Partial Purification and Characterization of Adenosine- and Guanosine-3′,5′-Monophosphate Phosphodiesterases from Human Lung Tissue†

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ABSTRACT: Crude extracts of human lung tissue were examined for cyclic adenosine- and guanosine-3′,5′-monophosphate (cAMP and cGMP) phosphodiesterase activities. Nonlinear reciprocal plots were observed for each substrate. DEAE-Sephadex chromatography of the extracts revealed four main fractions of activity, which were further purified by Sephadex gel filtration. The phosphodiesterase activity of the resulting individual fractions was partially characterized with respect to substrate specificity, kinetic parameters, apparent molecular weight (gel filtration), thermal stability at 30 and 37 °C, effect of the cyclic nucleotide not utilized as substrate, and the possible influence of Ca^{2+}-dependent protein activator. The results indicate that the tissue contains phosphodiesterases with strict specificity and a high apparent affinity for each of the two cyclic nucleotides (the K_m values determined were approximately 0.3–0.4 μM). The high affinity cAMP phosphodiesterase activity was enriched in two of the purified fractions; both activities probably represent fragments of the native high affinity cAMP specific enzyme. A third purified phosphodiesterase showed mixed substrate specificity. The K_m value recorded for hydrolysis of either substrate with this enzyme was approximately 25 μM. A fourth, irregularly occurring, phosphodiesterase activity also showed mixed substrate specificity. The K_m value registered for hydrolysis of either substrate with this fraction was approximately 0.4 μM. There was no evidence for a Ca^{2+}-dependent specific activation by a boiled lung tissue supernatant of any of the purified enzymes.

Adenosine-3′,5′-monophosphate phosphodiesterase and, more recently, guanosine-3′,5′-monophosphate phosphodiesterase are considered very important regulatory enzymes whose activities influence a number of aspects of cell function. The work of several laboratories suggests that the total phosphodiesterase activity in crude extracts of a variety of tissues results from a number of enzyme forms with differing substrate specificities and kinetic characteristics (for references, see reviews by Appleman et al., 1973, and by Amer and Krieghbaum, 1975). It has become increasingly clear that the phosphodiesterase activity pattern differs in various tissues (Kakiuchi et al., 1975) and probably also in different cell lines (see, e.g., Uzunov et al., 1974). The present communication deals with the phosphodiesterase activity of human lung tissue extracts, the aim primarily being to examine the number and general characteristics of the enzyme forms present in that tissue.

Material and Methods

Chemicals. [^{3}H]cAMP[1] (specific activity 20–40 Ci/mmol), [^{3}H]cGMP (specific activity 2–10 Ci/mmol), [^{3}H]adenosine (specific activity 30.5 Ci/mmol), and [^{3}H]-5′-AMP (specific activity 11.25 Ci/mmol) were obtained from New England Nuclear Corp. Snake venom (*Ophiophagus hannah*) and bovine serum albumin (fraction V powder) were purchased from Sigma Chemical Co. Bio-Rad AG 1-X2 (200–400 mesh) was delivered by AB Kemila-Preparat, Stockholm. Other chemicals were of analytical grade. All reagents were used without further purification. Water, double distilled from glass, was used throughout.

Buffers. Two regularly employed buffer systems will be referred to as: buffer A, 0.04 M Tris-HCl, pH 8 (4 °C), made 5 mM in $MgCl_2$ and 3.75 mM in 2-mercaptoethanol; and buffer B, buffer A made 0.1 M in NaCl.

Tissue. Human lung tissue (kindly supplied by Dr. Hans Schüller, and Dr. Hans Henriksson, University Hospital, Lund) was obtained within a few hours postoperation from patients suffering from carcinoma of the lung. Care was taken to free the tissue from as much remaining blood as possible. Generally, the tissue was lyophilized and stored at −20 °C until further processing. It was then reconstituted with the appropriate amount of water (4 °C).

Homogenization of Tissue. One part of tissue was homogenized at 4 °C with two parts (w/w) of buffer B for approximately 3 min in a Sorvall Omnimixer at high speed. The homogenate was centrifuged for 30 min at 200 000*g*. The obtained supernatant, containing 15–20 mg of protein per ml, is referred to as the crude tissue extract. Heating this supernatant for 3 min at 100 °C followed by centrifugation produced solutions referred to as boiled supernatants. When examined for influence on phosphodiesterase activity, the boiled supernatant constituted 15% by volume of the reaction mixture.

Chromatographic Procedures. Standard procedures were used for ion-exchange chromatography and gel filtration. Details are given in the legends to pertinent figures. Pooled active fractions were concentrated by Amicon ultrafiltration (Membrane UM 10) followed by lyophilization. Treated in this way, fractions could be stored at −20 °C for months without appreciable loss of activity, in accordance with observations by Thompson and Appleman (1971b).

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[1] Abbreviations used: 5′-AMP, adenosine 5′-monophosphate; cAMP, cyclic adenosine 3′,5′-monophosphate; cGMP, cyclic guanosine 3′,5′-monophosphate; DEAE, diethylaminoethyl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

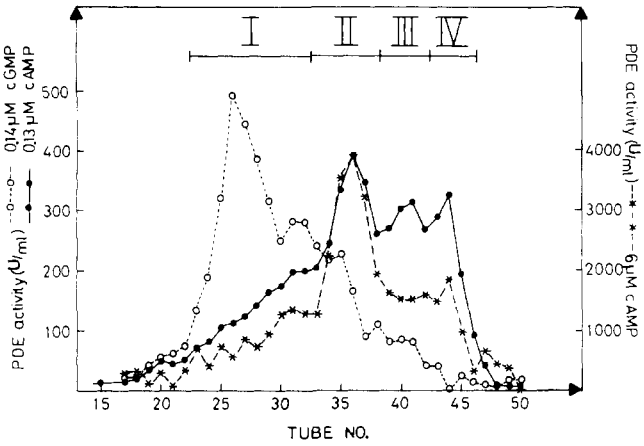

FIGURE 1: DEAE-Sephadex A-50 chromatography of a crude lung tissue supernatant (200 000*g*) from 25 g of human lung tissue homogenized in 50 ml of buffer B (column, 1.5 × 30 cm). Elution was performed with a linearly increasing concentration of NaCl in buffer A, the gradient being produced by feeding buffer B with buffer A made 0.5 M in NaCl (initial volume, 300 ml of each buffer). Flow rate was 8.8 ml/h. Fractions were collected each hour. Each fraction was assayed for phosphodiesterase activity at the indicated substrate concentrations. The majority of protein eluted within tubes 2–15. Total recoveries were: at 0.13 μM cAMP, 72% of the applied amount of enzyme activity; at 6 μM cAMP, 88%; and at 0.14 μM cGMP, 60%.

