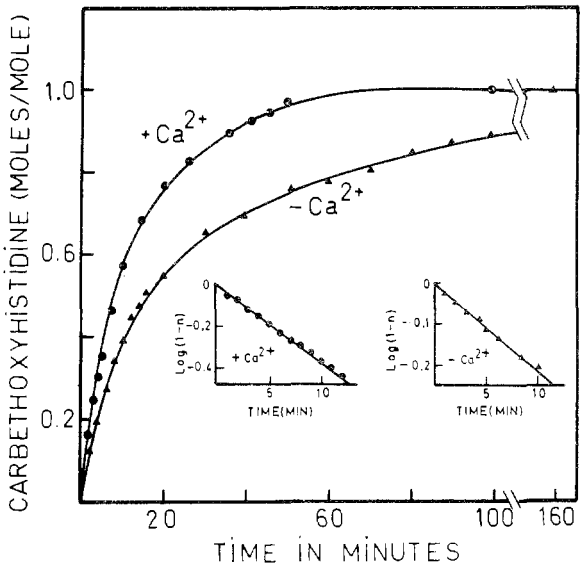

FIGURE 1: Kinetics of carbethoxylation of the histidine residue of the protein modulator by diethylpyrocarbonate. The protein modulator (1 mg/mL) containing endogenous Ca^{2+} was incubated at 23 °C, pH 6.0, with 0.588 mM diethylpyrocarbonate in the absence (●—●—●) or presence of 2 mM EGTA (▲—▲—▲). The carbethoxyimidazole content was calculated from the absorbance at 242 nm using a molar extinction coefficient of 3200 M^{-1} cm^{-1}. The inset illustrates the pseudo-first-order nature of the reaction; n is the number of carbethoxyhistidine residues in the modulator.

Carboxymethylation. Selective modification of methionine residues of the protein modulator was attempted by carboxymethylation with iodoacetic acid and iodoacetamide by the method of Gundlach et al. (1959). The modulator (5 mg/mL) was dissolved in 0.2 M sodium acetate buffer, pH 5.5, containing either 2 mM $CaCl_2$ or 2 mM EGTA; to this solution was added an equal volume of a freshly prepared 15 mM solution of iodoacetic acid (or iodoacetamide) in the same buffer to yield a 54-fold molar excess of alkylating agent over protein. The reaction mixture was incubated at 37 °C in the dark for up to 7 days. Aliquots of the reaction mixture were withdrawn at intervals for assay of modulator activity. The reaction was terminated by addition to an equal volume of 80 mM cysteine and the reaction mixture was dialyzed extensively against water. The mixture was lyophilized and the extent of modification determined by amino acid analysis after performic acid oxidation and acid hydrolysis. Carboxymethylation was also carried out in a similar fashion at pH 2.5 (0.05 M potassium hydrogen phthalate-HCl) and at pH 6.9 (0.1 M phosphate buffer).

Mild Oxidation. Methionine residues of the modulator were oxidized to methionine sulfoxides by treatment with *N*-chlorosuccinimide (and Chloramine-T) essentially as described by Shechter et al. (1975). Oxidation was initiated by addition to 0.5 mL of protein solution (0.5 mg/mL in 0.1 M Tris-HCl buffer, pH 8.5, containing 2 mM $CaCl_2$ or 2 mM EGTA) of 35 μL of 10 mM aqueous *N*-chlorosuccinimide (threefold molar excess over methionine residues) or 35 μL of 20 mM aqueous Chloramine-T (sixfold molar excess over methionine residues). The reaction mixture was kept at 23 °C for 20 (in the absence of Ca^{2+}) or 60 min (in the presence of Ca^{2+}). Aliquots of the reaction mixture were withdrawn for assay of modulator activity. At the end of the reaction excess reagent was removed by exhaustive dialysis against water and the sample was lyophilized and the resulting oxidized protein cleaved with cyanogen bromide as described above. Cyanogen bromide digests were subjected to peptide mapping and also acid hydrolyzed in the presence of 10 mM dithioerythritol prior to amino acid analysis to determine the extent of methionine modification as suggested by Shechter et al. (1975).

