Ed., Amsterdam, Elsevier, p 237.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Mandaron, P. (1976), *Wilhelm Roux' Arch. Entwicklungsmech. Org. 179,* 185.
Marks, E., and Sowa, B. (1976), in The Insect Integument, H. R. Hepburn, Ed., Amsterdam, Elsevier, p 339.
Mills, R., Greenslade, F., Fox, F., and Nielsen, D. (1967), *Comp. Biochem. Physiol. 22,* 327.
Mitchell, H. K., Weber-Tracy, U., and Schaar, G. (1971), *J. Exp. Zool. 176,* 429.
Rudall, K. M. (1976), in The Insect Integument, H. R. Hepburn, Ed., Amsterdam, Elsevier, p 22.
Rudall, K. M., and Kenchington, W. (1973), *Biol. Rev. 48,* 597.
Yamamoto, K. R., and Alberts, B. M. (1976), *Annu. Rev. Biochem. 45,* 721.
Yund, M. A., and Fristrom, J. W. (1975a), *Dev. Biol. 43,* 287.
Yund, M. A., and Fristrom, J. W. (1975b), in Developmental Biology: Pattern Formation, Gene Regulation, McMahon, D., and Fox, C. F., Ed., Menlo Park, W. A. Benjamin, p 404.
Zweidler, A., and Cohen, L. (1971), *J. Cell Biol. 51,* 240.

Chemical Modification Studies on the Ca^{2+}-Dependent Protein Modulator: The Role of Methionine Residues in the Activation of Cyclic Nucleotide Phosphodiesterase†

Michael Walsh and Frits C. Stevens*

Appendix: Circular Dichroism Studies on Ca^{2+}-Dependent Protein Modulator Oxidized with *N*-Chlorosuccinimide

Michael Walsh, Frits C. Stevens,* Kim Oikawa, and Cyril M. Kay

ABSTRACT: Methionine residues have been implicated in the activation of cyclic nucleotide phosphodiesterase by the Ca^{2+}-dependent protein modulator [Walsh, M., & Stevens, F. C. (1977) *Biochemistry 16,* 2742–2749]. Treatment of the modulator with *N*-chlorosuccinimide in the presence of Ca^{2+} resulted in selective oxidation of methionine residues at positions 71, 72, 76, and, possibly, 109 in the modulator sequence. These residues lie on the surface of the molecule exposed to solvent. This modification has several effects on the modulator protein: (1) the Ca^{2+}-binding properties of the oxidized modulator are changed with apparent loss of high-affinity binding sites, (2) the oxidized protein no longer interacts with phosphodiesterase, and (3) troponin C like activities, viz., Ca^{2+}-dependent change in mobility on urea–polyacrylamide gel electrophoresis and formation of a urea-stable complex with troponin I, are lost upon oxidation of the modulator. The phosphodiesterase binding domain of the modulator protein appears to be located between the second and third Ca^{2+}-binding loops, a region of the molecule known from previous partial proteolysis studies [Walsh, M., Stevens, F. C., Kuznicki, J., & Drabikowski, W. (1977), *J. Biol. Chem. 252,* 7440–7443] to be exposed in the presence of Ca^{2+}.

Considerable evidence has been provided in recent years that a ubiquitous Ca^{2+}-binding protein, originally discovered as an activator of a cyclic nucleotide phosphodiesterase by Cheung (1970, 1971) and Kakiuchi et al. (1970) in rat brain and by Goren & Rosen (1971) in bovine heart, may serve as the central regulator which couples Ca^{2+} to the regulation of cyclic nucleotide metabolism and nonmuscle contractile and secretory processes. This so-called modulator protein exhibits many Ca^{2+}-dependent regulatory activities in addition to its stimulation of phosphodiesterase: (1) activation of a specific adenylate cyclase (Brostrom et al., 1975; Cheung et al., 1975); (2) troponin C like activity with a reconstituted Ca^{2+}-sensitive actomyosin ATPase system (Amphlett et al., 1976; Dedman et al., 1977); (3) activation of smooth-muscle myosin light-chain kinase (Dabrowska et al., 1978), skeletal-muscle protein kinase (Yagi, et al., 1978), and brain protein kinase (Schulman & Greengard, 1978); (4) activation of erythrocyte membrane (Ca^{2+},Mg^{2+})ATPase (Jarrett & Penniston, 1977; Gopinath & Vincenzi, 1977); and (5) stimulation of erythrocyte-membrane Ca^{2+} transport (Macintyre & Green, 1977). Furthermore, an unidentified protein, which appears to be an additional modulator-regulated enzyme, has been isolated from brain and shown to interact with the modulator in a Ca^{2+}-dependent manner (Wang & Desai, 1976, 1977). These modulator-regulated enzymes may contain a common modulator-binding domain.

The modulator protein exhibits none of the above-mentioned activities in the absence of Ca^{2+} ions. The protein binds four Ca^{2+} ions per mole, and the binding of Ca^{2+} is accompanied

† From the Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada, R3E OW3 (M.W. and F.C.S.), and the Medical Research Council Group of Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7 (K.O. and C.M.K.). *Received April 10, 1978.* This work was supported by grants from the Medical Research Council of Canada. M.W. was the recipient of a Medical Research Council of Canada studentship. The results reported here are taken from a thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for a Ph.D. degree

0006-2960/78/0417-3924$01.00/0

by a substantial conformational change in the modulator, whereupon the protein assumes an active form. The Ca^{2+}-binding properties of the modulator indicate that it functions as a physiological regulator alternating between active and inactive forms with fluctuations in the intracellular Ca^{2+} concentration. This Ca^{2+}-mediated conformational change, therefore, may serve a crucial role in the regulation of numerous Ca^{2+}-dependent physiological processes.

