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Conformation-Dependent Nitration of the Protein Activator of Cyclic Adenosine 3′,5′-Monophosphate Phosphodiesterase†

Paul G. Richman* and Claude B. Klee

ABSTRACT: The conformational transition observed upon binding of Ca^{2+} to the Ca^{2+}-dependent activator protein of cyclic adenosine 3′,5′-monophosphate phosphodiesterase is reflected in a change in reactivity of its two tyrosine residues toward chemical modification by tetranitromethane (TNM). In the presence of Ca^{2+} both tyrosine residues (99 and 138) are nitrated by TNM, although at different rates, while in the presence of [ethylenebis(oxoethylenenitrilo)]tetraacetic acid (EGTA), only one tyrosine residue (99) is nitrated. The nitrotyrosines produced in the presence of Ca^{2+} exhibit apparent pKs of 7.3 and 8.6 by spectrophotometric titration. The tyrosine nitrated in the presence of EGTA only exhibited the lower pK. The rate of nitration of activator protein by TNM is enhanced by increasing the ionic strength (maximum employed was 1.0). Increasing ionic strength to 1.0 (0.33 M Na_2SO_4) does not lead to any significant change in secondary structure as measured by circular dichroic studies. The apparent rate of reaction of *N*-acetyltyrosinamide with TNM is also enhanced by increased ionic strength but to a much smaller extent than is observed with the protein. The results presented here together with other data recently reported from this laboratory indicate that tyrosine-138 (the one nitrated only in the presence of Ca^{2+}) is in an unusual microenvironment reflected by the high apparent pK of its phenol hydroxyl group (11.9 unmodified and 8.6 as the nitrophenol). The apparent K_m of activator protein for phosphodiesterase is unchanged upon nitration of both tyrosine residues.

The Ca^{2+}-dependent activator protein (also called modulator protein) of cyclic adenosine 3′,5′-monophosphate phosphodiesterase was first detected by Cheung (1967) and later characterized by Cheung (1970, 1971) and Kakiuchi et al. (1970). It was subsequently purified in several laboratories from a number of sources (Teo et al., 1973; Lin et al., 1974a,b; Watterson et al., 1976; Klee, 1977; Beale et al., 1977). The experimental evidence indicates that the mechanism by which the protein activates the enzyme requires an initial binding of Ca^{2+}

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to the free activator and a subsequent binding of a Ca^{2+}-activator complex to the phosphodiesterase (Kakiuchi et al., 1973; Teo & Wang, 1973; Lin et al., 1974a,b; Wolff et al., 1977).

Upon binding of Ca^{2+}, activator protein undergoes alterations in its secondary and tertiary structure as seen by changes in its fluorescence, circular dichroism, and UV[1] absorption spectra, as well as by resistance to trypsin (Wang et al., 1975; Liu & Cheung, 1976; Klee, 1977; Wolff et al., 1977). The negative difference spectrum generated in the 270–290-nm region upon binding of Ca^{2+} has been interpreted in terms of increased exposure to solvent of the tyrosine residues. Spectrophotometric titration of the two tyrosine residues has indicated that they are in different microenvironments (Klee, 1977).

In this paper the reaction of activator protein with tetranitromethane, a chemical modification reaction specific for tyrosine under certain conditions (Riordan & Vallee, 1972), was investigated in some detail. Other amino acids known to react with this reagent (cysteine and tryptophan) are absent in this protein.

Materials and Methods

Nitrotyrosine was a product of ICN. *N*-Acetyltyrosinamide was a product of Schwarz/Mann. Tetranitromethane, a product of Sigma, was purified by extracting three times with equal volumes of water (Riordan & Vallee, 1972). Cyanogen bromide was a product of Fluka.

Activator protein was purified from bovine brain acetone powder (Pel-Freez) as previously described (Klee, 1977). The purified activator protein was dialyzed against 0.05 M ammonium bicarbonate (4 °C), repeatedly lyophilized from water, and stored desiccated at −20 °C.

Activator-dependent cyclic adenosine 3′,5′-monophosphate phosphodiesterase from bovine brain (specific activity 3 μmol per min per mg at 30 °C) was purified by ammonium sulfate fractionation, DEAE-cellulose chromatography, and affinity chromatography on activator protein coupled to Sepharose (Klee & Krinks, 1978).

All other chemicals were either obtained as previously described (Klee, 1977) or were the purest grade commercially available.

Nitration Reactions. Nitration reactions were performed at ambient temperature (24 °C) in Tris-HCl buffer (0.05 M, pH 8.0). Reactions were initiated by the addition of 0.5 M TNM in ethanol to give a final concentration of 8.5×10^{-3} M (50-fold molar excess to tyrosine). The course of the reactions was followed spectrally at 428 nm where the nitrophenolate anion has a large absorbance (Sokolovsky et al., 1966).

Analytical scale reactions were done in microcuvettes in a total volume of 0.20 mL. The final concentration of activator protein was 8.5×10^{-5} M. Other additions were as indicated in the legends to the figures. In the experiment with *N*-acetyltyrosinamide, the concentration of the amide was 17×10^{-5} M.