Carbamoylation. The amino groups of the modulator were modified by carbamoylation according to Stark (1972). To a solution (5.0 mg/mL in 0.05 M Tris-HCl, pH 7.5, containing 2 mM $CaCl_2$ or 2 mM EGTA) of the modulator was added an equal volume of 0.4 M potassium isocyanate in the same buffer. The reaction mixture was kept at 23 °C for 2 weeks and samples were withdrawn at appropriate time intervals for assay of modulator activity. The reaction was terminated by the addition of an equal volume of 1 M glycine and the reaction mixture dialyzed extensively against water prior to determination of the extent of carbamoylation as judged from the residual lysine and the newly formed homocitrulline as determined by amino acid analysis.

Guanidination. Amino groups in the protein modulator were also modified by guanidination using the method described by Habeeb (1972). Protein modulator containing endogenous Ca^{2+} was dissolved (5 mg/mL) in 0.05 M phosphate buffer, pH 11, with or without 2 mM EGTA. Guanidination was initiated by the addition of an equal volume of 1.0 M *O*-methylisourea in the same buffer. The reaction mixture was incubated at 23 °C and aliquots were withdrawn at selected intervals for assay. The reaction was terminated by rapid freezing and the mixture lyophilized prior to acid hydrolysis and amino acid analysis.

Chemical Modification of Carboxyl Groups. The total free carboxyl content of the protein modulator was determined by the method of Hoare and Koshland (1967). The protein was dissolved (1 mg/mL) in 3 mL of 1 M glycine methyl ester in 7.5 M urea. The pH was adjusted to pH 4.75 with 1 M HCl and the reaction initiated by the addition of 0.1 mL of 3 M 1-ethyl-3-dimethylaminopropylcarbodiimide in 7.5 M urea to give a final reagent concentration of 0.1 M and the reaction allowed to proceed at 25 °C. During the reaction the pH was kept constant by addition of 1 M HCl using a Radiometer pH-stat; 1-mL aliquots were withdrawn after 1, 2, and 4 h of incubation and added to 5 mL of 1 M sodium acetate buffer, pH 4.75, to quench the reaction with the diimide. The samples were then dialyzed extensively against 1 mM HCl in the cold and lyophilized prior to acid hydrolysis and amino acid analysis. The extent of carboxyl group modification was estimated from the increase in glycine content of the modulator.

For modification of carboxyl groups in the native protein, the modulator (0.25 mg) was dissolved in 1 mL of 0.25 M glycine methyl ester hydrochloride containing 2 mM $CaCl_2$ or 2 mM EGTA and the solution was adjusted to pH 4.75 with 0.2 M NaOH. The reaction was initiated by the addition of solid 1-ethyl-3-dimethylaminopropylcarbodiimide to a final concentration of 1.44 mM. The reaction mixture was incubated at 25 °C and the pH maintained at 4.75 using the pH-stat with 0.1 M HCl. Aliquots were withdrawn at intervals for assay and after 60 min of incubation the remaining reaction mixture was diluted into a large excess of 1 M acetate buffer, pH 4.75, and treated as described above.

Results and Discussion

Carbethoxylation of Histidine. Diethylpyrocarbonate has been shown to be relatively specific for modification of histidine residues at pH 6 or above (Grouselle et al., 1973; Vincent et al., 1975). The protein modulator contains a single histidine residue (Stevens et al., 1976) which, as shown in Figure 1, can

TABLE II: Carboxymethylation of Protein Modulator at pH 5.5.[a]

Reaction time (h)	Sample + Ca^{2+}		Sample − Ca^{2+}	
	Residual act. (%)	Residual Met[b]	Residual act. (%)	Residual Met[b]
0	100	9.65	100	9.58
2	90		92	
4	82		85	
6	72		73	
10	57	8.80	70	8.85
26	34	7.51	50	8.68