Wang et al. (1975) originally observed that the mechanism of Ca^{2+} activation of phosphodiesterase closely resembles the mechanism of Ca^{2+} regulation of muscle actomyosin ATPase, which is also modulated by a Ca^{2+}-binding protein, troponin. Stevens et al. (1976) and Watterson et al. (1976) demonstrated that the protein modulator and troponin C, the Ca^{2+}-binding subunit of troponin, exhibit striking similarities in physical and chemical properties. Comparison of the tryptic fragments obtained by controlled enzymatic digestion of the protein modulator in the presence and absence of Ca^{2+} (Walsh et al., 1977) with similar fragments of troponin C (Drabikowski et al., 1977) lent further support to the postulate that these two Ca^{2+}-binding proteins are homologous proteins. The amino acid sequence determination of the bovine brain protein modulator has recently been completed by Vanaman et al. (1977). Comparison of this sequence with that of rabbit skeletal muscle TN-C[1] (Collins et al., 1977) indicated 80% sequence homology between the two proteins, taking into account conservative replacements. Furthermore, as mentioned above, Amphlett et al. (1976) and Dedman et al. (1977) demonstrated that the protein modulator could substitute for Tn-C in a reconstituted Ca^{2+}-sensitive actomyosin ATPase system. Waisman et al. (1975) showed the protein modulator to be ubiquitous in the animal kingdom and suggested that it may have been one of the earliest Ca^{2+}-binding proteins to emerge with a defined function and be a precursor of Tn-C.

We have been interested for some time in the structure–function relations of the protein modulator, particularly with respect to its activation of phosphodiesterase. An earlier survey of the effects of various chemical modifications of functional groups in the protein modulator implicated methionine and lysine residues as being essential for the expression of modulator activity (Walsh & Stevens, 1977). We report here the results of detailed investigations of the role of methionine residues of the modulator protein in its activation of phosphodiesterase. The Appendix describes circular dichroism studies on the *N*-chlorosuccinimide-oxidized modulator protein.

Materials and Methods

Materials. The bovine brain protein modulator of cyclic nucleotide phosphodiesterase was isolated and purified by a modification of the procedure described by Teo et al. (1973) for the purification of the bovine heart modulator, as described in detail elsewhere (Walsh & Stevens, 1978). The preparation was judged to be homogeneous by the criteria of 15% polyacrylamide gel electrophoresis, $NaDodSO_4$–polyacrylamide gel electrophoresis in the presence of urea, and isoelectric focusing. Modulator-deficient phosphodiesterase was prepared from bovine heart as previously described (Teo et al., 1973). Bovine cardiac troponin I was a generous gift from Dr. C. M. Kay of the University of Alberta.

Cyclic 3′,5′-adenosine monophosphate (cAMP), *N*-chlorosuccinimide, and $NaDodSO_4$ were obtained from Sigma Chemical Co. (St. Louis, Mo.). Formic acid was purchased from Aldrich Chemical Co. (Milwaukee, Wis.), and EGTA was 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 & 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; 1 unit of modulator activity is defined as the amount giving 50% maximal activation of the enzyme (Teo et al., 1973).

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

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 achieved by the method of Gross & Witkop (1961), as modified by Steers et al. (1965). The protein was dissolved in 70% (v/v) aqueous formic acid at a concenttration of 1 mg/mL. An equal volume of 70% (v/v) formic acid containing cyanogen bromide (2 mg/mL) was added. The reaction mixture was kept at room temperature in a stoppered tube in the dark for 40–48 h. Deionized water (at least 4 volumes) was then added, and the mixture was lyophilized, redissolved in water, and again lyophilized.

Electrophoresis. Polyacrylamide gel electrophoresis was performed on slab gels employing the discontinuous buffer system of Davis (1964) either with 6 M urea (11.4% polyacrylamide) or without urea (15% polyacrylamide). Gels were stained overnight with 0.25% Coomassie brilliant blue G-250 in 7.5% acetic acid and destained electrophoretically with 7.5% acetic acid.

Mild Oxidation of the Modulator Protein. Selective oxidation of methionine residues of the protein modulator was achieved by treatment with *N*-chlorosuccinimide (NCS) essentially as described by Shechter et al. (1975). The modulator (0.5 mg/mL) was dissolved in 0.1 M Tris-HCl, pH 8.5, containing 4 mM $CaCl_2$ or 4 mM EGTA. Oxidation was initiated by the addition of a freshly prepared, concentrated solution of NCS (10 mM) to yield a threefold molar excess of NCS over methionine residues. The reaction mixture was incubated at 23 °C. Aliquots of the reaction mixture were withdrawn at appropriate time intervals, and the reaction was terminated by the addition of an equal volume of 40 mM methionine. Part of the mixture was assayed for modulator activity, and the remainder was dialyzed extensively against water and lyophilized prior to: (1) amino acid analysis after cyanogen bromide digestion and acid hydrolysis in the presence of 0.2 M dithioerythritol (unoxidized methionines will show up as homoserine and oxidized methionines are reconverted to methionine) and (2) urea–polyacrylamide gel electrophoresis.

In order to characterize the modified derivatives in detail, samples of modulator protein were oxidized on a preparative scale as described above starting with about 20 mg of protein. The reaction in the presence of Ca^{2+} was allowed to proceed for 90 min and that in the absence of Ca^{2+} was stopped after 15 min. In each case the reaction was terminated by the addition of an equal volume of 40 mM methionine followed by extensive dialysis against water and lyophilization.