Gel filtration columns were calibrated with human γ-globulin and bovine serum albumin (monomers and dimers). A linear relation was observed between log molecular weight and elution volume. The apparent molecular weights given below for the phosphodiesterase activities are obtained from this empirical relation. They are thus roughly correct only if the enzymes show shapes and physicochemical characteristics similar to the proteins chosen for calibration. Goren and Rosen (1972) reported that "molecular weights" determined for phosphodiesterases using this procedure in fact do not conform to figures obtained with other techniques. All chromatographic procedures were performed at 4 °C.

Protein concentrations were estimated according to the procedure described by Lowry et al. (1951) using bovine serum albumin as standard protein.

Assay of Phosphodiesterase Activity. The Thompson–Appleman procedure (1971a; cf. Thompson et al., 1974) was followed with some modifications. Reaction mixtures contained: 50 μl of a suitable concentration of [^{3}H]cAMP in buffer A (see above), 400 μl of buffer A, made 0.5% in bovine serum albumin, and 50 μl of the enzyme preparation suitably diluted in the last mentioned buffer. The reaction, performed in a disposable plastic tube, was initiated by substrate addition. After incubation (usually for 12 min) at 30 °C, the reaction was terminated by immersing the tube in a boiling water bath for 2 min. After cooling to approximately 30 °C, 100 μl of snake venom (1 mg/ml) was added. Incubation was continued for another 15 min followed by the addition of 1 ml of a Bio-Rad–H_2O suspension (1:3, v/v). The tubes were shaken for approximately 10 min and centrifuged, and 0.5 ml of the supernatant, mixed with 10 ml of Instagel (Packard), was assayed for radioactivity. Blanks were run under identical conditions with the appropriate buffer replacing the enzyme addition. Specific activity was determined on an aliquot of the substrate preparation. Incomplete recovery of radioactivity (approximately 70%) after a supposedly complete degradation of added [^{3}H]cAMP was noted. Therefore, each preparation of Bio-Rad–H_2O suspension (after extensive washing with 0.5 M NaOH, H_2O, 0.5 M HCl, and H_2O) was calibrated for adsorption of adenosine with the aid of [^{3}H]adenosine. The calibration procedure was checked and found reliable and highly reproducible, by treating suitable concentrations of [^{3}H]-5′-AMP with snake venom under conditions mimicking those of the second step of the phosphodiesterase assay. The recovery of adenosine varied from 60 to 70% depending on resin preparations (cf. Rutten et al., 1973; Boudreau and Drummond, 1975a; Ferre et al., 1975; Lynch and Cheung, 1975). Results given below for phosphodiesterase activity are corrected for this adsorption. Assays for cGMP phosphodiesterase activity were performed analogously. Adsorption of guanosine to the ion-exchange resin was usually slightly lower than that of adenosine (recovery varied from 70 to 78%). The initial velocity of the phosphodiesterase catalyzed reaction, v, was expressed as nmol of cAMP or cGMP degraded/min × mg protein of enzyme solution. One unit (U) of enzyme activity is the amount capable of hydrolyzing 1 pmol of cAMP or cGMP/min at the pertinent substrate concentration.

Linearity of velocity v with enzyme concentration and with time was found to prevail for both cAMP and cGMP hydrolysis until approximately one-fifth of the substrate had been converted if extended reaction times were avoided. If, however, bovine serum albumin was omitted from buffers employed for enzyme dilution and assay, pronounced losses of activity were observed with increased enzyme dilutions and reaction times for hydrolysis of cGMP by crude supernatants and for hydrolysis of both cAMP and cGMP by all of the purified phosphodiesterase fractions.

Kinetic examinations were performed at fixed reaction times (mostly 12 min) under circumstances where less than 20% of substrate hydrolysis occurs. Figures for K_m and V_{max} were calculated according to the procedure described by Wilkinson (1961). All calculations utilized initial substrate concentrations. They were performed with a Hewlett-Packard No. 9810A desk calculator.

Results

Phosphodiesterase Activities in Crude Supernatants. The total phosphodiesterase activity of crude lung tissue supernatants (200 000 *g*) varied somewhat in different preparations. It was determined to approximately 2–3 × 10^3 U/ml supernatant (i.e., specific activity 100–200 U/mg protein) when assayed at 0.13 μM cAMP, to 7–15 × 10^3 U/ml supernatant at 6 μM cAMP, to 1–3 × 10^3 U/ml supernatant at 0.14 μM cGMP, and 10–30 × 10^3 U/ml at 7 μM cGMP. For each substrate and concentration, approximately 80–90% of the total phosphodiesterase activity in the homogenate could be recovered in the soluble fraction. Nonlinear Lineweaver–Burk (1934) plots were observed with both substrates; with cAMP, the results suggested three different K_m values—0.4 μM when assayed at substrate concentrations between 0.5 and 0.09 μM, 2–4 μM at substrate concentrations between 1 and 10 μM, and 50–100 μM at substrate concentrations from 20 to 150 μM cAMP. With cGMP as substrate, two slopes were observed at double-reciprocal plots. The corresponding K_m values calculated according to Wilkinson (1961) were: 0.5 μM at substrate concentrations between 0.5 and 0.09 μM, and 50 μM at substrate concentrations between 3 and 15 μM.