[a] The modulator (2.5 mg/mL) was incubated at pH 5.5, 37 °C, with 8 mM iodoacetic acid in the presence of 2 mM Ca^{2+} (sample + Ca^{2+}) or 2 mM EGTA (sample − Ca^{2+}); details are described under Materials and Methods. [b] Determined as methionine sulfone after performic acid oxidation and acid hydrolysis as described under Materials and Methods. The color value for aspartate was used in calculating the methionine sulfone content from amino acid analysis (Spackman et al., 1958).

be fully modified by carbethoxylation both in the presence and absence of Ca^{2+}. The kinetics of the reaction are pseudo-first-order; the half-time for carbethoxylation in the presence of Ca^{2+} was 8 min, whereas, in the absence of Ca^{2+}, it was 15.5 min, indicating that the imidazole ring becomes more accessible to or more reactive with diethylpyrocarbonate as the protein binds $Ca.^{2+}$. Modification of the single histidine residue is without effect on the ability of the modulator to stimulate cyclic nucleotide phosphodiesterase; it appears therefore that this residue is not involved in interaction with the enzyme.

Nitration of Tyrosine Residues. The protein modulator contains 2 tyrosine residues (Stevens et al., 1976). Figure 2 illustrates the kinetics of the nitration of these residues by tetranitromethane. In the presence of Ca^{2+} stoichiometric modification of both tyrosine residues was obtained, whereas in the absence of Ca^{2+} 1.5 residues of 3-nitrotyrosine were formed per mole of modulator as revealed by both spectrophotometric methods and amino acid analysis. Nitration in the presence of a sixfold higher concentration of tetranitromethane for 24 h in the absence of Ca^{2+} led to the formation of only 1.6 mol of 3-nitrotyrosine per mol of protein as estimated by amino acid analysis. Clearly, the rate of nitration was greater in the presence of Ca^{2+}, indicating that one or possibly both tyrosine residues are more accessible to the modifying agent in the active form of the modulator. In this connection it is interesting to note that, in the model proposed by Kretsinger and Barry (1975) for troponin C, a protein which we believe to be homologous to the modulator (Stevens et al., 1976; Watterson et al., 1976), the peptide oxygen of one of the tyrosine residues (Tyr-109) coordinates one of the bound Ca^{2+} ions while the hydroxyphenyl ring is within 5 Å of another bound Ca^{2+} (Kretsinger and Barry, 1975). The proposed proximity of the aromatic side chain of a tyrosyl group to a bound Ca^{2+} is supported by a study of the circularly polarized emission of terbium substituted bovine cardiac troponin C (Brittain et al., 1976).

Both in the presence and absence of Ca^{2+} the progress curve for the nitration of the modulator (Figure 2) appears to be monophasic, indicating that the reactivity of the two tyrosine residues differs by less than an order of magnitude, which will make selective modification of one tyrosine residue rather difficult.

Whether in the presence or absence of Ca^{2+}, modification of the tyrosine residues of the protein modulator by nitration with tetranitromethane was found to be of no consequence to its ability to stimulate cyclic nucleotide phosphodiesterase in the presence of Ca^{2+}.

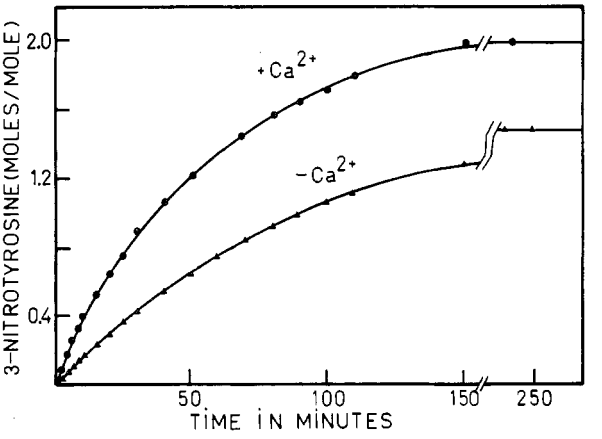

FIGURE 2: Kinetics of nitration of the protein modulator. The modulator (1 mg/mL) was incubated at 23 °C, pH 8.0, with tetranitromethane (0.6 mM) in the presence of 2 mM Ca^{2+} (●—●—●) or 2 mM EGTA (▲—▲—▲). The nitrotyrosine content was estimated from the absorbance at 428 nm using a molar extinction coefficient of 4100 M^{-1} cm^{-1}.