[1] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; NCS, *N*-chlorosuccinimide; PDE, cyclic nucleotide phosphodiesterase (EC 3.1.4.17); $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; TN-C, troponin C; TN-I, troponin I.

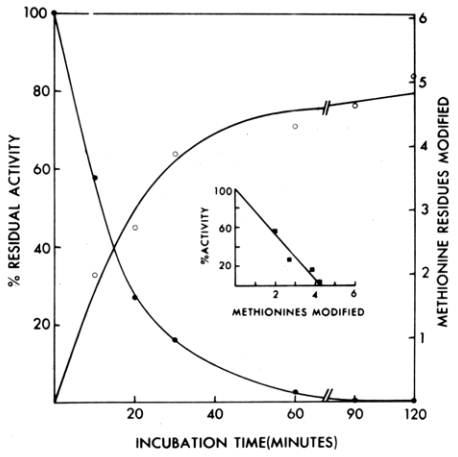

FIGURE 1: Time course of mild oxidation of the protein modulator with NCS in the presence of Ca^{2+}. The modulator (0.5 mg/mL) was treated with NCS (3 equiv/methionine residue) at 23 °C, pH 8.5, in the presence of 4 mM Ca^{2+}. Aliquots were withdrawn from the reaction mixture at the indicated times for assay of modulator activity (●–●) and determination of the extent of methionine modification (○–○) as described under Materials and Methods. The inset (■–■) illustrates the relationship between loss of activity and modification of methionine residues.

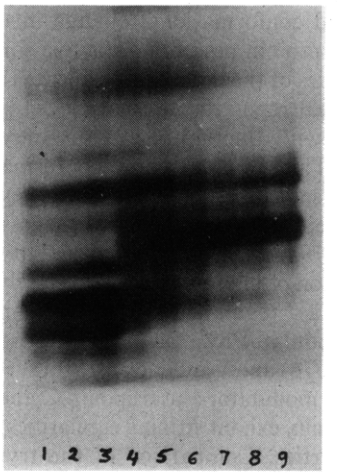

FIGURE 2: Monitoring of the mild oxidation reaction in the presence of Ca^{2+} by electrophoretic analysis (15% polyacrylamide gel electrophoresis) of cyanogen bromide digests: (1 and 2) digests of native modulator; (3–9) digests of modulator incubated with NCS for 0, 10, 20, 30, 60, 90, and 120 min, respectively. Approximately 100 μg of protein was applied to each slot.

Isolation of Modified Cyanogen Bromide Peptide. Electrophoretic analysis of cyanogen bromide digests of native and oxidized modulator protein (see below) revealed that a single peptide, which contains all the oxidized methionine residues, obtained from oxidized modulator is not obtained by cyanogen bromide digestion of the native protein. This peptide was isolated by preparative gel electrophoresis employing 15% polyacrylamide slab gels in batches of 1 mg of digest. The first gel was stained and destained in the usual way to check the efficiency of the separation, and the R_f value of the modified peptide was determined and found to be 0.59, in agreement with previously obtained values. The location of the modified peptide in all subsequent preparative gels was identified by this R_f value, and this portion of each gel was cut out. The gel slices were cut into 1-mm cubes and homogenized in a minimum volume of 0.1 M Tris-HCl, pH 8.1, in order to elute the peptide from the gel. The homogenate was stirred overnight and then centrifuged at 27 000*g* for 30 min. The supernatant was decanted into a lyophilization flask, and the pellet was resuspended in a minimum volume of 0.1 M Tris-HCl, pH 8.1, and centrifuged as before. The resultant supernatant was combined with the first supernatant, and the mixture was lyophilized. The residue was dissolved in 3.5 mL of water and applied to a Sephadex G-25 column (2.5 × 90 cm) previously equilibrated with deionized water and elution achieved with deionized water. Fractions (6.4 mL) were collected at a flow rate of 108 mL/h. The absorbance at 280 nm of each fraction was measured in a Beckman Model 25 spectrophotometer. The peptide eluted between fractions 38 and 48, which coincided with the void volume as determined in an independent filtration using blue dextran. These fractions were pooled and lyophilized.

The preparation, shown to be homogeneous by 15% polyacrylamide gel electrophoresis, was dissolved in 5 mL of water. Aliquots of 0.5, 1.0, and 1.5 mL were lyophilized in hydrolysis tubes and acid hydrolyzed in the presence of 0.2 M dithioerythritol prior to amino acid analysis.