Separation of Various Phosphodiesterase Activities. DEAE-Sephadex Chromatography. The crude extract was immediately chromatographed on a column of DEAE-Sephadex A-50. The elution profile of the phosphodiesterase

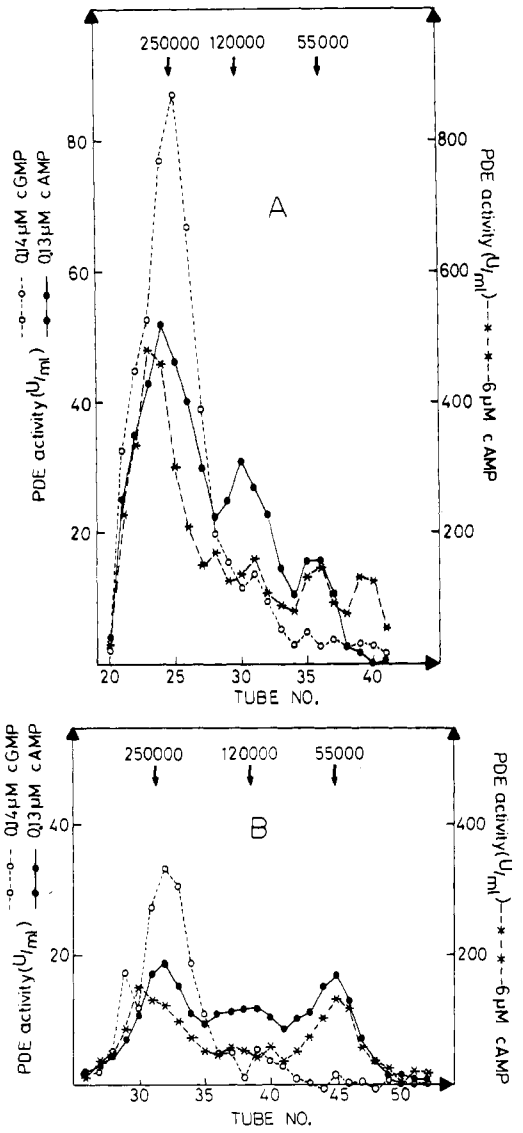

FIGURE 2: Gel filtration on Sephadex G-200 (2.5 × 95 cm) in buffer B of aliquots of a lung tissue supernatant incubated for 30 min at 0 °C, pH 8 (A), or at room temperature, pH 6 (B). (Determinations of pH were performed at 4 °C). Apparent molecular weights are indicated. Flow rate was 9.5 ml/h. Fractions were collected each hour. Total recovery of enzyme activity subjected to incubation, lyophilization, reconstitution, and gel filtration: At pH 8, 40 (0.13 μM cAMP), 43 (6 μM cAMP), 41% (0.14 μM cGMP); at pH 6, 11 (0.13 μM cAMP), 10 (6 μM cAMP), and 15% (0.14 μm cGMP).

activity and the total recovery varied somewhat from preparation to preparation, but four distinct fractions were regularly obtained (see Figure 1).

Gel Filtration. A crude lung tissue supernatant was separated into three parts. One was continuously iced, the second was incubated at room temperature for 30 min, and the third was treated analogously after having had pH adjusted to 6. Incubation was followed by lyophilization. After reconstitution, the samples were gel filtered individually on the same column of Sephadex G-200. The results (see Figure 2) show that the phosphodiesterase activity profile in samples incubated at room temperature and pH 6 differs from those kept at 0 °C and pH 8. A major part of the total activity is lost after incubation at pH 6 and room temperature. However, in that event, the relative proportion of phosphodiesterase activity with high elution volumes increases. The elution pattern of the sample incubated at pH

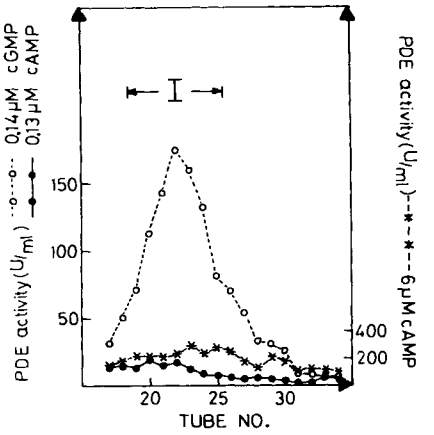

FIGURE 3: DEAE-Sephadex rechromatography of fraction I previously subjected to Sephadex G-200 gel filtration. Chromatography conditions as in Figure 1.

8 and room temperature resembled that of the iced one. Similar results were obtained when 2-mercaptoethanol was omitted from the homogenizing medium and eluent or when homogenization and gel filtration were performed at a fivefold increased concentration of 2-mercaptoethanol (i.e., 0.02 M).

Further Purification and Characterization of Individual DEAE-Sephadex Fractions. The four main fractions obtained by DEAE-Sephadex chromatography were further purified by Sephadex gel filtration. The resulting peaks of phosphodiesterase activity were rechromatographed on DEAE-Sephadex and/or Sephadex gels until reasonably homogeneous activity profiles were observed. The final purification steps resulted in rather heavy losses of enzyme activity.

Fraction I. The phosphodiesterase activity of fraction I primarily hydrolyzed cGMP. Gel filtration revealed a major component which eluted at an apparent molecular weight of about 240 000. Extended purification was achieved by repeated gel filtration and ion-exchange chromatography (Figure 3). The resulting phosphodiesterase activity hydrolyzed cGMP at 0.14 μM more than ten times faster than a corresponding concentration of cAMP (see Figure 3). The overall yield of this fraction was usually less than 5%; specific activity in relation to that of the crude supernatant was increased 20–30 times. The K_m value of the purified cGMP phosphodiesterase of fraction I was determined to be 0.30 ± 0.05 μM (mean of six experiments ± SEM); Lineweaver–Burk plots were linear over the range of substrate concentrations utilized (from 28 to 0.09 μM cGMP). The kinetic parameters were unaffected by 0.1 or 1 μM cAMP, but at 10 μM cAMP, V_{max} was reduced to 60%, at 100 μM cAMP to 45%, and at 1000 μM cAMP to 14% of the figure observed in the absence of cAMP. None of these concentrations of cAMP affected the apparent affinity of the enzyme for cGMP. Furthermore, none of the kinetic parameters were affected by the addition of a boiled supernatant with or without supplementary addition of Ca^{2+} (final concentration: 10^{-5} or 10^{-6} M); nor did EGTA (final concentration 10^{-4} M) influence the activity. Gel filtration of fraction I with buffer B made 0.1 mM in cAMP, cGMP, or Ca^{2+}, 0.01 mM in EGTA, 1 mM in adenosine, 1 M in urea, 0.02% in sodium dodecyl sulfate (each agent was used separately), or in buffer B made pH 6 (4 °C) did not significantly change the activity profile in any case, although urea and sodium dodecyl sulfate markedly reduced the total ac-

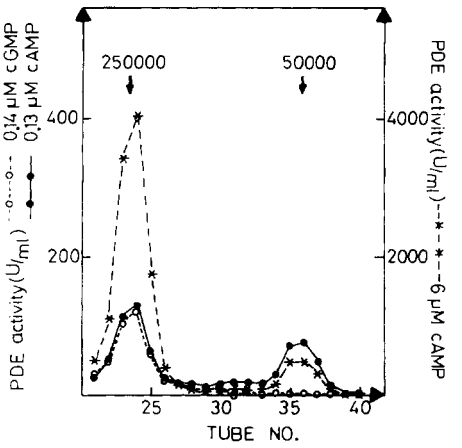

FIGURE 4: Gel filtration of DEAE-Sephadex fraction II on Sephadex G-200 (2.5 × 95 cm). Elution was performed with buffer B. Flow rate was 9.5 ml/h. Fractions were collected each hour. Tubes 22–25 constitute purified fraction II. Recovery at 6 μM cAMP was 63% of activity applied to the column.

