Welgus, H. G., Campbell, E. J., Bar-Shavit, Z., Senior, R. M., & Teitelbaum, S. L. (1985a) *J. Clin. Invest. 76*, 219–224.
Welgus, H. G., Connolly, N. L., & Senior, R. M. (1985b) *Clin. Res. 33*, 825A.
Welgus, H. G., Connolly, N. L., & Senior, R. M. (1986) *J. Clin. Invest. 77*, 1675–1681.
Werb, Z., & Gordon, S. (1975) *J. Exp. Med. 142*, 346–360.
Werb, Z., & Reynolds, J. J. (1975) *Biochem. J. 151*, 645–653.

Use of Lectin Affinity Chromatography for the Purification of Collagenase from Human Polymorphonuclear Leukocytes

James E. Callaway, Joseph A. Garcia, Jr., Carol Lipsey Hersh, Raymond K. Yeh, and Maureen Gilmore-Hebert*
International Genetic Engineering, Inc., Santa Monica, California 90404
Received December 26, 1985; Revised Manuscript Received April 14, 1986

ABSTRACT: Polymorphonuclear leukocytes (PMNLs) store collagenase in an inactive form in secretory granules. The enzyme can be activated in vitro by limited proteolysis or by sulfhydryl-modifying agents such as *N*-ethylmaleimide (NEM). We have enriched NEM-activated collagenase 820-fold using granule isolation, gel filtration, and wheat germ agglutinin (WGA)–agarose chromatography. The use of WGA–agarose resulted in a 55-fold enrichment of collagenase in a single step with very little loss of activity. The chromatographic behavior of collagenase on other lectin matrices was explored and gave information about the type of complex asparagine-linked oligosaccharide found on collagenase isolated from PMNLs.

Mammalian collagenase (EC 3.4.24.7) cleaves native triple-helical collagen (types I, II, and III) between residues 772 and 773 to produce $^3/_4$- and $^1/_4$-length fragments. This cleavage site in type I collagen is a Gly–Ile bond in the α-1 chain and a Gly–Leu bond in the α-2 chain [for review, see Harper (1980)]. The triple-helical conformation of the cleavage fragments is unstable at physiological temperatures and upon unfolding becomes susceptible to general proteolysis. This implies that the initial cleavage of collagen by collagenase is the rate-limiting step in collagen degradation.

Collagenases are metalloenzymes that require both zinc and calcium for enzymatic activity. Mammalian collagenases capable of cleaving collagen types I, II, and III have been isolated from human fibroblasts (Stricklin et al., 1977, 1978; Wilhelm et al., 1984), rat uterus (Roswit et al., 1983), rabbit synovial fibroblast cells (Vater et al., 1981), and mouse bone cultures (Sakamoto et al., 1978). The collagenases from these sources have apparent molecular masses ranging from 45 to 65 kDa[1] and are synthesized in an inactive form. The process of activation of the procollagenase zymogen and the presence of both specific and nonspecific collagenase inhibitors results in precise regulation of enzyme activity (Cawston et al., 1983; Macartney & Tschesche, 1983b,c; Stricklin & Welgus, 1983; Stricklin et al., 1983; Vater et al., 1983; Welgus & Stricklin, 1983).

Human polymorphonuclear leukocytes (PMNLs, or neutrophils)[1] store collagenase in secretory granules as an enzymatically inactive molecule (Lazarus et al., 1968; Murphy et al., 1982; Macartney & Tschesche, 1983a; Hasty et al., 1984). This enzyme can be activated by mild proteolytic digestion or by compounds that modify sulfhydryl residues such as NEM (Murphy et al., 1980; Macartney & Tschesche, 1983a; Hasty et al., 1984; Weiss et al., 1985). Attempts have been made to isolate this enzyme from PMNLs by using conventional chromatographic procedures (Uitto et al., 1980; Christner et al., 1982; Murphy et al., 1982; Macartney & Tschesche, 1983a). These preparations had relatively low specific activities when compared to collagenases purified from other sources (Harris & Vater, 1982). This report describes the use of lectin chromatography to rapidly achieve a high degree of purification of the enzyme and provide information about its carbohydrate structure.