Results and Discussion

Oxidation of Protein Modulator with NCS. Figure 1 illustrates the effect of mild oxidation of the protein modulator with NCS in the presence of Ca^{2+}. In agreement with our previous preliminary observations (Walsh & Stevens, 1977), oxidation under these conditions results in the rapid loss of modulator activity. Since modulator protein does not contain either cysteine or tryptophan residues, the reaction is specific for methionine. The extent of methionine oxidation can be determined by quantitative modification of the unoxidized methionine residues with cyanogen bromide, followed by acid hydrolysis and amino acid analysis. In this way, methionine residues unaffected by oxidation are determined as homoserine or homoserine lactone, whereas methionine residues oxidized to methionine sulfoxide are unaffected by the cyanogen bromide treatment and are converted back to methionine during acid hydrolysis (Schechter et al., 1975). In every instance, the sum of methionine, homoserine, and homoserine lactone accounted, within experimental error, for the nine methionine residues known to be present in modulator protein, and the methionine value obtained was taken as representing the number of modified methionine residues. As shown in Figure 1, loss of activity occurs concomitant with methionine modification and from the inset it can be seen that the activity decreases linearly as a function of methionine modification; complete loss of activity after 60–90 min of reaction correlates with the apparent oxidation of four methionine residues per mole of modulator. A control sample treated in an identical manner, with the exception that NCS was omitted from the reaction mixture, retained full modulator activity. Amino acid analysis of the control sample after cyanogen bromide treatment and acid hydrolysis revealed the presence of one methionine residue even at zero time; this can be explained by the observation (Richman & Klee, 1978) that the Met-109–Thr-110 peptide bond in modulator protein is very resistant to cyanogen bromide cleavage. It is thus possible that complete loss of modulator activity occurs with the oxidation of three rather than four methionine residues, since our analytical technique cannot inform us about the oxidation state of methionine-109.

Figure 2 shows the electrophoretic pattern on a 15% polyacrylamide slab gel of the cyanogen bromide digests of native modulator protein and modulator protein after different times of incubation with NCS. The peptide pattern (slots 1–3) for the modulator protein is compatible with its amino acid composition and changes as a function of reaction time (slots 4–7); after 60 min of incubation with NCS, which results in totally inactive material, the peptide pattern no longer changes (slots

7-9). The inactive material has fewer peptide bands than the native protein and all but one of the peptide bands appear to correspond to peptides obtained from the native protein. This peptide pattern is compatible with the modification of three to four methionine residues only if the modification is specific and if these oxidized residues are in a sequence uninterrupted by unmodified methionines (see below).

A series of parallel experiments to the ones described above was performed in a study of the effect of oxidation of the protein modulator with NCS in the absence of Ca^{2+}. Complete loss of activity occurs much faster (within 10 min) and correlates well with the oxidation of three methionine residues. However, the cyanogen bromide peptide pattern of the modified derivatives in this case was much more complex than for modification in the presence of Ca^{2+}, indicating that the reaction was less specific and that more methionine residues are available for reaction.

Further Characterization of Protein Modulator Inactivated by Oxidation with NCS in the Presence of Ca^{2+}. Inactive modulator protein, obtained by incubation with NCS in the presence of Ca^{2+} for 90 min as described under Materials and Methods, was further characterized in terms of its secondary structure, as determined by circular dichroism, its Ca^{2+}-binding ability, its TN-C-like activities, and the location of the modified methionine residues.

Studies described in the Appendix show that the oxidized modulator protein is still able to undergo Ca^{2+}-dependent conformational changes and that in the presence of a sufficient Ca^{2+} concentration the CD spectrum of the oxidized modulator is virtually indistinguishable from that of the native molecule. In addition, the CD titration studies, also described in the Appendix, show that, whereas the native modulator contains both high- ($K_d = 1.9 \times 10^{-7}$ M) and low-affinity (4×10^{-4} M) binding sites, the oxidized modulator appears to possess only low-affinity binding sites ($K_d = 3.8 \times 10^{-4}$ M). Even at Ca^{2+} concentrations well above (6 mM) those required to elicit the full conformational change in the oxidized modulator (as measured by circular dichroism), the oxidized modulator not only fails to activate phosphodiesterase but a more than 20-fold excess of inactive, oxidized modulator is also without consequence in an assay mixture containing enough native modulator to give 50% maximal stimulation of the enzyme. Thus, not only is the oxidized modulator inactive but it also can probably no longer bind to the enzyme, since it does not seem to interfere with the interaction between native modulator and phosphodiesterase.

Two properties of troponin C which are shared by modulator protein are a Ca^{2+}-dependent change in mobility on urea-polyacrylamide gel electrophoresis (Perry et al., 1972; Amphlett et al., 1976) and the Ca^{2+}-dependent formation of a complex with troponin I which is stable in 6 M urea (Perry et al., 1972; Amphlett et al., 1976) as demonstrated by urea-polyacrylamide gel electrophoresis. These TN-C-like properties of the modulator are lost upon oxidation with NCS.

In an attempt to identify the actual residues modified by oxidation with NCS, the new cyanogen bromide peptide (Figure 2) was isolated by preparative slab gel electrophoresis as described under Materials and Methods. Its amino acid composition is shown in Table I, together with the theoretical amino acid composition of residues 52-124 as calculated from the sequence data (Vanaman et al., 1977). Residues 51 and 124 are methionine residues, and the peptide composed of residues 52-124 would represent a cyanogen bromide peptide in which the methionines at positions 71, 72, 76, and 109 were not subject to cleavage by cyanogen bromide. The agreement between actual and theoretical amino acid compositions is within experimental error, and the results are in accord with the previously discussed observations that complete inactivation occurs concomitant with the modification of three to four methionine residues (Figure 1) and that these oxidized methionine residues occur in a sequence uninterrupted by unmodified methionine residues.

TABLE I: Amino Acid Composition of Methionine-Oxidized Cyanogen Bromide Peptide of the Protein Modulator.

	amino acid comp[a]		
amino acid	av[a]	integer	res 52-124[b]
Lys	4.52	4-5	4
His	0.60	1	1
Arg	3.28	3	4
Asp	11.91	12	13
Thr	5.07	5	5
Ser	1.91	2	2
Glu	15.11	15	11
Pro	trace	0-1	1
Gly	5.79	6	5
Ala	5.74	6	5
Cys	0.00	0	0
Val	4.00	4	4
Met	2.98	3[c]	5
Ile	3.89	4	4
Leu	4.58	4-5	4
Tyr	1.16	1	1
Phe	3.82	4	4

[a] The results represent amino acid analyses of three independent hydrolyses of the peptide. [b] Values taken from the amino acid sequence as determined by Vanaman et al. (1977). [c] Only cyanogen bromide resistant (oxidized) methionine residues would have been analyzed as methionine and this value therefore does not include the carboxy-terminal homoserine residue.