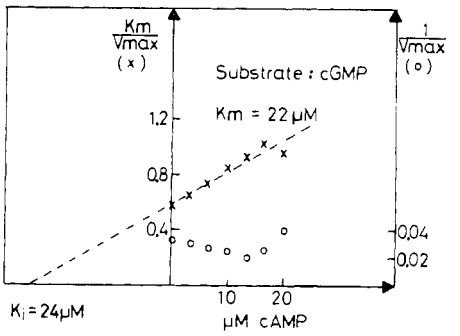

FIGURE 5: Hydrolysis of cGMP by purified fraction II in the presence of various concentrations of cAMP. Data were analyzed according to Wilkinson (1961) giving a pair of K_m and V_{max} values for each inhibitor concentration. The figure shows replots of the quotients K_m/V_{max} and $1/V_{max}$ against concentration of cAMP. The indicated K_m value is calculated from the figures $(K_m/V_{max})_{I=0}$ and $(1/V_{max})_{I=0}$. Substrate concentrations ranged from 28 to 5 μM cGMP (six different concentrations).

tivity. Irregularly, the activity pattern at DEAE-Sephadex chromatography of the crude supernatant showed a second peak in connection with the main peak of fraction I (see Figure 1, tubes 30–33). A comparable degree of activity toward cAMP and cGMP at 0.13/0.14 μM concentrations characterized this component. When purified by DEAE-Sephadex rechromatography, the last mentioned activity eluted ahead of the remaining cGMP specific phosphodiesterase activity. Gel filtration revealed an apparent molecular weight of about 160 000. The K_m value was determined to 0.4 μM and the V_{max} value to 18 nmol/min × ml of enzyme for hydrolysis of either substrate. Hill plots were normal (Hill coefficients: 0.92 for cAMP hydrolysis and 0.96 for cGMP hydrolysis). The activity of this fraction, when assayed at 0.14 μM cGMP, was unaffected by EGTA (10^{-3} M) or a boiled supernatant.

Fraction II. Fraction II showed phosphodiesterase activity of comparable degree when assayed with cGMP and cAMP. This fraction could be identified during purification because the ratio of the activities observed at 6 μM and at 0.13 μM cAMP was higher than the corresponding ratios for other fractions (see Figures 1, 4, and 6). At gel filtration, the phosphodiesterase activity of fraction II eluted corresponding to an apparent molecular weight of approximately 260 000 (Figure 4). The elution volume remained unchanged when gel filtration was performed with buffer B containing either 0.1 mM cAMP, 1 mM Ca^{2+}, 0.01 mM EGTA, or 1 M urea (although urea reduced the total activity), or if it was performed at pH 6. Based on the increase in V_{max}, fraction II has been purified 200-fold. The K_m value for cAMP hydrolysis was determined to be 26 ± 5 μM (mean of six experiments ± SEM). Hydrolysis of cGMP at concentrations below 0.2–0.1 μM followed upward concave Lineweaver–Burk plots. With higher substrate concentrations, the K_m value was determined to approximately 20 μM (see Figure 5). The Hill coefficient (Hill, 1910; cf. Hammes and Wu, 1974) for hydrolysis of cGMP at substrate concentrations between 35 μM and 0.12 μM was calculated to 1.00. cAMP at comparably high concentrations inhibited the hydrolysis of cGMP. When the K_m and V_{max} values for the hydrolysis of cGMP obtained at each concentration of cAMP according to the procedure of Wilkinson (1961) are replotted as K_m/V_{max} or $1/V_{max}$ against concentration of cAMP and straight lines were fitted to these points, the intercept with the x axis should give the K_i value and the intercept with the y axis the K_m/V_{max} value or the $1/V_{max}$ value (see Cleland, 1963). The K_i for cAMP calculated in this way was 24 μM (see Figure 5). A competitive type of inhibition is suggested because the plot of K_m/V_{max} against concentration of cAMP shows significant regression ($p < 0.001$), whereas the plot of $1/V_{max}$ against concentration of cAMP fails to do so ($p > 0.05$). Dixon (1953) plots of the data also suggest a competitive type of inhibition. Completely analogous results to those recorded above were obtained when the effect of cGMP on the hydrolysis of cAMP was examined (K_m for the cAMP phosphodiesterase activity, 29 μM; K_i for inhibition of that activity by cGMP, 24 μM, the inhibition being competitive). cGMP at lower concentrations (0.01, 0.1, or 1 μM) did not influence the kinetic parameters of the cAMP phosphodiesterase of this fraction. There was no indication of a Ca^{2+}-dependent activation of the phosphodiesterase activity of this fraction by a boiled lung tissue supernatant in experiments similar to those recorded for fraction I above; nor did EGTA at 10^{-4} M concentration affect the activity.