Preparative scale reactions were carried out as described above with 7.0 mg of activator protein in a final volume of 4.8 mL in the presence of 1.0 M NaCl and the following additions: (preparation 1) 5×10^{-4} M $CaCl_2$ (60 min reaction time); (preparation 2) 1.4×10^{-3} M EGTA (60 min reaction time); (preparation 3) 5×10^{-4} M $CaCl_2$ (10 min reaction time). After the indicated reaction times, the protein was separated from the other components of the reaction mixture by gel filtration on a column (Sephadex G-25 fine, 1.6 × 23 cm) equilibrated with ammonium bicarbonate (0.05 M, pH 8.0) at room temperature. In preparation 3, the reaction was terminated by bringing the pH to 5.5 with 2 M acetic acid prior to gel filtration as above. The extent of nitration was determined spectrally on the material obtained after gel filtration using an $\epsilon_{381nm}^{M} = 2200$ (Riordan & Vallee, 1972). Protein concentration was determined as described by Lowry et al. (1951) using bovine serum albumin as the standard. The extent of nitration and the protein concentration were also determined by amino acid analysis as described below. The two methods of protein determination were in good agreement. Protein concentrations of solutions of native activator protein were calculated utilizing an $\epsilon_{230nm}^{1\%} = 25$ (Klee, 1977). A molecular weight of 16 500 (Klee, 1977) was utilized in calculating molar concentrations of activator protein (or nitrated activator protein).[2]

Amino Acid Analysis. Amino acid analyses were performed as previously described (Klee, 1977). In this system nitrotyrosine emerges just after, but completely resolved from, phenylalanine.

Spectrophotometric Titrations. Samples were prepared for titration by dialysis against 0.1 M KCl (4 °C). Samples of 2.0 mL were titrated in 1-cm cuvettes at 23 °C with NaOH and back titrated with HCl. Measurements of pH were made directly in the cuvette, 3 min after addition of base or acid, on a Radiometer Model PHM 64 pH meter equipped with a combined GK2322C electrode. Titration was followed by the absorbance change at 422 nm. The values were corrected for dilution caused by addition of acid or base. The total absorbance change observed upon going from pH 5.0 to 10.5 was considered equivalent to the complete ionization of all the nitrotyrosine residues in the protein. This absorbance change agreed well with the value calculated from the molar extinction coefficient of the nitrophenolate ion at its maximum (Riordan & Vallee, 1972). Absorbance changes were not time dependent.

Cyanogen Bromide Cleavage of Nitro-Activator Protein and Separation of the Resulting Peptides. Nitro-activator protein (2.2 mg) was reacted with CNBr (Gross & Witkop, 1962) in 70% formic acid in a closed flask at room temperature for 24 h (total volume, 0.5 mL). The molar ratio of CNBr:methionine residues was 80:1. The reaction mixture was evaporated in vacuo, redissolved in 0.4 mL of 0.2 M $(NH_4)HCO_3$ (pH 8.5), and chromatographed on a column (0.9 × 43 cm) of Sephadex G-50 (superfine) equilibrated with 0.2 M $(NH_4)HCO_3$ (pH 8.5). A sample was taken for amino acid analysis prior to chromatography. Fractions of 0.55 mL were collected and the absorbances at 425, 380, and 230 nm were measured. The recovery of nitrotyrosine from the reaction and subsequent column chromatography was over 90% based on the A_{380nm}. The extent of the CNBr cleavage reaction was greater than 90% of completion based on the loss of methionine (see Table II). High voltage electrophoresis was performed on Whatman 3MM paper (pH 2, 1000 V, 2 h, 10 °C). Peptides containing nitrotyrosine were located by their yellow color after exposure to ammonia.

Absorption. Measurements of absorption were carried out

[1] Abbreviations used: UV, ultraviolet; MRW, mean residue weight; CD, circular dichroism; cAMP, cyclic adenosine 3′,5′-monophosphate; EGTA, [ethylenebis(oxoethylenenitrilo)]tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Tyr(NO_2), 3-nitrotyrosine; TNM, tetranitromethane; $NaDodSO_4$, sodium dodecyl sulfate.

[2] This molecular weight value was determined in our laboratory by $NaDodSO_4$-polyacrylamide gel electrophoresis. This value agrees well with the molecular weight recently reported by Vanaman et al. (1977) based on sequence determination.

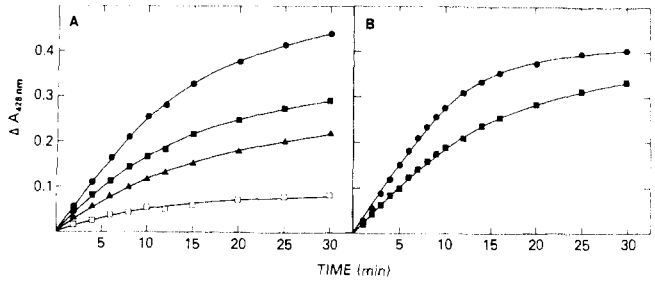

FIGURE 1: Effect of ionic strength on the course of nitration. (A) Activator protein (8.5×10^{-5} M) was reacted with TNM (8.5×10^{-3} M) at pH 8 in the presence of Ca^{2+} (5×10^{-4} M) and the following concentrations of NaCl: none (□—□), 0.05 M (▲—▲), 0.20 M (■—■), or 1 M (●—●). (B) *N*-Acetyltyrosinamide (17×10^{-5} M) was reacted with TNM (8.5×10^{-3} M) at pH 8 in the presence of either 0 (■—■) or 1 M (●—●) NaCl as indicated.