Modification of Arginine Residues with 1,2-Cyclohexanedione. Both in the presence and absence of Ca^{2+}, it was possible to modify 4 out of a total of 6 arginine residues (as determined by amino acid analysis) in the modulator by treatment with 1,2-cyclohexanedione in borate buffer, pH 9.0. This degree of arginine modification was without effect on the activity of the activator.

Carboxymethylation. Iodoacetic acid and iodoacetamide have been mainly used as alkylating agents to modify histidine, cysteine, and methionine residues in proteins (Means and Feeney, 1971). Since the protein modulator does not contain any cysteine residues, we originally intended to attempt specific alkylation of the sole histidine residue. However, from initial experiments under a variety of conditions of pH (pH 2.5, 5.5, and 6.9) and reagent concentration, it became obvious that the histidine residue was resistant to modification by either iodoacetic acid or iodoacetamide. For example, incubation of the modulator with iodoacetic acid at pH 5.5 for 168 h either in the presence or absence of Ca^{2+} resulted in a total loss of activity, whereas controls treated in an identical fashion, except that the reagent was omitted, retained full activity during the same period. As judged by amino acid analysis after acid hydrolysis, the only residues affected by alkylation were methionine residues; hence, no reaction has occurred with residues of histidine or lysine which are known to yield acid-stable carboxymethyl derivatives. Table II shows the time course of

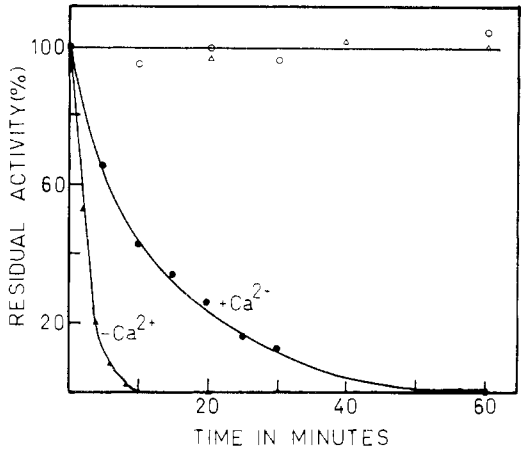

FIGURE 3: Time course of inactivation of the protein modulator by mild oxidation. The modulator (0.5 mg/mL) was incubated at 23 °C, pH 8.5, with 3 equiv of *N*-chlorosuccinimide per methionine residue in the presence of 2 mM Ca^{2+} (●—●—●) or 2 mM EGTA (▲—▲—▲). Open symbols represent controls treated the same way except that *N*-chlorosuccinimide was omitted.

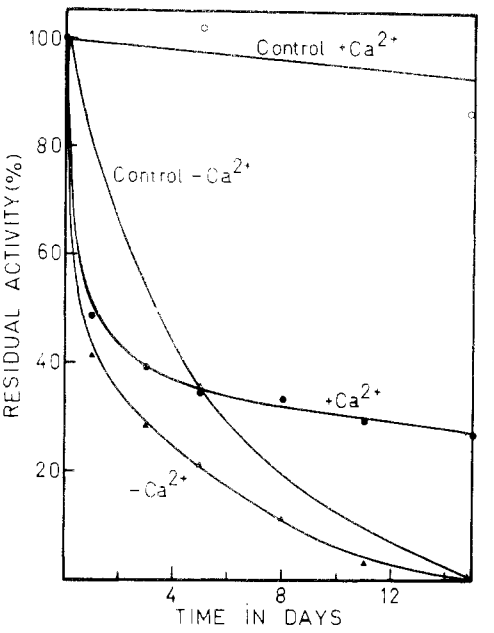

FIGURE 4: Time course of inactivation of protein modulator by carbamoylation. The modulator (2.5 mg/mL) was treated with potassium isocyanate (0.2 M) at 23 °C, pH 7.5, in the presence of 2 mM Ca^{2+} (●—●—●) or 2 mM EGTA (▲—▲—▲). Open symbols represent controls treated the same way, except that potassium isocyanate was omitted.