Fraction III. The phosphodiesterase activity of fraction III predominantly hydrolyzed cAMP. It could regularly be separated by gel filtration into at least two main components (IIIb and IIIc) eluting at volumes corresponding to apparent molecular weights of about 110 000 and 55 000, respectively (Figure 6). Fraction IIIc is probably identical with fraction IV (see below). The gel filtration pattern of fraction III also exhibited a third, usually small, peak of phosphodiesterase activity (IIIa). Irregularly, the amount of phosphodiesterase activity in fraction IIIa could exceed that of fractions IIIb and IIIc. The activity of fraction IIIa eluted at a volume corresponding to an apparent molecular weight of 220 000. The K_m value for fraction IIIa was determined to be 0.4–0.5 μM when substrate concentrations below 0.5 μM cAMP were employed. At higher substrate concentrations, nonlinear double-reciprocal plots were encountered. A purified aliquot of fraction IIIa was rechromatographed on Sephadex G-200 in buffer B. Part of the activity now eluted at a position corresponding to an apparent molecular weight of 110 000. Similar results were obtained when gel filtration of fraction IIIa was performed with buffer B made 0.6 M in NaCl, with buffer B depleted of Mg^{2+} but made 1 mM in EDTA, or with buffer B adjusted to pH 6. None of the last mentioned three eluents

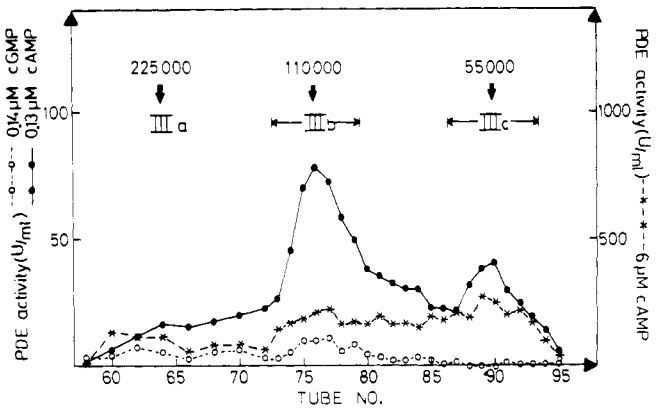

FIGURE 6: Gel filtration of a partially purified preparation of fraction III on Sephadex G-150 superfine (3.2 × 90 cm) in buffer B. Apparent molecular weights are indicated. Flow rate was 6 ml/h. Fractions were collected each hour.

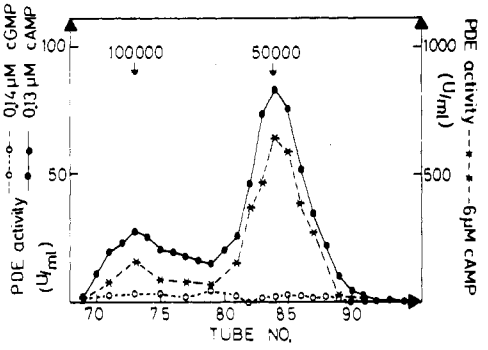

FIGURE 7: Gel filtration of fraction IV on Sephadex G-150 superfine (3.2 × 90 cm) in buffer B. Apparent molecular weights are indicated. Flow rate was approximately 6 ml/h. Fractions were collected each hour.

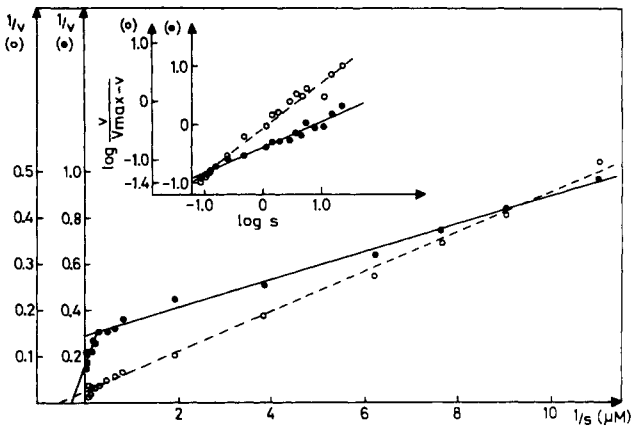

FIGURE 8: Lineweaver-Burk plots of kinetic data with purified fractions IIIb and IV. Each point represents the mean of four (fraction IIIb) or two (fraction IV) determinations. K_m and V_{max} calculated according to Wilkinson (1961) with substrate concentrations below 0.5 µM: 0.16 µM and 3 nmol/min × mg (fraction IIIb); 2.3 µM and 50 nmol/min × mg (fraction IV). Insert: Hill plot of corresponding data. Hill coefficients: 0.43 ± 0.02 (fraction IIIb); 0.75 ± 0.03 (fraction IV). Filled symbols represent results with fraction IIIb; open ones are results with fraction IV.

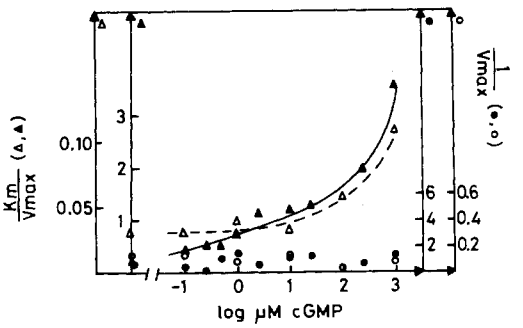

FIGURE 9: Effect of different concentrations of cGMP on the kinetic parameters of the cAMP phosphodiesterase activity of fractions IIIb and IV. Substrate concentrations ranged from 0.52 to 0.09 µM (six different concentrations). Concentration of cGMP plotted on a logarithmic scale. Points on y axis represent results obtained in absence of cGMP. Filled symbols represent results with fraction IIIb; open ones are results with fraction IV.

seemed to significantly change the activity pattern from that obtained at gel filtration with buffer B alone.

The phosphodiesterase activity pattern at gel filtration of fractions IIIb and IIIc with buffer B made 1 mM in adenosine, with buffer B made 1M in urea, or with buffer B made 0.1 mM in either of the two cyclic nucleotides was similar to that obtained with buffer B alone. Mixing aliquots of purified fractions IIIb and IIIc, respectively, with equal parts of a boiled lung tissue supernatant or with the protein-rich fraction of the lung tissue supernatant that elutes ahead of the phosphodiesterase activity at DEAE-Sephadex chromatography did not change their elution characteristics.

The activity of fraction IIIb could be purified to 20–30 times increased specific activity, but the yield after extensive purification was low. The K_m value was determined to be 0.37 ± 0.09 µM (mean of six experiments ± SEM) when assayed at substrate concentrations below 0.5 µM; at higher substrate concentrations, nonlinear double-reciprocal plots were obtained (Figure 8). The Hill coefficient was calculated to be 0.43 (Figure 8). cGMP inhibited the activity in a nonlinear manner (Figure 9), suggesting a dualistic mode of inhibitory action. At 0.1 µM cGMP, the initial velocity at a substrate concentration of 0.13 µM cAMP was reduced to half that observed in the absence of cGMP. Dixon plots of data suggested a competitive type of inhibition either if low concentrations of cGMP were examined (estimated K_i = 0.5–0.1 µM) or if high concentrations were employed (estimated K_i = 300 µM). However, fraction IIIb displayed only small amounts of phosphodiesterases activity when cGMP was utilized as a substrate (see Figure 6). The cAMP phosphodiesterase activity of this fraction was unaffected by the presence of a boiled lung tissue supernatant.