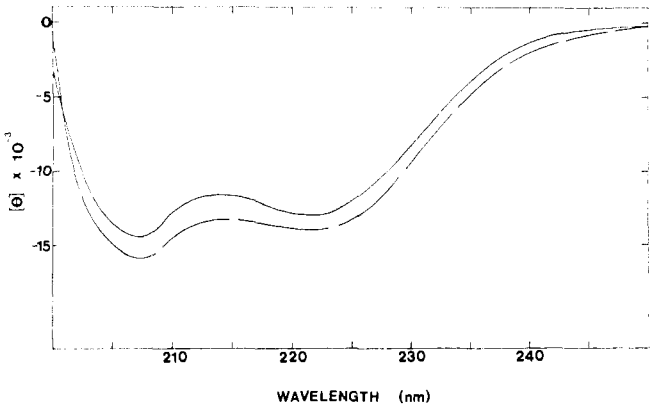

FIGURE 2: Effect of ionic strength on the CD spectrum of activator protein. CD spectra were performed at pH 8 as described under Materials and Methods on activator protein samples (0.2 mg/mL) at ionic strengths of 0 (—), 0.1 or 1.0 (- - -).

on a Cary 118C spectrophotometer equipped with a microcell holder (Model 1643400) and a reference beam attenuator (Model 14473475).

Circular Dichroism. Circular dichroic spectra were carried out utilizing a Cary 6001 attachment to a Cary 60 spectropolarimeter. Low speed scans were performed in duplicate at 26 °C using a cell with a 1-mm light path. A MRW of 112.9 was used (Klee, 1977).

Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was performed in slabs according to the method of Laemmli (1970) using a 12.5% gel. Protein was stained with Coomassie blue.

Assay of Activator Protein. Activator protein was assayed by its ability to activate partially purified bovine brain phosphodiesterase as previously described (Klee, 1977). The concentration of cAMP utilized in the assay was 2×10^{-4} M.

Results

Reaction of Activator Protein with TNM on an Analytical Scale. The reaction conditions were investigated on an analytical scale. The course of nitration was monitored spectrophotometrically at 428 nm, where the nitrophenolate ion absorbs maximally ($\epsilon_{428nm}^{M} = 4100$ at pH 8) (Sokolovsky et al., 1966). The increase in absorbance at this wavelength is only a semiquantitative guide to the true reaction course since it contains a small contribution from the trinitromethane anion ($\lambda_{max} = 350$ nm) produced in the reaction (see below). Absorbance is also dependent on the complete ionization of the nitrophenol produced in the reaction.

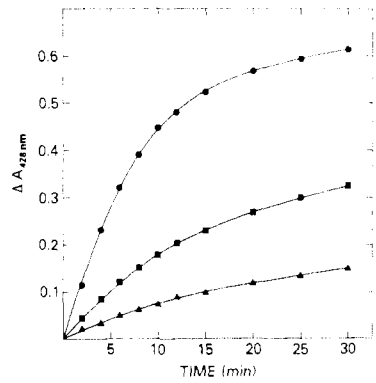

FIGURE 3: Effect of Ca^{2+} or guanidine hydrochloride on the course of nitration of activator protein. Activator protein (8.5×10^{-5} M) was reacted with TNM (8.5×10^{-3} M) at pH 8 in the presence of guanidine HCl (2 M) (●—●), Ca^{2+} (5×10^{-4} M) plus NaCl (1 M) (■—■), or EGTA (1.4×10^{-3} M) plus NaCl (1 M) (▲—▲).

The reaction of proteins with TNM as originally described by Sokolovsky et al. (1966) utilized 1 M NaCl in the reaction mixture, and investigations since that time have utilized conditions of salt encompassing the whole concentration range from 0 to 2 M NaCl (see references cited in Riordan & Vallee, 1972; Riordan & Sokolovsky, 1971). When activator protein was treated with TNM in the presence of Ca^{2+} and various concentrations of NaCl, the results shown in Figure 1A were obtained. A similar rate enhancement by NaCl was seen in the absence of Ca^{2+}. The addition of 1 M KCl or 0.33 M Na_2SO_4 gave the same apparent rate enhancement observed with 1 M NaCl (data not shown). Thus, the dramatic increase in the apparent reaction rate is an effect of ionic strength and not some specific effect of NaCl.

In order to differentiate between the effect of ionic strength on the availability of the tyrosines of activator protein for reaction and its possible effect on the rate of reaction itself, the reaction of TNM with *N*-acetyltyrosinamide also was examined in the absence of added salt and in 1 M NaCl (Figure 1B). The experiment with the model compound was done at the same concentration (17×10^{-5} M) of tyrosine used in the experiment with activator protein (Figure 1A). The presence of salt gave a large increase in the apparent initial rate of nitration of activator protein (5-fold) as opposed to the small enhancement of initial rate seen with the model compound (1.3-fold). Note that the apparent initial rate of reaction with activator protein in 1 M NaCl and Ca^{2+} is similar to that with *N*-acetyltyrosinamide in 1 M NaCl.

The large increase in rate of nitration of activator protein in the presence of salt does not seem to be due to a large unfolding of the protein. When the circular dichroic spectrum of native activator protein was determined at pH 8 at ionic strengths of $\mu = 0, 0.1$, or 1.0 (Na_2SO_4), the results shown in Figure 2 were obtained. Only a small increase in negative ellipticity and therefore presumably secondary structure is seen upon addition of salt. A similar increase has previously been observed upon addition of Ca^{2+} (Liu & Cheung, 1976; Klee, 1977). Since no special precautions were taken to remove Ca^{2+} from the salt solutions utilized in this experiment, it seems possible that the change observed may be attributed to Ca^{2+}.