inactivation for the first 26 h both in the presence and absence of Ca^{2+}. In both cases activity is lost gradually and after 26 h is reduced to 50% in the absence of Ca^{2+} and 35% in the presence of Ca^{2+}. It thus appears activity is lost faster in the presence of Ca^{2+}. Carboxymethylation of methionine residues in proteins yields *S*-carboxymethylmethionylsulfonium salts and upon acid hydrolysis of the protein these modified residues are degraded to give a mixture of methionine, homoserine lactone, and *S*-carboxymethylhomocysteine (Gurd, 1972). Consequently the exact amount of carboxymethylmethionine cannot be determined by amino acid analysis after acid hydrolysis. Treatment of the carboxymethylated protein with performic acid prior to acid hydrolysis can be used to distinguish between methionine residues unaffected by carboxymethylation and those regenerated during acid hydrolysis (Vithayathil and Richards, 1960). Performic acid treatment quantitatively converts methionine residues to methionine sulfone but leaves the *S*-carboxymethylmethionylsulfonium residues unaffected. Subsequent acid hydrolysis is without effect on the methionine sulfone residues and the *S*-carboxymethylmethionylsulfonium residues breakdown products do not include methionine sulfone. Thus the unmodified methionine content of the carboxymethylated protein is obtained from the methionine sulfone content after performic acid oxidation and acid hydrolysis. The analytical results are given in Table II; in the presence of Ca^{2+}, approximately 2 methionine residues have been modified in 26 h, whereas in the absence of Ca^{2+} approximately 1 methionine residue was modified. Control samples treated in identical fashion except for the omission of iodoacetic acid retained full activity and showed no change in methionine content over the same time period.

Modification of Methionine Residues by Mild Oxidation. We also investigated the role of methionine residues for the function of the modulator using the procedure recently reported by Shechter et al. (1975). These authors demonstrated that exposed methionine residues in proteins were oxidized to methionine sulfoxides by mild oxidizing reagents such as Chloramine-T and *N*-chlorosuccinimide at neutral or slightly alkaline pH. With Chloramine-T oxidation of cysteine and with *N*-chlorosuccinimide oxidation of cysteine and tryptophan were found to occur as side reactions; our protein modulator contains neither cysteine nor tryptophan and thus the reaction should be specific for methionine. The extent of methionine oxidation can be determined by quantitative modification of the unoxidized methionine residues with cyanogen bromide (methionine sulfoxide residues remain intact) followed by acid hydrolysis and amino acid analysis (Shechter et al., 1975). Figure 3 shows the time course of inactivation of protein modulator, in the presence and absence of Ca^{2+}, by oxidation with *N*-chlorosuccinimide. In the presence of Ca^{2+}, the activity is completely lost after 60 min and analysis of the modified sample indicates that 3 methionine residues have been modified at that stage. In the absence of Ca^{2+}, the inactivation is much faster and all activity is lost after 10 min at which time analysis indicates that approximately 4 methionine residues have been oxidized. Peptide maps of cyanogen bromide digests of *N*-chlorosuccinimide oxidized modulator were consistent with these observations. Using Chloramine-T as the oxidizing agent in the presence of Ca^{2+}, the modulator activity is completely lost in less than 2 min. These results are consistent with those obtained by carboxymethylation in that they confirm that modification of one or more methionine residues results in inactivation of the modulator. In contrast to the results obtained by carboxymethylation, loss of modulator activity by oxidation of methionine residues is faster in the absence of Ca^{2+}. Evidence from peptide maps indicates that there are some differences in the particular methionine residues which are oxidized and thus exposed in the presence and absence of Ca^{2+}.