Fraction IV was purified by Sephadex G-150 gel filtration (Figure 7). The specific activity of purified preparations was usually increased 10–20 times in relation to that of the crude supernatant. Efforts at extended purification were connected with heavy losses of activity. The K_m value was estimated to be 1–2 µM; nonlinear kinetics were less apparent than for fraction IIIb (Figure 8). cGMP at 0.1, 1, or 10 µM concentrations did not influence the kinetic parameters of the cAMP phosphodiesterase activity. At higher concentrations (100–1000 µM), cGMP acted as a competitive inhibitor according to the Dixon plot; the K_i value was in the order of 500 µM. Addition of a boiled lung tissue supernatant did not affect activity. The cGMP phosphodiesterase activity of fraction IV was usually very low (see Figure 7).

Thermal Inactivation of Phosphodiesterase Activity. Samples of purified fractions I, II, IIIb, and IV were incubated at 30 or 37 °C. Aliquots of the fractions were removed after a specified time of incubation and immediately assayed for phosphodiesterase activity.

Table I: Schematic Summary of Some Properties of the Phosphodiesterase Fractions Purified from Human Lung Tissue.

	Fraction Designation			
	I	II	IIIb	IV
Substrate	cGMP	cGMP cAMP	cAMP	cAMP
Approx K_m (μM)	~0.3	~25 ~25	~0.35	~1-2
Effect of cyclic nucleotide not utilized as substrate	Slight noncomp (?) inhib by cAMP	Comp inhib; App K_i similar to K_m above	Clearcut inhib even at low concn of cGMP	Significant inhib only at high concn of cGMP
Evidence for cooperativity	None	Slightly downward concave Lineweaver-Burk plot at low concn of cGMP; Hill coeff, 1.00	Nonlinear Lineweaver-Burk plot; Hill coeff, 0.43	Slightly nonlinear Lineweaver-Burk plot; Hill coeff, 0.78
Thermal inactivation (37 °C)	Very slight	Very slight	Profound	Profound
App mol wt	240 000	260 000	110 000	55 000
Influence of boiled supernatant and Ca^{2+}	None	None	None	None

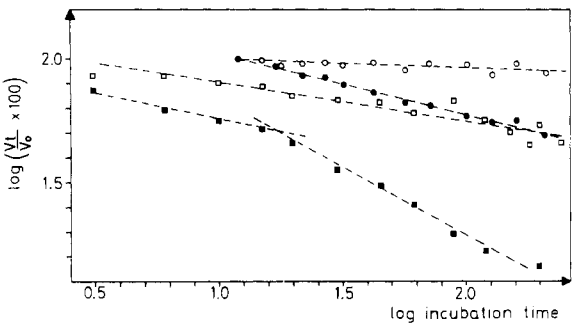

FIGURE 10: Thermal inactivation of the phosphodiesterase activity of purified fractions. A sample of the purified fraction was incubated at 30 or 37 °C. Aliquots were removed after specified times of incubation and immediately assayed for phosphodiesterase activity at 30 °C (reaction time: 12 min). Activity after a specified time of incubation is expressed as percentage of the initial activity. The figure shows the plot of log activity vs. log time of incubation at 37 °C for fraction IIIb (substrate: 0.13 μM cAMP; filled squares) and fraction II (substrate: 6 μM cAMP; open squares) and similar plots for activity decay at 30 °C with fraction IIIb (incubation performed at pH 6, filled circles) and fraction II (open circles). Incubation times at 30 °C include time of assay of activity. However, no effort was made to correct data obtained at 37 °C for inactivation occurring during the following assay of activity at 30 °C.

At 30 °C, i.e., the temperature chosen for assay of the phosphodiesterase activity, prolonged incubation of fractions IIIb and IV resulted in partial losses of activity. Plots of activity or log activity against time resulted in upward concave curves. However, when log activity was plotted against log time of incubation, a linear relation seemed to exist (see Figure 10). The activity of fraction II (substrate: 6 μM cAMP) seemed to decay less pronouncedly but according to a similar linear relation (see Figure 10). At 37 °C, thermal inactivation of fraction I (substrate: 0.14 μM cGMP) and fraction II (substrate: 6 μM cAMP (see Figure 10) or 7 μM cGMP) also seemed to follow a linear double logarithmic relation, revealing a low degree of thermal decay. However, inactivation of fractions IIIb (Figure 10) and IV was nonlinear according to this plot. At short incubation times, the rates of inactivation seemed to correspond to those observed at 30 °C. Inactivation proceeded more rapidly after extended incubation at 37 °C. The kinetic parameters of purified fraction IV were examined before and after thermal inactivation at 37 °C for 90 min. The results (K_m = 5.4 μM before treatment and 3.3 μM after treatment; V_{max} = 2.34 nmol/min × mg before treatment and 0.52 nmol/min × mg after treatment) indicate a major effect of heat treatment on the V_{max} value.

Influence of EGTA and a Boiled Lung Tissue Supernatant on the Phosphodiesterase Activity of a Crude Supernatant. EGTA (final concentration 1 mM) did not affect the phosphodiesterase activity of a 200-fold diluted crude supernatant (substrate concentrations: 6 and 0.13 μM cAMP, and 7 and 0.14 μM cGMP). Exclusion of bovine serum albumin from the buffer used for dilution of enzyme and assay of activity decreased the activity toward cGMP, but EGTA produced no further inhibition. Addition of a boiled, centrifuged supernatant prepared either from human lung or from rat brain tissue was also without effect in the presence of bovine serum albumin. In its absence, a boiled supernatant increased cGMP hydrolytic activity to the level observed with bovine serum albumin. Similar results were obtained when a crude supernatant prepared from guinea-pig lung immediately after removal of the tissue was employed as an enzyme source. With a crude supernatant analogously prepared from rat brain, EGTA (1 mM) reduced the basal activity assayed at 7 μM cGMP threefold, whereas the addition of a boiled brain supernatant increased it threefold (i.e., to nine times the activity obtained in the presence of EGTA). Inclusion of bovine serum albumin in the buffer system did not influence these effects, although activity in each case was higher in the presence of bovine serum albumin than in its absence.