It has been previously shown by Richman & Klee (1978) that the Met-109-Thr-110 peptide bond is very resistant to cyanogen bromide cleavage. Our own (see above) results show that even in native modulator protein our analysis shows one "modified" methionine. It is thus not unlikely that in the oxidized modulator, Met-109 is actually not oxidized but simply resistant to cyanogen bromide cleavage; if that is indeed the case, then only residues 71, 72, and 76 were oxidized by treatment with NCS in the presence of Ca^{2+}. Under the reaction conditions used, NCS will only oxidize exposed methionine residues (Shechter et al., 1975), and thus we can conclude that methionine-71, -72, and -76 (and possibly -109) are exposed on the surface of modulator protein in the presence of Ca^{2+} and are probably important for its interaction with phosphodiesterase. The remaining methionine residues are either partially or completely buried within the hydrophobic interior of the molecule. Our finding that methionine-71, -72, and -76 are exposed is in agreement with our earlier observation (Walsh et al., 1977) that, in the presence of Ca^{2+}, the peptide bond between Lys-77 and Asp-78 is readily available to tryptic cleavage. Also, one would expect the site involved in protein-protein interaction to be located on the surface of the molecule.

It is widely believed that protein-protein interaction involves an initial recognition which occurs via charged side chains and provides an initial relatively weak interaction, followed by the formation of strong intermolecular binding which occurs via hydrophobic residues (Chothia & Janin, 1975). We observed earlier (Walsh & Stevens, 1977) that neutralization of the positive charge of the ϵ-amino group of two or three lysine residues of the modulator resulted in a loss of ~60% of PDE-

stimulating activity. It is conceivable that the charged side chains of Lys-75 and/or Lys-77, and perhaps other neighboring side-chains, are involved in the initial recognition of PDE by the modulator and that, once weak binding occurs via ionic interactions, the binding is strengthened by the formation of hydrophobic bonds involving any one or more of Met-71, -72, and -76. This PDE-binding domain is disrupted upon removal of Ca^{2+} ions from the modulator. Based on the sequence homology with troponin C (Vanaman et al., 1977) and the predicted structure of troponin C (Kretsinger & Barry, 1975), the proposed phosphodiesterase-binding domain would be located in the region of the sequence joing the second and third calcium-binding loops.

Acknowledgment

We are grateful to P. Ngai for technical assistance and amino acid analyses.

References

Amphlett, G. W., Vanaman, T. C., & Perry, S. V. (1976) *FEBS Lett. 72,* 163-168.

Brostrom, C. O., Huang, Y.-C., Breckenridge, B. M., & Wolff, D. J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 64-68.

Butcher, R. W., & Sutherland, E. W. (1962) *J. Biol. Chem. 237,* 1244-1250.

Cheung, W. Y. (1970) *Biochem. Biophys. Res. Commun. 38,* 533-538.

Cheung, W. Y. (1971) *J. Biol. Chem. 246,* 2859-2869.

Cheung, W. Y., Bradham, L. S., Lynch, T. J., Lin, Y. M., & Tallant, E. A. (1975) *Biochem. Biophys. Res. Commun. 66,* 1055-1062.

Chothia, C., & Janin, J. (1975) *Nature (London) 256,* 705-708.

Collins, J. H., Greaser, M. L., Plotter, J. D., & Horn, M. J. (1977) *J. Biol. Chem. 252,* 6356-6362.

Dabrowska, R., Sherry, J. M. F., Aromatorio, D. K., & Hartshorne, D. J. (1978) *Biochemistry 17,* 253-258.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121,* 404-427.

Dedman, J. R., Potter, J. D., & Means, A. R. (1977) *J. Biol. Chem. 252,* 2437-2440.

Drabikowski, W., Grabarek, Z., & Barylko, B. (1977) *Biochim. Biophys. Acta 490,* 216-224.

Gopinath, R. M., & Vincenzi, F. F. (1977) *Biochem. Biophys. Res. Commun. 77,* 1203-1209.

Goren, E. N., & Rosen, O. M. (1971) *Arch. Biochem. Biophys. 142,* 720-723.

Gross, E., & Witkop, B. (1961) *J. Am. Chem. Soc. 83,* 1510-1511.

Howard, S. M., & Pierce, J. G. (1969) *J. Biol. Chem. 244,* 6468-6476.

Jarrett, H. W., & Penniston, J. T. (1977) *Biochem. Biophys. Res. Commun. 77,* 1210-1216.

Kakiuchi, S., Yamazaki, R., & Nakajima, H. (1970) *Proc. Jpn. Acad. 46,* 587-592.

Kretsinger, R. H., & Barry, C. D. (1975) *Biochim. Biophys. Acta 405,* 40-52.

Macintyre, J. D., & Green, J. W. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* 271.

Perry, S. V., Cole, H. A., Head, J. F., & Wilson, F. J. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37,* 251-262.

Richman P. G., & Klee, C. B. (1978) *Biochemistry 17,* in press.

Schulman, H., & Greengard, P. (1978) *Nature (London) 271,* 478-479.