Chemical Modification of Amino Groups. Since the modulator is believed to contain a blocked amino terminal (Stevens et al., 1976; Watterson et al., 1976), reaction with potassium isocyanate under the conditions used should be specific for the carbamoylation of lysine residues to yield homocitrulline (Stark, 1972). Figure 4 shows the time course of inactivation by carbamoylation. In the absence of Ca^{2+} both the sample containing the reagent and the control sample were completely inactive after 2 weeks of incubation, indicating that the modulator is unstable under those conditions even in the absence

TABLE III: Carbamoylation of Protein Modulator.[a]

Reaction time (days)	Sample + Ca^{2+} (mol/mol)			Sample − Ca^{2+} (mol/mol)		
	Lys	Homocitrulline	Total	Lys	Homocitrulline	Total
2	6.53	2.32	8.85	5.28	3.60	8.88
7	5.60	3.32	8.92	3.15	5.89	9.04
15	2.41	5.80	8.21	2.26	6.30	8.56

[a] Reaction conditions are as described in the text and the legend to Figure 5. The lysine and homocitrulline contents were determined after acid hydrolysis and the values have not been corrected for possible hydrolysis of homocitrulline back to lysine (Stark, 1972).

TABLE IV: Guanidination of Protein Modulator.

Reaction time (days)	Residual act. (%)				Lys (mol/mol)	
	$+Ca^{2+}$ [a]		$-Ca^{2+}$ [b]			
	Control[c]	Sample	Control[c]	Sample	$+Ca^{2+}$ [a]	$-Ca^{2+}$ [b]
0	100	100	100	100	9	9
7	81	79	32	42	7	7
20	68	75	0	0-25	6	7

[a] Reaction with modulator containing endogenous Ca^{2+} (see Materials and Methods). [b] Reaction with 2 mM EGTA added to reaction mixture (see Materials and Methods). [c] Controls are the same as the samples except that *O*-methylisourea was omitted.

of reagent. However, it can be seen from Figure 4 that the initial loss of activity in the absence of Ca^{2+} is much more rapid in the sample containing the potassium isocyanate than in the control sample and this together with the analytical data in Table III would indicate that modification of one or more lysine residues results in a decrease of modulator activity. The observation is even more striking if the reaction is carried out in the presence of Ca^{2+} (Figure 4, Table III) since in this case the control sample retains full activity over the entire time period, whereas the sample incubated with isocyanate shows a substantial initial decrease in modulator activity which levels out at 30-35% of the original value after 4 days of incubation. Thus, reaction of modulator with isocyanate in the presence of Ca^{2+} results in modification of 1 or 2 lysine residues with a concomitant 60-70% decrease in activator activity, but further modification of the remaining lysine residues does not result in a further decrease in activity. If one takes into account that the figures in Table III should be corrected for some hydrolysis of homocitrulline back to lysine during acid hydrolysis before amino acid analysis (17-30%, according to Stark, 1972), it can be concluded that, after 2 weeks of incubation, essentially all the lysine residues have been modified; such a sample still retains about 30% of its original activity. In the absence of Ca^{2+}, the rate of carbamoylation is faster than in the presence of Ca^{2+} and this is probably due to the fact that, as has been suggested before (Ho et al., 1975), the structure of the modulator in the absence of Ca^{2+} is less rigid and more prone to denaturation as evidenced by the observation that under these conditions activity was also lost from the control sample.

Selective modification of the ϵ-amino groups was also attempted by guanidination with *O*-methylisourea. This lysine modification has one advantage over carbamoylation in that it will retain the positive charge on the modified residue whereas this charge is abolished by carbamoylation. From the results given by Table IV it can be seen that both in the presence of Ca^{2+} and in the absence of Ca^{2+} the modulator activity of guanidinated sample is not significantly different from that of control samples even though 2 to 3 lysines have been modified. It is clear from the controls that the modulator is not stable under the conditions of the incubation, especially in the absence of Ca^{2+}, and this makes interpretation of the data difficult. It may be that modification of some lysine residues with retention of the positive charge (as is the case in guanidination) is of no consequence for the activity of the protein whereas modification of the same residues with loss of the positive charge (as is the case in carbamoylation) results in partial loss of activity. The data in Table IV also again show that the protein is more stable in the presence of Ca^{2+} since, after 20 days of reaction, it retains 70% of its original activity, whereas the corresponding sample reacted in the absence of Ca^{2+} and with approximately the same degree of modification is totally inactive.