Table I gives a schematic summary of some properties of each of the purified phosphodiesterases.

Discussion

The present report on fractionation by ion-exchange chromatography and gel filtration of cyclic nucleotide phosphodiesterases from crude human lung tissue reveals the presence of at least three different enzyme forms. This pattern of multiple phosphodiesterase activities recalls those previously described for bovine liver and rat heart by Rus-

sell et al. (1973), Schröder and Rickenberg (1973), and Terasaki and Appleman (1975).

One enzyme (DEAE-Sephadex fraction I) seems to be specific for cGMP. The small amounts of cAMP phosphodiesterase activity in this fraction could be caused by contamination, as the cGMP phosphodiesterase activity is unaffected by low concentrations of cAMP. The reason why high concentrations of cAMP affect only V_{max} but not the apparent affinity for cGMP is not clear. Specific as well as trivial explanations are plausible. cGMP phosphodiesterases with characteristics similar to that of fraction I have previously been described by others: e.g., Russell et al. (1973), Schröder and Rickenberg (1973), Thompson et al. (1973), (liver); Amer and Mayol (1973), Hidaka et al. (1974), Asano and Hidaka (1975), Patterson et al. (1975) (blood platelets or sera); Marks and Raab (1974) (epidermis); and Russell and Pastan (1974) (chicken fibroblasts).

The second main form of phosphodiesterase activity purified from lung tissue supernatants (DEAE-Sephadex fraction II) hydrolyses both cyclic nucleotides with roughly similar kinetic characteristics, indicating that one single enzyme is responsible for both cAMP and cGMP hydrolysis. The apparent molecular weight of this phosphodiesterase is approximately 260 000. Similar phosphodiesterase enzymes have been described for other tissues, e.g., Menahan et al. (1969), Thompson and Appleman (1971b), Hrapchak and Rasmussen (1972), Klotz et al. (1972), Bevers et al. (1974), and more recently, Terasaki and Appleman (1975). The kinetic characteristics of these enzymes seem to be complex. An upward concave Lineweaver-Burk plot for the hydrolysis of cAMP or cGMP, suggesting positive cooperativity, has been reported (Russell et al., 1973; Bevers et al., 1974; Sakai et al., 1974; Terasaki and Appleman, 1975). However, we found the Hill plot for hydrolysis of cGMP to be normal with purified fraction II. Stimulation of the hydrolytic activity toward cAMP by low concentrations of cGMP (cf. Beavo et al., 1971; Franks and MacManus, 1971; Klotz and Stock, 1972; Russell et al., 1973; Sakai et al., 1974; Terasaki and Appleman, 1975) and noncompetitive inhibition of it by higher concentrations of cGMP have been observed with crude enzyme or with purified low affinity phosphodiesterases (Klotz and Stock, 1972; Russell et al., 1973; Sakai et al., 1974). None of these findings were recorded with the phosphodiesterase activity of fraction II under the present circumstances. Inhibition of cAMP hydrolysis at higher concentrations of cGMP was observed, but it was of competitive nature, as was also reported by Terasaki and Appleman (1975).

A Ca^{2+}-dependent protein activator augments part of the phosphodiesterase activity in a number of tissues, especially brain (cf. Cheung, 1971; Goren and Rosen, 1972; Lagarde and Colobert, 1972; Miki and Yoshida, 1972; Kakiuchi et al., 1973, 1975; Teo et al., 1973; Teo and Wang, 1973; Brostrom and Wolff, 1974; Wells et al., 1975). In the absence of bovine serum albumin, apparent "activation" of diluted lung tissue supernatants or purified phosphodiesterases occurs at the addition of boiled supernatants. However, we consider this effect nonspecific because: (i) under these circumstances, the initial reaction velocity is not linear with enzyme concentration; (ii) EGTA does not influence this activation; (iii) the activation mechanism demonstrated in control experiments with rat brain tissue occurred independent of the presence of bovine serum albumin. We have tried without success to demonstrate the existence of a Ca^{2+}-dependent type of activation mechanism for the purified lung tissue cGMP phosphodiesterases. There are several possible explanations for this failure. First, lung tissue could lack the pertinent enzyme form. Phosphodiesterases from other tissues have previously been reported to lack a Ca^{2+}-dependent activation mechanism (Amer and Mayol, 1973; cf. Asano and Hidaka, 1975; Chader et al., 1974; Sheppard and Tsien, 1974; Rutten et al., 1973; Lemon and Bhoola, 1975: Boudreau and Drummond, 1975b). Secondly, the activity of the responsive enzyme could have been destroyed or blocked during preparation (Kakiuchi et al., 1973, 1975; Lin et al., 1974). This explanation is unlikely, as we failed to demonstrate significant influence of the phosphodiesterase activity of a freshly prepared guinea-pig lung tissue supernatant treated with either EGTA or a boiled brain supernatant (active with brain enzyme). Thirdly, the activator could be so firmly bound to the lung enzyme that EGTA treatment at 10^{-3} M is insufficient for dissociation to occur (cf. Teo et al., 1973; Cheung and Lin, 1974; Pledger et al., 1974, 1975; Kakiuchi et al., 1975). The apparent molecular weight and the kinetic properties reported for the activator modulated enzyme (Brostrom and Wolff, 1974; Teshima and Kakiuchi, 1974; Kakiuchi et al., 1975; Lin et al., 1975) recall those we have observed for the irregularly occurring minor component of DEAE-Sephadex fraction I. However, even this fraction, when purified, was unaffected by EGTA and a heated supernatant. The demonstration of a phosphodiesterase that was not affected by Ca^{2+} in rat liver and heart (Kakiuchi et al., 1975) and the suggestion that rat lung tissue (Campbell and Oliver, 1972) and bovine brain (Kakiuchi et al., 1971) contain at least two phosphodiesterases with comparable activity toward cAMP [the major enzyme form of lung apparently being the minor one of the brain (Campbell and Oliver, 1972)] agree with our interpretation that a Ca^{2+}-dependent phosphodiesterase does not play a major role in lung tissue (see also Hitchcook, 1973).