Shechter, Y., Burstein, Y., & Patchornik, A. (1975) *Biochemistry 14,* 4497-4503.

Steers, E., Jr., Craven, R. G., Anfinsen, C. B., & Bethune, J. L. (1965) *J. Biol. Chem. 240,* 2478-2484.

Stevens, F. C., Walsh, M., Ho, H. C., Teo, T. S., & Wang, J. H. (1976) *J. Biol. Chem. 251,* 4495-4500.

Teo, T. S., Wang, T. H., & Wang, J. H. (1973) *J. Biol. Chem. 248,* 588-595.

Vanaman, T. C., Sharief, F., & Watterson, D. M. (1977) in *Calcium Binding Proteins and Calcium Function* (Wasserman, R. H., et al., Eds.) Elsevier, North Holland, p 107.

Waisman, D., Stevens, F. G., & Wang, J. H. (1975) *Biochem. Biophys. Res. Commun. 65,* 975-982.

Walsh, M., & Stevens, F. C. (1977) *Biochemistry 16,* 2742-2749.

Walsh, M., & Stevens, F. C. (1978) *Can. J. Biochem. 56,* in press.

Walsh, M., Stevens, F. C., Kuznicki, J., & Drabikowski, W. (1977) *J. Biol. Chem. 252,* 7440-7443.

Wang, J. H., & Desai, R. (1976) *Biochem. Biophys. Res. Commun. 72,* 926-932.

Wang, J. H., & Desai, R. (1977) *J. Biol. Chem. 252,* 4175-4184.

Wang, J. H., Teo, T. S., Ho, H. C., & Stevens, F. C. (1975) *Adv. Cyclic Nucleotide Res. 5,* 179-194.

Watterson, D. M., Harrelson, W. G., Jr., Keller, P. M., Sharief, F., & Vanaman, T. C. (1976) *J. Biol. Chem. 251,* 4501-4513.

Yogi, K., Yazawa, M., Kakiuchi, S., Ohshima, M., & Wenishi, K. (1978) *J. Biol. Chem. 253,* 1338-1340.

Appendix: Circular Dichroism Studies on Ca^{2+}-Dependent Protein Modulator Oxidized with *N*-Chlorosuccinimide

ABSTRACT: The structural features and Ca^{2+}-binding properties of native and *N*-chlorosuccinimide-oxidized modulator protein were compared by circular dichroism. In the presence of Ca^{2+}, the far-UV spectra of native and oxidized modulator protein are virtually indistinguishable, indicating that oxidation of surface methionine residues does not alter the overall conformation of the molecule. In the absence of Ca^{2+}, however, the circular dichroism spectra of native and oxidized modulator are different with calculated helical contents of 40% and 26%, respectively. As judged by circular dichroism titration studies, the native modulator contains both high- ($K_d = 1.9 \times 10^{-7}$ M) and low-affinity ($K_d = 4 \times 10^{-4}$ M) Ca^{2+}-binding sites, whereas the modified modulator appears to possess only low-affinity sites ($K_d = 3.8 \times 10^{-4}$ M). The reduced secondary structure in Ca^{2+}-free oxidized modulator protein may account for the absence of high affinity Ca^{2+} binding sites.

Circular dichroism provides a convenient method for determining the gross conformational properties of a protein in solution and is particularly suitable for detecting changes in conformation brought about by chemical modification of amino acid residues. We report here the results of comparative circular dichroism studies on the structure and Ca^{2+}-binding properties of native modular protein and modulator protein, in which methionine-71, -72, -76 and, possibly, -109 were oxidized by mild oxidation with *N*-chlorosuccinimide.

Materials and Methods

Materials. Native and NCS-oxidized modulator were prepared as described in the main paper.

Circular Dichroism. The circular dichroism (CD) measurements were made on a Cary Model 6001 circular dichroism attachment whcih was connected to a Cary 60 re-

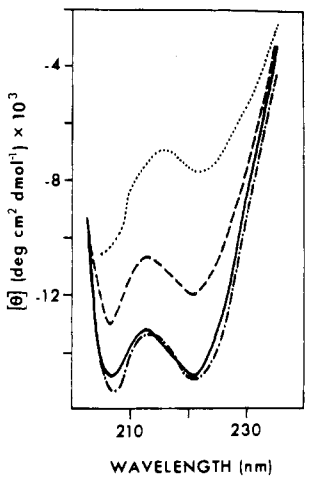

FIGURE 3: Far-UV spectra of native and oxidized bovine brain modulator protein in 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH 7.5, with or without 3 mM $CaCl_2$. Protein concentration was 1.05 mg/mL for the native protein and 1.17 mg/mL for the oxidized protein and the pathlength was 0.050 cm: Native protein without Ca^{2+} (- - -); native protein with Ca^{2+} (—); oxidized protein without Ca^{2+} (· · ·); oxidized protein with Ca^{2+} (- • -).