Experimental Procedures

Materials

All radiochemicals and related material were obtained from New England Nuclear. The protein molecular weight standards and protein staining kit were purchased from Bio-Rad. Miracloth was from Calbiochem, and Cytodex beads were from Pharmacia. The SDS was from Bethesda Research Laboratories, and all other reagents were purchased from Sigma Chemical Co.

Methods

Except for the isolation of PMNLs from the cellular components in blood and the collagenase assay, all procedures were performed at 4 °C. The pH of all buffers was adjusted while the solution was at 4 °C.

Isolation of Polymorphonuclear Leukocytes (PMNLs). Leukophoresis was performed on 2 units of human blood (from Hemacare, Van Nuys, CA) to enrich for white blood cells (recovery was about 10^{10} cells). Each of these leukophoresis concentrates was subsequently subjected to discontinuous density gradient centrifugation on Ficoll-Hypaque to isolate the PMNLs (English & Anderson, 1974).

[1] Abbreviations: Brij-35, poly(oxyethylene) 23-lauryl ether; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; E-PHA, erythroagglutinating phytohemagglutinin; kDa, kilodalton; L-PHA, leukoagglutinating phytohemagglutinin; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PMNL, polymorphonuclear leukocyte; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/86/0425-4757$01.50/0

Granule Preparations. The frozen PMNLs were pulverized by using a mortar and pestle and allowed to thaw. Sucrose buffer (0.34 M sucrose, 50 mM Tris-HCl, pH 7.5) was added at a ratio of 50 mL per leukophoresis concentrate, and the cells were lysed by nitrogen cavitation at 750 psi for 30 min followed by a release of pressure over a 15-min period. The lysate was centrifuged at 1000*g* for 10 min to remove nuclei and unbroken cells, and the supernatant was filtered through Miracloth to remove lipids. The nuclear pellet was resuspended in 25 mL of sucrose buffer per leukophoresis concentrate and centrifuged at 1000*g* for 10 min; this was repeated twice. The three postnuclear supernatants were combined and centrifuged at 25000*g* for 20 min. This pellet containing granules was suspended in 10 mL of granule lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 15 mM $CaCl_2$, 0.1% Triton X-100, 2 mM PMSF, and 2 mM NEM) per leukophoresis concentrate, and the granules were lysed by using a type B dounce homogenizer (10 strokes). The lysate was centrifuged at 27000*g* for 10 min to remove debris and unbroken granules. The supernatant was saved, and the pellet was washed twice more with the same buffer. The granule supernatants were combined and clarified before column chromatography. This material was incubated for at least 1 h at 4 °C before chromatography was initiated. Collagenase activated by this method was determined to be fully activated because (a) no increase in collagenase activity was observed when NEM was added to assay mixtures containing this material and (b) a time course of the activation process showed that activation was completed in 1 h under these conditions. For isolation of latent collagenase, NEM was not added to the lysis buffer when the granules were lysed.

Activation. To compare methods of collagenase activation, 2 g of frozen cell pellet were suspended in 1 mL of granule lysis buffer and were lysed by using a type B Dounce homogenizer. This procedure was done either in the presence of 2 mM PMSF or 2 mM PMSF and 2 mM NEM or in the absence of both reagents (in the latter case, the lysate was taken through five cycles of freezing and thawing). The insoluble material was removed by centrifugation, and 5 μL of each lysate was assayed (without addition of NEM to the assay mixture) for 17 h at room temperature.

Column Chromatography. The lysate from PMNL granules isolated from five leukophoresis concentrates was activated with NEM and concentrated to 10 mL by using an Amicon YM-30 filter. This sample was dialyzed against the column buffer (1 M NaCl and 50 mM Tris-HCl, pH 7.8) overnight at 4 °C, adjusted to 20% glycerol, and applied to a Sephacryl S-200 column (90 × 4.5 cm) at a flow rate of 45 mL/h.