Binding of Ca^{2+} to the activator protein causes conformational changes which may be associated with its ability to interact with and activate cAMP phosphodiesterase. As shown in Figure 3, the apparent rate of nitration of activator protein is faster in the presence of Ca^{2+} than in its absence (presence

TABLE I: Summary of Preparative Scale Nitrations of Activator Protein.[a]

Prep no.	Reaction conditions: Additions	Reaction conditions: Time (min)	Spectral anal.[b] of Tyr(NO_2) (residues/mole)	Amino acid anal. (residues/mole)[c]: Tyr(NO_2)	Amino acid anal. (residues/mole)[c]: Tyr
1	Ca^{2+}	60	1.92	1.54	Trace
2	EGTA	60	1.11	1.02	0.87
3	Ca^{2+}	10	1.23	1.02	0.78

[a] Nitration reactions were performed as described under Materials and Methods in the presence of the indicated additions (1 M NaCl was present in each reaction). After reacting for the indicated times, activator protein was separated from the other components on Sephadex G-25 as described under Methods. [b] Calculated from the A_{381nm} (isosbestic point) of the protein obtained after gel filtration. Protein concentration was determined as described under Materials and Methods (mol wt 16 500). [c] The number of residues per mole were normalized to 8 phenylalanine per 16 500 mol wt polypeptide chain.

of EGTA) by a factor of two- to threefold. This experiment was done in the presence of 1 M NaCl. Ca^{2+} also increases the apparent rate of reaction at low ionic strength (data not shown). The presence of 2 M guanidine hydrochloride gave the fastest apparent rate. A similar rate enhancement was caused by 2 M guanidine hydrochloride in the reaction of *N*-acetyltyrosinamide with TNM (data not shown). The mechanism underlying the effect of guanidine hydrochloride on the apparent reaction rate is not clear from the available data.

Preparative Scale Reactions of Activator Protein with TNM. Preparative scale reactions of activator protein were performed in the presence of 1 M NaCl under the conditions indicated in Table I. After gel filtration of the reaction mixtures, the extent of nitration was determined spectrally ($\epsilon_{381nm}^{M} = 2200$) and by amino acid analyses. The spectrally determined values of nitrotyrosine content agreed well with the loss of tyrosine determined by amino acid analysis (nitrotyrosine plus tyrosine should equal 2 mol/mol of activator protein). However, the values of nitrotyrosine obtained by amino acid analyses were always lower than the spectrally determined values. This has been observed by others also (Bustin, 1971; Sokolovsky et al., 1966). A sample of authentic nitrotyrosine subjected to the hydrolysis conditions used in the amino acid analyses was quantitatively recovered. Amino acid analyses showed that the other amino acids (including the single histidine residue) were unaltered in each of the three preparations of nitro-activator proteins (data not shown). The nitration of phenols by TNM is often accompanied by the formation of polymers (Bruice et al., 1968). Polymer formation has also been observed in the reaction of proteins with this nitrating reagent (Riordan & Sokolovsky, 1971; Glazer, 1976). The three preparations of nitro-activator protein were therefore examined by $NaDodSO_4$ gel electrophoresis (Figure 4) to determine if any high molecular weight polymers were formed during the reaction. Most of the protein in each of the three preparations of nitro-activator protein comigrates with native activator protein. However, there is a small amount of high molecular weight species in each of the preparations (the higher molecular weight species may not be clearly seen in the reproduction).

The trinitromethane produced in each of the reactions was quantitated after gel filtration. The molar ratios of trinitromethane to nitrotyrosine as determined spectrally were 1.7, 1.5, and 1.8 for preparations 1, 2, and 3, respectively.

In agreement with the analytical scale reactions, the extent of nitration is greater in the presence of Ca^{2+} (preparation 1)

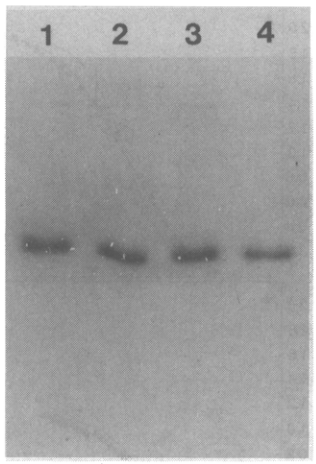

FIGURE 4: $NaDodSO_4$-polyacrylamide gel electrophoresis. Samples of 5 μg were prepared for electrophoresis by incubation at 100 °C for 1 min in the presence of 2% $NaDodSO_4$, 7 M urea, and 0.2 M dithioerythritol. Bromophenol blue was utilized as the marker dye. (1) Preparation 1; (2) preparation 2; (3) preparation 3; (4) "native" activator protein.

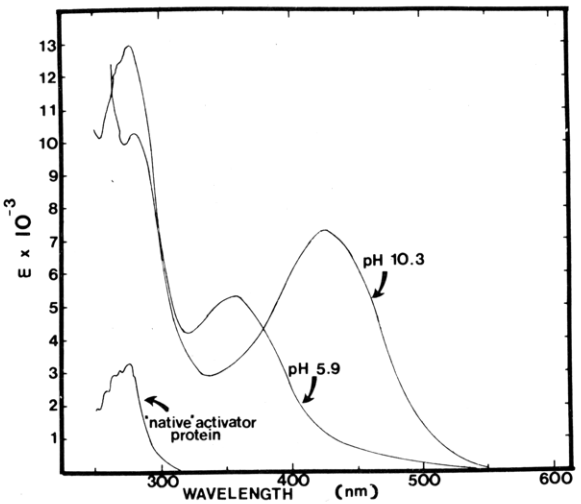

FIGURE 5: Visible and near-UV absorption spectrum of nitro-activator protein (mol wt 16 500). A sample of completely nitrated activator protein (preparation 1) was dialyzed against 0.1 M KCl overnight (4 °C) followed by centrifugation at 24 000*g* for 45 min. Samples were titrated to their final pH values with HCl or NaOH. Protein concentration was 0.6 mg/mL. The spectra were performed in the presence of 5×10^{-4} M $CaCl_2$. The spectrum of "native" activator protein was performed at pH 7.5 in the presence of 0.05 M NaCl.