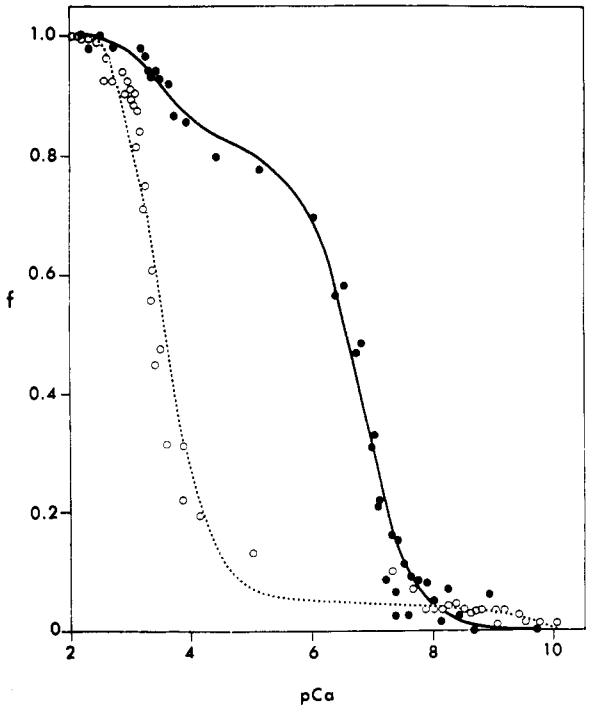

FIGURE 4: CD calcium titration of the conformational change in native (●) and oxidized protein modulator (○). Protein was dissolved in 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH 7.47. $CaCl_2$ was added incrementally and $[\theta]_{222nm}$ recorded after each addition. f is the fraction of completion of the conformational change, and pCa = $-\log [Ca^{2+}]$. The lines represent computer-calculated theoretical titration curves which best fit the data points.

cording spectropolarimeter in accordance with previously described methodology (Oikawa et al., 1968).

Protein Concentrations. Modulator concentration was measured by ultraviolet absorption employing an $E^{1\%,1\ cm}_{275-278\ nm}$ of 1.9 (Stevens et al., 1976).

Calcium Ion Concentration. The level of free Ca^{2+} ions in solution was adjusted by means of an EGTA-containing buffer, employing principles discussed by Perrin & Dempsey (1974), and the concentration of free Ca^{2+} ions was calculated as described by Burtnick & Kay (1977).

Results and Discussion

Circular Dichroism Spectra of Native and Oxidized Modulator Protein. Figure 3 shows the far-UV circular dichroism spectra of native and oxidized modulator protein in the presence and absence of Ca^{2+}. Experimental details are given in the figure legend. As previously shown by others using both bovine brain modulator protein (Wolff et al., 1977; Liu & Cheung, 1977) and modulator protein from other sources (Kuo & Coffee, 1976; Klee, 1977; Dedman et al., 1977), Ca^{2+} binding to the native protein induces a conformational change in the molecule with a substantial increase in helical content. Calculations of helical content from the observed ellipticities by the method of Fasman (1976) show that in our hands the native protein exhibits 49% α-helical content in the presence of Ca^{2+} and 40% α-helical content in the absence of Ca^{2+}. The circular dichroism spectrum of the oxidized modulator in the presence of Ca^{2+} is essentially indistinguishable from that of the native protein in the presence of Ca^{2+}, indicating that the average degree of secondary structure in the molecule is unaffected by the oxidation of surface methionine residues. Thus, the loss of phosphodiesterase-stimulating activity upon the oxidation of modulator protein with NCS is probably not the result of gross conformational changes in the molecule. It is clear from Figure 3 that removal of Ca^{2+} ions has much more of an effect on the oxidized modulator protein than on the native protein; the oxidized modulator in the absence of Ca^{2+} exhibits only 26% helical content, compared to 40% helical content for the native protein under the same conditions.

Circular Dichroism Titration Studies of Ca^{2+} Binding. The Ca^{2+}-binding properties of the oxidized modulator were compared to those of native modulator by observing the effect of incremental additions of Ca^{2+} on far-UV spectra of the proteins. Such a technique can identify different classes of Ca^{2+}-binding sites within the molecule and yield the corresponding dissociation constants for Ca^{2+}; it is particularly well suited for comparing the Ca^{2+}-binding properties of the chemically modified form of the modulator protein with those of the native molecule. The results are shown in Figure 4. The fraction of completion of the CD spectral change, f, is plotted against the negative logarithm of the free Ca^{2+} concentration in solution, pCa. The solid curve in Figure 4 is a best-fit computer-calculated theoretical titration curve for the native modulator calculated according to the method of Hincke et al. (1978) assuming the native protein possess two classes of Ca^{2+}-binding sites with apparent dissociation constants for Ca^{2+} of $1.89 \pm 0.19 \times 10^{-7}$ M and $4.03 \pm 2.28 \times 10^{-4}$ M. This theoretical curve is constructed on the assumption that the binding sites in any class are equivalent, independent, and noninteracting (Hincke et al., 1978). The theoretical curve is not steep enough for the data in the region above pCa ~6; it is possible that a better fit of this portion of the curve would be obtained by assuming cooperativity in the state function change. Ca^{2+} binding to the high-affinity sites ($K_d = 1.89 \times 10^{-7}$ M) of the native modulator elicits 80% of the observed CD spectral change, the remainder occurring upon binding of Ca^{2+} to the low-affinity site(s) with $K_d = 4 \times 10^{-4}$ M. Similar CD titration studies on the native modulator protein have been performed by Dedman et al. (1977) and by Wolff et al. (1977). Our results differ from theirs in that our mathematical treatment of the data assumes two classes of sites. It is generally accepted that the modulator contains four Ca^{2+}-binding sites divisible into two classes of sites: a high- and a low-affinity class. The reported apparent dissociation constants for Ca^{2+} range from 2×10^{-7} (Wolff et al., 1977) to 4×10^{-6} M (Klee,

1977) for the high-affinity class of sites and from 1×10^{-6} (Wolff et al., 1977) to 8.6×10^{-4} M (Watterson et al., 1976) for the low-affinity class of sites.