DEAE-Sephadex fractions IIIb and IV displayed a high apparent affinity and specificity for cAMP. The apparent molecular weight of these enzyme forms suggests that they are closely related to each other. Moreover, both fractions showed a pronounced thermal sensitivity in accordance with properties previously described for the high affinity part of the phosphodiesterase activity (cf. Jard and Bernard, 1970; Goren and Rosen, 1972; Bevers et al., 1974). The activity of fraction IIIb was found to follow nonlinear kinetics. This property could be due to negative cooperativity regulation, although lack of demonstration of enzymatic homogeneity precludes that conclusion (cf. Russell et al., 1972). Whether the complex inhibitory action of cGMP on the activity of fraction IIIb depends on interference with such an allosteric regulation mechanism remains to be established.

Thus there seems to be no doubt that the activity of fractions IIIb and IV, in one way or another, corresponds to the high affinity, probably membrane-associated, enzyme previously described in other tissues by a number of workers (for references, see Appleman et al., 1973; Amer and Krieghbaum, 1975). This enzyme apparently exists in several forms, which at gel filtration elute in positions corresponding to apparent molecular weights of some 400 000 (Klotz et al., 1972; Russell et al., 1973), 200 000–250 000 (Thompson and Appleman, 1971a,b; Schröder and Rickenberg, 1973), 120 000 (Schröder and Rickenberg, 1973) or less than 100 000 (Jard and Bernard, 1970; Kakiuchi et al., 1975; Pichard and Kaplan, 1975; and Tisdale, 1975). However, discrepancies seem to exist regarding the issue of re-

versible interconversion between these forms. The use of DEAE-Sephadex purified preparations for examination could be significant in this respect (cf. Thompson and Appleman, 1971b; Amer and McKinney, 1972; Russell et al., 1973). This is supported by our observation (see also Schröder and Rickenberg, 1973) that gel filtration of a crude supernatant reveals the majority of high affinity cAMP phosphodiesterase at an elution volume corresponding to a high molecular weight. We therefore suspect that fractions IIIb and IV are fragments of the naturally occurring high affinity cAMP phosphodiesterase. In what way could these fragments then have been derived? The work of Russell and Pastan (1973, 1974) and Russell et al. (1973) indicates that limited proteolytic modification of the high affinity cAMP phosphodiesterase could influence both its kinetic and physicochemical characteristics. The altered gel filtration behavior of a brain cAMP phosphodiesterase after trypsin treatment, described by Cheung and Lin (1974), and the present demonstration that incubation of a crude supernatant for only 30 min at pH 6 and room temperature drastically changes the elution pattern of the phosphodiesterase activity agree with that interpretation. To clarify this question, we are currently examining the effect of limited proteolytic degradation of purified phosphodiesterase in our laboratory.

Acknowledgment

The authors are grateful to Dr. T. Nilsson for valuable discussions and support and to Dr. J. Kristoffersson for expert help with computer programming.

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The Chemical Modification of Papain with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide†

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ABSTRACT: The reaction of the water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), with active papain in the presence of the nucleophile ethyl glycinate results in an irreversible inactivation of the enzyme. This inactivation is accompanied by the derivatization of the catalytically essential thiol group of the enzyme (Cys-25) and by the modification of 6 out of 14 of papain's carboxyl groups and up to 9 out of 19 of the enzyme's tyrosyl residues. No apparent irreversible modification of histidine residues is observed. Mercuripapain is also irreversibly inactivated by EDC/ethyl glycinate, again with the concomitant modification of 6 carboxyl groups, up to 10 tyrosyl residues, and no histidine residues; but in this case there is no thiol derivatization. Treatment of either modified native papain or modified mercuripapain with hydroxylamine results in the complete regeneration of free tyrosyl residues but does not restore any activity. The competitive inhibitor benzamidoacetonitrile substantially protects native papain against inactivation and against the derivatization of the essential thiol group as well as 2 of the 6 otherwise accessible carboxyl groups. The inhibitor has no effect upon tyrosyl modification. These findings are discussed in the context of a possible catalytic role for a carboxyl group in the active site of papain.

A recent comprehensive review (Glazer and Smith, 1971) of the literature concerning the structure and function of the sulfhydryl proteinase papain (EC 3.4.22.2) cites extensive evidence for the following generally accepted features of the mechanism of papain-catalyzed hydrolyses of amides or esters of α-*N*-acyl-L-amino acids. After an initial reversible substrate-binding step, the carbonyl group of the scissile amide or ester linkage of the substrate undergoes a nucleophilic attack by the thiol group of Cys-25 in the papain active site. This leads to the displacement of the amine or alcohol leaving group from the amide or ester substrate and the concomitant formation of a thiol ester, the so-called acyl-enzyme intermediate. Hydrolysis of this intermediate then occurs, completing the acylation–deacylation cycle of catalysis. The pH dependence of the acylation step rate constant is generally attributed to two catalytically essential functional groups in the papain active site with apparent pK_a's of about 4 and about 8. The latter pK_a is most readily assigned to the aforementioned thiol group of Cys-25, and the former pK_a is usually attributed to a group which functions as a general base (Brubacher and Bender, 1966) in the acylation step.

Opinion varies as to the identity of this putative general base. Early speculation (Smith and Kimmel, 1960; Whitaker and Bender, 1965) centered on a carboxyl group, a reasonable assignment based upon the normally expected pK_a values of aspartate or glutamate residues in proteins (Tanford and Hauenstein, 1956) and supported by the finding (Smith and Parker, 1958) that a low apparent heat of ionization, typical of carboxylic acid dissociation, is associated with the acidic limb of the pH–rate profile for papain catalysis. On the other hand, much recent speculation (see, for example, Lowe and Whitworth, 1974) has focused upon the imidazole group of His-159 in the active site of papain, an assignment first suggested by Lowe and Williams (1965) on the basis of chemical modification studies. X-ray crystallography (Drenth et al., 1968, 1971) tends to support the proponents of the imidazole group, showing that at least in the crystal the imidazole moiety of His-159 is in fact immediately adjacent to the thiol group of Cys-25 in the papain active site, whereas the only free carboxyl group of the active site (Asp-158) is several angstroms farther away in a position which makes direct interaction with the thiol group difficult to visualize. Be that as it may, recent evidence has

† From the Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061. *Received January 20, 1975.* This work was supported by grants from the National Science Foundation (GB 38238 and BMS-74-13750). A preliminary account of some of this work was presented at the 168th National Meeting of the American Chemical Society, Atlantic City, New Jersey, September 1974.