Chemical Modification of Carboxyl Groups. The acid hydrolysate of the protein modulator contains 25 Asp and 30 Glu for a total of 55 acidic residues (Stevens et al., 1976). The total free carboxyl group content of the modulator was estimated from the increase in glycine content after treatment of the urea denatured protein with glycine methyl ester in the presence of water-soluble carbodiimide (Hoare and Koshland, 1967). Reaction for 1, 2, and 4 h gave values of 38, 40, and 41 carboxyl groups, respectively. This value of approximately 40 free carboxyls and 15 amides agrees, within experimental error, with the values (38 and 16) obtained by Liu and Cheung (1976) for the modulator prepared from bovine brain; these authors obtained their values by estimating the amide content of the protein by determining on the amino acid analyzer the NH_3 liberated from the protein by hydrolysis in 2 N HCl at 100 °C.

Figure 5 shows the effect of carboxyl group modification on native protein modulator in the presence and absence of Ca^{2+}. Our results are qualitatively in agreement with those of Liu and Cheung (1976). In the presence or absence of Ca^{2+} complete loss of modulator activity occurs concomitant with the modification of 22 carboxyl groups per mol. The loss of activity is faster in the absence of Ca^{2+} than in the presence of Ca^{2+}. In the presence of Ca^{2+} modification of 2 carboxyl groups occurred within the first minute, with only an 8% loss in activity; Liu and Cheung (1976) reported the rapid modification

TABLE V: Summary of Chemical Modification Studies on Bovine Heart Protein Modulator.

Modification	Experimental evidence	Modified function	No. of residues in native protein	No. of residues modified $+Ca^{2+}$	No. of residues modified $-Ca^{2+}$	Residual act. (%) $+Ca^{2+}$	Residual act. (%) $-Ca^{2+}$	Effect of Ca^{2+} on rate[a]
Carbethoxylation	Change in absorption, 242 nm; no change in absorption, 278 nm	His	1	1	1	100	100	+
Nitration	Change in absorption, 428 nm; amino acid analysis	Tyr	2	2	1.5	100	100	+
1,2-Cyclohexanedione treatment	Amino acid analysis	Arg	6	4	4	100	100	0
Carboxymethylation	Amino acid analysis after performic acid oxidation	Met	9	2	1	34	50	+
N-Chlorosuccinimide	Peptide mapping and amino acid analysis after CNBr treatment	Met	9	3-4	3-4	0	0	−
Carbamoylation	Amino acid analysis	Lys	9	2-3	4	45	35	−
				3-4	6	35	20	
Guanidination	Amino acid analysis	Lys	9	2	2	80	40	−
				3	2-3	75	0	−
Amide formation with Gly methyl ester	Amino acid analysis (increase in Gly)	Carboxyl	40	2	/	90	/	−
				22	22	0	0	

[a] In the case of carbethoxylation, nitration and arginine modification rate refers to the rate of modification of those residues. In the other cases rate refers to the rate at which modulator activity is lost. A positive sign (+) indicates that the reaction or loss of activity is faster in the presence of Ca^{2+}, and a negative sign (−) indicates the loss of activity is slower in the presence of Ca^{2+}.

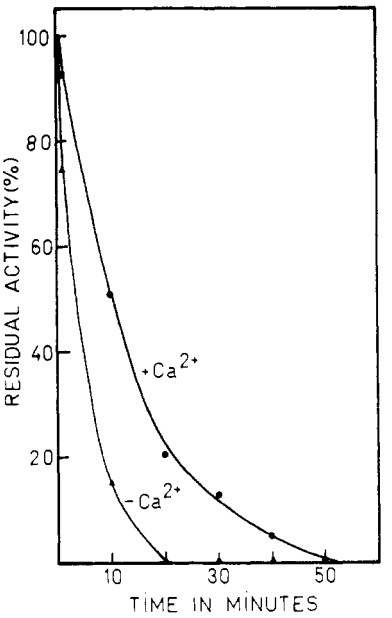

FIGURE 5: Time course of inactivation of protein modulator by blocking of the carboxyl groups with glycine methyl ester. The modulator (0.25 mg/mL) was incubated at 25 °C, pH 4.75, with 0.25 M glycine methyl ester, 1.44 mM 1-ethyl-3-dimethylaminopropylcarbodimide in the presence of 2 mM $CaCl_2$ (●—●—●) or 2 mM EGTA (▲—▲—▲).