than in the presence of EGTA (preparation 2) under otherwise identical conditions. Thus, in the presence of Ca^{2+} both tyrosine residues are nitrated, whereas in the presence of EGTA only one tyrosine residue is nitrated.

Properties of Nitro-Activator Protein. The visible and near-UV absorption spectrum of nitro-activator (preparation 1) is shown in Figure 5. At low pH (5.9) the visible spectrum of nitro-activator protein has a maximum at 357 nm which shifts to 425 nm upon ionization of the nitrophenol groups at high pH (10.3), with an isosbestic point at 379 nm.

In order to assess which of the two tyrosine residues in activator protein were nitrated under each of the various reaction conditions, spectrophotometric titrations of the nitrotyrosine groups were performed. It was previously reported from this laboratory that the two tyrosine residues in activator protein have apparent p*K*s of 10.4 and 11.9, respectively, with a slight decrease in the low apparent p*K* (10.4 to 10.1) upon binding of Ca^{2+} (Klee, 1977). The titration curve of nitro-activator

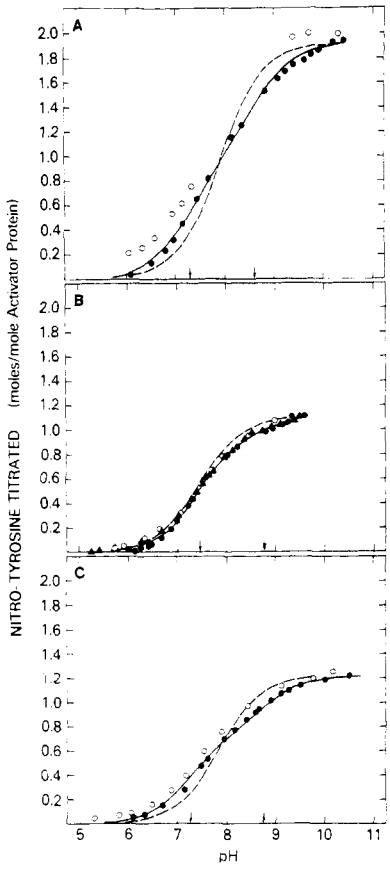

FIGURE 6: Spectrophotometric titrations of nitro-activator proteins. Samples of nitro-activator protein were titrated as described under Materials and Methods. Protein concentrations were 0.5–0.6 mg/mL. The titrations were performed in the presence of 5×10^{-4} M $CaCl_2$ (forward (●—●); back (○—○). Preparation 2 was also titrated (forward) in the presence of 1.4×10^{-3} M EGTA (▲—▲). See text for explanation of the various curves. (A) Preparation 1; (B) preparation 2; (C) preparation 3.

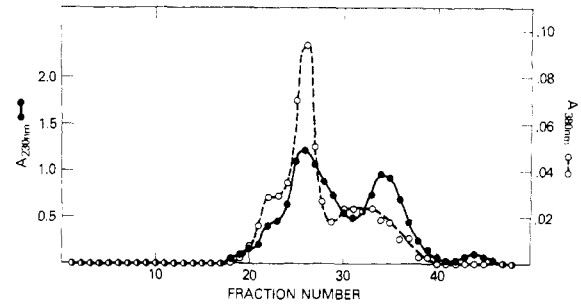

FIGURE 7: Sephadex G-50 chromatography of CNBr digest. A sample of nitro-activator protein was reacted with CNBr prior to chromatography on Sephadex G-50 (see Materials and Methods). The recovery of nitrotyrosine (A_{380nm} units) from the column was 100%.

protein (preparation 1) is shown in Figure 6A. The back-titration (open circles) indicates that some irreversible denaturation has occurred. The data agree well with a calculated curve (Henderson–Hasselbalch equation) for 2 pKs of 7.3 (1 mol) and 8.6 (0.92 mol) (solid line). The broken line represents the calculated curve for a single apparent pK (7.8). Nitrotyrosine gave an apparent pK of 6.9 when titrated under these conditions (data not shown) which agrees well with the literature value of 6.8 (Riordan et al., 1967). Thus, the difference in the apparent pKs of two nitrotyrosine residues is similar to that of the apparent pKs of the tyrosines in native activator protein. This difference in the apparent pKs of the nitrotyrosines enabled us to estimate the extent of reaction of each tyrosine in the other preparations (2 and 3) where only partial reactions were obtained.

When nitro-activator prepared in the presence of EGTA (preparation 2) was titrated the results shown in Figure 6B were obtained. The data fit a calculated curve (solid line) assuming 1 mol of nitrotyrosine with an apparent pK of 7.4 and 0.11 mol with an apparent pK of 8.7. The other line (broken line) is a calculated curve assuming a single apparent pK of 7.4 for 1.11 mol. Similar results were obtained when the titration was performed in the presence of EGTA (closed triangles).