The dashed line in Figure 4 represents a best-fit computer-calculated curve for the oxidized modulator, calculated assuming the oxidized protein possesses two classes of Ca^{2+}-binding sites with apparent dissociation constants for Ca^{2+} of $4.25 \pm 1.54 \times 10^{-10}$ and $3.83 \pm 0.46 \times 10^{-4}$ M. In view of the negligible conformational change associated with it, the high-affinity ($K_d = 4.25 \times 10^{-10}$) site(s) are probably an artifact of the mathematical treatment of the data and do not truly exist. Thus, the affinity for Ca^{2+} of sites whose occupancy results in the major conformational change in the molecule has been reduced by two to three orders of magnitude in the oxidized modulator protein. In view of the circular dichroism spectra in Figure 3, this change in affinity probably reflects the conformational differences between the Ca^{2+} free forms of native and oxidized modulator rather than any conformational difference between the Ca^{2+}-bound states.

References

Burtnick, L. D., & Kay, C. M. (1977) *FEBS Lett. 75,* 105-110.

Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D., & Means, A. R. (1977) *J. Biol. Chem. 252,* 8415-8422.

Fasman, G. D. (1976) *Proteins 3,* 138-142.

Hincke, M. T., McCubbin, W. D., & Kay, C. M. (1978) *Can. J. Biochem. 56,* in press.

Klee, C. B. (1977) *Biochemistry 16,* 1017-1024.

Kuo, I. C. Y., & Coffee, C. J. (1976) *J. Biol. Chem. 251,* 6315-6319.

Liu, Y. P., & Cheung, W. Y. (1976) *J. Biol. Chem. 251,* 4193-4198.

Oikawa, K., Kay, C. M., & McCubbin, W. D. (1969) *Biochim. Biophys. Acta 168,* 164-167.

Perrin, D. D., & Dempsey, B. (1974) in *Buffers for pH and Metal-Ion Control,* Chapman and Hall, Ltd., London.

Stevens, F. C., Walsh, M., Ho, H. C., Teo, T. S., & Wang, J. H. (1976) *J. Biol. Chem. 251,* 4495-4500.

Watterson, D. M., Harrelson, W. G., Jr., Keller, P. M., Sharief, F., & Vanaman, T. C. (1976) *J. Biol. Chem. 251,* 4501-4513.

Wolff, D. J., Poirier, P. G., Brostrom, C. O., and Brostrom, M. A. (1977) *J. Biol. Chem. 252,* 4108-4117.

Structural Organization of the Lipoprotein HDL_c from Atherosclerotic Swine. Structural Features Relating the Particle Surface and Core†

D. Atkinson,* A. R. Tall, D. M. Small, and R. W. Mahley‡

ABSTRACT: The plasma lipoprotein HDL_c from miniature swine fed a high-cholesterol, saturated-fat diet exhibits a thermal transition (temperature range 25-45 °C) of its core-located cholesterol esters. This transition from an ordered, smectic-like structure to a more disordered structure is similar to that described for human plasma low-density lipoprotein (LDL). Small-angle X-ray scattering measurements demonstrate that HDL_c is a spherical particle (~180-Å diameter) intermediate in size between human LDL (~220 Å) and normal high-density lipoprotein (~100 Å). The electron-density profile of HDL_c below the transition (10 °C) exhibits a single core-located electron-density peak associated with a region of overlapping steroid moieties of the cholesterol esters arranged in a layered structure in the particle core. This electron-density profile may be compared to that for human LDL (below the transition temperature) which exhibits two core-located electron-density peaks due to layered cholesterol esters. Thus, the smaller size of HDL_c results in one fewer molecular units in the repeating, layered cholesterol ester organization. Comparison of the electron-density profiles for HDL_c and LDL demonstrates a common structural feature. The region of overlapping steroid moieties juxtaposed to the surface-located phospholipids and apoproteins is positioned at a constant distance from the particle surface in HDL_c and LDL. This constant structural feature relating the core-located cholesterol esters and the particle surface suggests a common interaction between the phospholipid and proteins at the surface and the initial layer of cholesterol esters in the particle core.

Human plasma low-density lipoprotein (LDL[1]) undergoes a reversible thermal transition between 20 and 45 °C (Deckelbaum et al., 1975, 1977). This transition is associated with a change from an ordered to a more disordered organization of the cholesterol esters localized in the core of the quasi-spherical LDL particle. Recently, we have used models of the molecular organization of the cholesterol esters in LDL to interpret the X-ray small-angle scattering profiles obtained from these assemblies and, hence, derive information on the molecular packing of the cholesterol esters below and above the transition (Atkinson et al., 1977). At 10 °C, the cholesterol esters are arranged in the core of the LDL particle in a radially repeating organization with a molecular packing similar to that of the smectic phase exhibited by the isolated esters. At 45 °C, this regular radial repeating organization is absent and the organization of the cholesterol esters is less ordered.

The lipoprotein designated HDL_c, which appears in the plasma of cholesterol-fed miniature swine, has α_2 mobility and an apoprotein composition including the arginine-rich and A-1 apoproteins (LDL apo-B is absent) (Mahley et al., 1975).

† From the Biophysics Division, Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02118. *Received March 21, 1978.* This work was supported by United States Public Health Service Research Grant HL-18623 and Training Grant HL-07291.

‡ Laboratory of Experimental Atherosclerosis, Heart, and Lung Institute, Bethesda, Md. 20014.

[1] Abbreviations used: LDL, low-density lipoprotein; HDL, high-density lipoprotein.

0006-2960/78/0417-3930$01.00/0