of 5 carboxyl groups with no loss of activity, followed by the modification of a less reactive group of another 5 carboxyl groups and simultaneous loss of activity. It has been pointed out (Caraway and Koshland, 1972; Liu and Cheung, 1976) that the coupling agent carbodiimide is known to cause intra- or intermolecular cross-linkage between activated carboxyl and amino groups and also may react with tyrosine to form an *O*-arylisourea derivative. Liu and Cheung (1976) have observed the formation of multiple bands in analytical gel electrophoresis after carboxyl group modification indicating the possible formation of cross-links. In the present study (Figure 5) a control containing carbodiimide and Ca^{2+} but from which glycine methyl ester was omitted was found to lose ~75% of its activity after 30 min incubation and ~85% after 60 min incubation and a similar control in the absence of Ca^{2+} resulted in a loss of ~80% of its activity after 10 min and ~90% after 60 min; these inactivations could be due to cross-linking of the protein molecules. It is difficult to assess the importance of this side reaction in the actual carboxyl group modification experiment; presumably, because of the relatively high concentration of glycine methyl ester utilized in the modification reactions, the observed activity losses are predominantly due to carboxyl group modification rather than cross-linking. In the reaction carried out in the presence of EGTA, the added possibility also exists that the carboxyl groups of EGTA become activated by the carbodiimide and could then react with the free amino groups of the protein modulator or of the glycine methyl ester; because of the greater than 10 000-fold molar excess of glycine methyl ester over the protein concentration, the latter is of course much more likely and would be without significant effect on our results. Control reactions in which only the water soluble carbodiimide was omitted from the reaction mixture retained full activity whether or not Ca^{2+} was present; such samples did not show an increase in glycine after acid hydrolysis, indicating the complete removal of the glycine methyl ester in the dialysis step.

General Conclusions

Table V summarizes our results in terms of the type and number of amino acid residues modified in the presence and absence of Ca^{2+} and the effect these modifications have on the ability of the modulator to activate cyclic nucleotide phosphodiesterase. It is clear from the results of the present study that the reactivities of many of the amino acid side chains in the protein modulator are affected by Ca^{2+}. This observation

lends further support to the suggestion (Teo and Wang, 1973; Teshima and Kakiuchi, 1974; Lin et al., 1974; Wang et al., 1975) that the modulator assumes an "active" conformation upon binding of Ca^{2+}. It has been previously shown that, in the presence of Ca^{2+}, the modulator becomes more resistant to proteolysis (Ho et al., 1975; Liu and Cheung, 1976) and heat (Liu and Cheung, 1976) and also more helical as determined by optical rotatory dispersion (Liu and Cheung, 1976). Thus there is no doubt that a conformational change takes place upon Ca^{2+} binding; the determination of the exact nature of this conformational change will have to await more detailed chemical studies and the determination of the three-dimensional structure of this protein in the presence and absence of Ca^{2+}.

The single histidine residue, both tyrosine residues and 4 out of 6 arginine residues in the modulator can be modified, either in the presence or absence of Ca^{2+}, without noticeable effect on its ability to stimulate cyclic nucleotide phosphodiesterase. On the other hand, modification of one or more methionine residues by oxidation or carboxymethylation, guanidination or carbamoylation of several $-NH_2$ groups, or blocking of several carboxyl groups with glycine methyl ester all result in loss of activity. At this stage it is not possible to conclude whether the loss of activity is due to the fact that these residues are functionally essential or because their modification results in conformational changes in the molecule. We are now in the process of further characterizing these modified proteins both with respect to their structure and with respect to which particular residues have been modified by the various treatments.

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