In preparation 3 conditions were chosen that gave only partial reaction (see Table I). The titration curve for this preparation is shown in Figure 6C. The data agree well with a calculated curve (solid line) assuming 0.74 mol with an apparent pK of 7.2 and 0.49 mol with an apparent pK of 8.7. The broken line represents a calculated curve assuming a single apparent pK (7.8). Even in the presence of Ca^{2+} the two tyrosine residues exhibit slightly different rates of reaction.

Identification of Tyrosine Residue Nitrated in the Presence of EGTA. Spectrophotometric titration of nitro-activator protein prepared in the presence of EGTA (preparation 2) suggests that a single tyrosine residue was nitrated under these conditions. Nitration of additional activator protein under similar conditions as in preparation 2 (55 min reaction time instead of 60 min) gave nitro-activator protein that contained 0.89 mol of Tyr(NO_2) per mol of polypeptide as determined spectrally. Cyanogen bromide cleavage of this preparation followed by separation of the peptides on a column of Sephadex G-50 (Figure 7) showed that 75% of the nitrotyrosine residues were associated with a peptide(s) eluted between fractions 23 and 28 (the shoulder preceding this peak probably represents incompletely cleaved protein).

Activator protein contains 9 methionine residues and they are located at positions 36, 51, 71, 72, 76, 109, 124, 144, and 145 of the sequence (Vanaman et al., 1977). Amino acid analyses of pooled fractions 23–28 (peak I) and fractions 30–38 (peak II) (see Figure 7) were performed. The analysis of peak I agreed with the composite amino acid compositions of CNBr peptides 1 (36 residues), 5 (33 residues), and 6 (15 residues) (numbering starts from the amino terminus containing peptide). The single histidine residue was in this peak and proline was absent. The analysis of peak II agreed with the composite amino acid compositions of CNBr peptides 2 (15 residues), 3 (15 residues), and 7 (20 residues). Both proline residues were in this peak. CNBr peptides 4 (4 residues) and 8 (3 residues) were presumably eluted later from the column.

Peak I (fractions 23–28) was resolved into two peptides by electrophoresis at pH 2 (see Materials and Methods). The nitrotyrosine residue was associated with the more basic of the two peptides. Amino acid analysis of this peptide, after elution from the paper, agreed well with the analysis expected from CNBr peptides 5 and 6 (residues 77–124, Table II). This sequence contains tyrosine residue 99. The high values obtained for glycine and histidine probably reflect contamination by extraneous material eluted from the paper. The value for tyrosine agrees well with the loss of tyrosine expected at the principal site of reaction of activator protein with TNM. The identity of the peptide was confirmed by end group analysis (Hartley, 1970) which showed an amino-terminal lysine. It seems that cleavage at methionine-109 did not occur under our conditions.

The recovery of nitrotyrosine from the CNBr digest and subsequent column chromatography was quantitative, based

TABLE II: Amino Acid Analyses of Products of CNBr Reaction with Nitro-Activator Protein.[a].

Amino acid	CNBr-treated nitro-activator protein	"Native" activator protein[b]	CNBr peptide containing Tyr(NO_2)[c]	CNBr peptides Residues 76-108[b]	CNBr peptides Residues 76-124[b]
Asp	23.2	23	7.4	5	8
Thr	11.7	12	2.1	1	3
Ser	3.4	4	1.5	2	2
Glu	29.1	27	8.8	5	9
Pro	1.9	2	0	0	0
Gly	12.7	11	3.3	1	2
Ala	10.4	11	3.2	4	4
Val	6.1	7	3.2	2	3
Met	0.8	9	0	0	1
Ile	8.1	8	2.0	2	2
Leu	9.2	9	2.9	1	3
Tyr	1.0	2	0.16	1	1
Phe	8.0	8	2.0	2	2
Tyr(NO_2)	0.4	0	0.46	0	0
His	0.9	1	2.1	1	1
Lys	8.2	7	*d*	2	2
Arg	5.8	6	3.2	3	3

[a] The protein (or peptide) was hydrolyzed as described under Materials and Methods (24 h). Values were normalized to 8 phenylalanine per 16 500 mol wt polypeptide chain for the whole protein and to 2 phenylalanine per polypeptide chain for the CNBr peptide. Homoserine, although present, was not sufficiently resolved from the glutamic acid peak to obtain quantitative data. [b] Data taken from Vanaman et al. (1977). [c] CNBr peptide containing Tyr(NO_2) eluted from paper after electrophoresis of pooled fractions 23-28 from Sephadex column (see text). [d] Lysine was not resolved from the large ammonia peak in this analysis.

on the visible spectra (350-500-nm region) at each step of the procedure. However, amino acid analysis gave values about 50% of those expected.

A preparation of nitro-activator (made in the presence of Ca^{2+}) containing 1.75 mol of nitrotyrosine per 16 500 mol wt polypeptide was reacted with CNBr prior to gel filtration under conditions identical with those used in Figure 7. In this case peaks 1 and 2 (A_{380nm}) were nearly identical in size (52% and 48% of the A_{380nm} units were in peak 1 and 2, respectively).

The data presented above indicates that tyrosine residue 99 is the principal site of nitration of activator protein in the presence of EGTA. This infers that tyrosine residue 138 is nitrated by TNM only in the presence of Ca^{2+}.

Effect of Nitration on Ability to Activate cAMP Phosphodiesterase. The effect of extent of nitration of the activator protein on its affinity for the enzyme was examined (Table III). There was no significant difference in the values of apparent K_m. The three preparations of nitro-activator protein all activated cAMP phosphodiesterase as well as native activator at saturating concentrations of activator (or nitro-activator) protein (10^{-6} M).

TABLE III: Effect of Nitration of Activator Protein on Its Apparent K_m for cAMP Phosphodiesterase.

Prep no.	Extent of nitration (mol of Tyr(NO_2)/mol of protein)	Apparent K_m $\times 10^9$ (M)
1	1.92	3.8 ± 0.9 (2)
2	1.11	2.6 ± 0.8 (2)
"Native" activator	0	3.6 ± 0.8 (5)

[a] The enzyme was assayed as described under Materials and Methods in the presence of 10^{-9} to 10^{-6} M of the indicated preparations of nitrated activator proteins (or native activator protein). The apparent K_m was taken as the concentration of activator protein that gave half-maximum activation. The number of determinations are indicated by the numbers in parentheses.

Discussion

The apparent reaction rate of activator protein with TNM is enhanced by increased ionic strength, a condition which does not decrease the α-helix content of the protein as measured by circular dichroic studies. The reactivity of both tyrosines is enhanced by increased ionic strength since the effect was similar both in the presence of Ca^{2+} (where both tyrosines react) and the absence of Ca^{2+} (where essentially one tyrosine residue reacts). Activator protein contains a large negative surface charge at pH 8 because of the preponderance of side chain carboxyl groups. The formation of a phenoxide ion, necessary for reaction with TNM (Bruice et al., 1968), is energetically unfavorable in a molecule already negatively charged and the apparent pKs of the tyrosine residues are therefore higher than normal. The data suggest that the enhanced reactivity produced by increased ionic strength may be related to charge dispersion. The rate of nitration of copolymers of tyrosine and negatively charged residues (aspartate or glutamate) is much slower than the rate of nitration of copolymers of tyrosine and lysine (Riordan & Vallee, 1972; Riordan & Sokolovsky, 1971).

The results presented here show that the availability of the tyrosine residues of activator protein for reaction with TNM is dependent on the conformational state of the protein. Tyrosine residue 99 reacts well both in the absence of Ca^{2+} (presence of EGTA) and in the presence of Ca^{2+} and gives rise to a nitrotyrosine residue with an apparent pK of 7.2-7.4. Tyrosine residue 138 is reactive only in the presence of Ca^{2+} and gives rise to a nitrotyrosine with an apparent pK of 8.6-8.7. This is in agreement with the increased exposure of the tyrosine residues to solvent upon binding of Ca^{2+} to the protein seen by UV difference spectroscopy (Wang et al., 1975; Klee, 1977).

The two tyrosine residues are in different microenvironments as reflected by their apparent pKs of 10.4 and 11.9. These microenvironments are apparently unchanged upon nitration since the apparent pKs of the nitrotyrosine residues of the modified activator protein exhibit the same pattern (i.e., one near "normal" apparent pK of 7.3 and one of 8.7). Tyrosine

residue 138 was postulated to be in the hydrophobic interior of the protein on the basis of a large negative difference spectrum generated by 6 M guanidine hydrochloride and its high apparent pK. The binding of Ca^{2+} to activator protein has no effect on the apparent pK (11.9) of this residue (Klee, 1977) but dramatically increases its reactivity toward TNM, a seemingly paradoxical situation. One possible explanation is that TNM is reacting with this residue in the interior of the protein. The binding of Ca^{2+} might put the protein in a conformation that gives TNM (a hybrophobic compound) access to the interior to react with this residue (Glazer, 1977). However, the fact that this nitrotyrosine residue can be titrated at a pH (8.7) where the protein is presumably not denatured (although its pK is high for a nitrophenol) suggests that it is not buried. The sequence adjacent to tyrosine-138 is -Val-Asx-Tyr-Glx-Glx- (Vanaman et al., 1977). Its high apparent pK in both the native state (11.9) and as the nitrophenol (8.7) may be accounted for by its location near a cluster of negative charges from side chain carboxyl groups[3] (Wetlaufer, 1961). The reactivity with TNM could be enhanced by Ca^{2+} because Ca^{2+} may partially neutralize some of the negative charges by binding to vicinal carboxyl groups within a Ca^{2+} binding site (Vanaman et al., 1977).

Activator protein has been reported to resemble troponin C (Wang et al., 1975; Stevens et al., 1976; Watterson et al., 1976). The sequence of bovine brain activator protein (Vanaman et al., 1977) shows that activator protein is highly homologous to both rabbit skeletal muscle troponin C (Collins et al., 1973; Collins, 1974) and bovine cardiac troponin C (Van Eerd & Takahashi, 1976). Skeletal muscle troponin C has two tyrosine residues. The tyrosine residue at position 110 aligns with tyrosine-99 in activator protein. Since the other tyrosine residue is at position 10 in troponin C and position 138 in activator protein, it is not surprising that activator protein and rabbit skeletal troponin C show differences in the chemical properties of their tyrosine residues. Thus Ca^{2+} binding to skeletal muscle troponin C results in a positive tyrosine difference spectrum (Head & Perry, 1974; McCubbin & Kay, 1975) in contrast to a negative difference spectrum caused by Ca^{2+} binding to activator protein (Wang et al., 1975; Klee, 1977). The two tyrosine residues of skeletal muscle troponin C titrate with a pK of 10.7 (Lehrer & Leavis, 1974) whereas those of activator protein are distinguishable one from the other. Similarly the tyrosine residues of skeletal muscle troponin C are both nitrated by TNM (in the presence or absence of Ca^{2+}) (McCubbin & Kay, 1975), whereas tyrosine-138 of activator protein is resistant to nitration in the presence of EGTA. Interestingly, although activator protein can substitute for skeletal muscle troponin C in binding to troponin T and troponin I (Amphlett et al., 1976; Dedman et al., 1977), skeletal muscle troponin C has been reported not to activate cAMP phosphodiesterase[4] (Wang et al., 1975) or to require high concentrations of troponin C for activation (Dedman et al., 1977).

It is notable that bovine cardiac troponin C, unlike skeletal muscle troponin C, has three tyrosine residues (residues 5, 111, and 150), two of which can be aligned with those of activator protein (Vanaman et al., 1977). The chemical properties of the tyrosine residues of cardiac troponin C have not been investigated. Therefore comparison of the type made with skeletal troponin C (see preceding paragraph) is not yet possible. It will be interesting to see if cardiac troponin C (which contains a tyrosine residue near the carboxyl terminus) will activate cAMP phosphodiesterase.

We speculate that the carboxyl terminal region of activator protein, which contains tyrosine-138, may be involved in the interaction of activator protein and cAMP phosphodiesterase. This region also includes the single trimethyllysine (residue 115) of activator protein (Amphlett et al., 1976; Vanaman et al., 1977). Since nitration of tyrosine-138 does not significantly affect the ability of activator protein to interact with phosphodiesterase, the nitrated activator protein may serve as a useful probe for studying this interaction.

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[3] This is consistent with a small negative tyrosine difference spectrum observed upon lowering the pH from 5.3 to 4.3. Protein precipitation prevented more substantial evaluation of this blue shift.

[4] Rabbit skeletal muscle troponin C has recently been reported to activate phosphodiesterase from rat brain (Dedman et al., 1977) with an apparent K_m 600-fold higher than activator protein. Under our assay conditions (10^{-6} M cAMP) we have found that, at concentrations up to 10^{-5} M, skeletal muscle troponin C did not activate bovine brain cAMP phosphodiesterase and did not inhibit activation by the Ca^{2+}-dependent activator protein. The troponin C utilized in this experiment was the generous gift of C. M. Kay.

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Magnetic Nonequivalence within the Fatty Acyl Chains of Phospholipids in Membrane Models: ^{1}H Nuclear Magnetic Resonance Studies of the α-Methylene Groups†

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ABSTRACT: The existence of a large chemical shift difference between the α-methylene groups of the two fatty acyl chains of phospholipids in Triton X-100/phospholipid mixed micelles has been demonstrated using ^{1}H NMR [Roberts, M. F., & Dennis, E. A. (1977) *J. Am. Chem. Soc. 99,* 6142]. This difference between the two α-methylene groups in the Triton mixed micelle system (0.09 ppm) is now compared with that observed for synthetic short-chain phospholipids which exist as monomers (0.03 ppm for dihexanoylphosphatidylcholine) and those which form micelles (0.09 ppm for dioctanoylphosphatidylcholine). Ionic and zwitterionic detergents, such as cetyltrimethylammonium bromide, sodium dodecyl sulfate, sodium deoxycholate, and 3-(dimethyltetradecylammonio)propane-1-sulfonate, which form mixed micelles with dipalmitoylphosphatidylcholine, also were found to promote the large chemical shift difference of the two phospholipid α-methylene groups. Phospholipid line widths are much narrower in these latter mixed micelles, and the magnetic nonequivalence of the two protons on the *sn*-2 α-methylene carbon is observable. The spectra of the α-methylene protons are analyzed by decoupling the β-methylene protons and treating the resultant *sn*-2 multiplet as an AB system. The absolute magnitude of J_{AB} is always about 16 Hz, while the degree of the magnetic nonequivalence of the two protons depends on the detergent. In micelles with Triton X-100, only phospholipids containing short-chain fatty acids display line widths narrow enough to show the AB pattern. These results suggest that phospholipid molecules adopt a unique conformation in all micellar environments, be they pure phospholipid micelles or mixed micelles with nonionic or ionic detergents. In this conformation, the *sn*-1 α-methylene protons have indistinguishable chemical shifts and are in a more hydrophobic (shielded) environment than the strongly differentiated protons of the *sn*-2 α-methylene group. This pronounced difference in the two chains, which is not observed for monomeric phospholipid, is discussed in terms of phospholipid conformation and susceptibility to phospholipase A_2, an enzyme which catalyzes the hydrolysis of phospholipids specifically at the *sn*-2 carbonyl.

Proton NMR spectroscopy has been used extensively to examine the structure and packing of phospholipids in model membrane systems such as multibilayers and sonicated vesicles (Finer et al., 1972; Lee et al., 1972; Feigenson & Chan, 1974; Michaelson et al., 1974). We have used this technique to study phosphatidylcholine in mixed micelles with the nonionic detergent Triton X-100, a polydisperse preparation of *p-tert*-octylphenylpolyoxyethylene ethers (Ribeiro & Dennis, 1975). In these mixed micellar structures, subtle differences in the environment or conformation of the α-methylene groups of the two fatty acyl chains of various phospholipids are detected as chemical shift differences in the ^{1}H NMR spectra (Roberts & Dennis, 1977). This finding is of particular interest because mixed micelles serve as ideal substrates for phospholipase A_2 (Deems et al., 1975). This enzyme acts specifically to catalyze the hydrolysis of phospholipid by reaction at the carbonyl

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