Wheat germ agglutinin–agarose (coupled at a concentration of approximately 10 mg of protein/mL of resin) was packed into a 12-mL column (7 × 1.5 cm) in 500 mM NaCl, 20 mM Tris-HCl, pH 7.8, and 0.05% Brij-35. The active fractions collected from the S-200 column (from 690 to 780 mL) were pooled, concentrated to 5.4 mL by using an Amicon YM-30 filter, and dialyzed against the column buffer for 24 h at 4 °C. The sample was clarified and applied to the column at a flow rate of 12.5 mL/h. After 200 mL of buffer had passed through the column, 0.25 M *N*-acetylglucosamine was added to the column buffer to remove tightly bound protein.

For analytical chromatography of collagenase on various lectin matrices, columns were poured in 1-mL plastic pipets. The bed volume of these columns was approximately 1 mL, and the flow rates were 2 mL/h. The volume of sample applied ranged from 200 to 400 μL, and 1-mL fractions were collected.

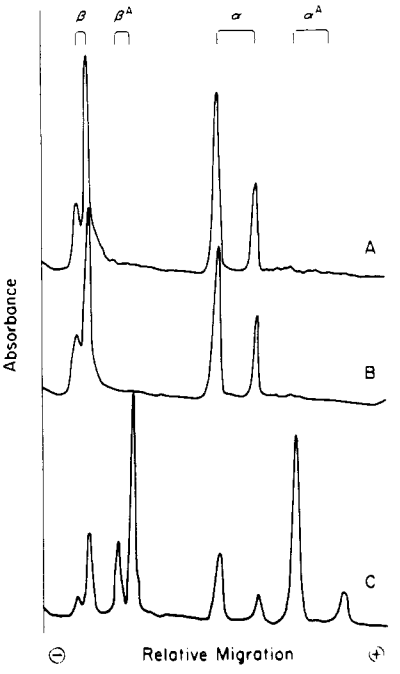

FIGURE 1: Densitometer tracings of a fluorogram of an SDS–PAGE of collagenase assay mixtures. Cells were lysed in the presence of PMSF (A), the absence of PMSF (B), or the presence of PMSF and NEM (C). Equal aliquots of the lysates were incubated with type I [^{3}H]collagen for 17 h at room temperature in the absence of NEM. Cleavage products of the type I polypeptides (α and β) are designated (α^A and β^A). See Methods for details.

Assay of Collagenase Activity. Collagenase activity was determined with 0.5–0.6 μg (0.3 μCi) of [^{3}H]collagen in each assay sample. Assay buffer (50 mM Tris-HCl, pH 7.8, and 1 mM $CaCl_2$) was added (50–90 μL) to bring the final reaction volume to 100 μL. When we worked with latent collagenase, 2 μL of 100 mM NEM was added to the assay mixture. The final concentration of collagen in these assays was 20 nM. The incubation was performed at room temperature for 1–3 h (the assay is linear over this time span), unless another incubation time is stated. The reactions were terminated by the addition of 5 μL of 500 mM EDTA (pH 7.5); the samples were then lyophilized and prepared for SDS–PAGE. Following electrophoresis, the gels were treated with ENHANCE for fluorography. The gels were exposed to X-ray film for 16–18 h. Calculations of specific activity were based on reactions that resulted in the cleavage of 40–60% of the substrate, and all quantitations of activity were based on integrated densitometer scans.

SDS–PAGE. Samples for electrophoresis were prepared by the method of Laemmli (1970) and run on a 10% acrylamide–1.3% bis(acrylamide) gel (Maniatis et al., 1982) at constant voltage. Protein species were detected by the Bio-Rad silver-staining procedure.

RESULTS

Activation. To compare the activation of PMNL collagenase by NEM or endogenous proteases, equal amounts of frozen cells were lysed in the presence of NEM and PMSF, PMSF, or no protease inhibitors. PMSF was used as the negative control because it was found to be an effective inhibitor of the endogenous proteases found in the cell lysate. In the presence of PMSF and the absence of NEM (Figure 1A), only the uncleaved α and β polypeptides of type I collagen were detected. In contrast, when cells were lysed in the presence of both PMSF and NEM, 70% of the α and β polypeptides were converted to the classical $^3/_4$- and $^1/_4$-length

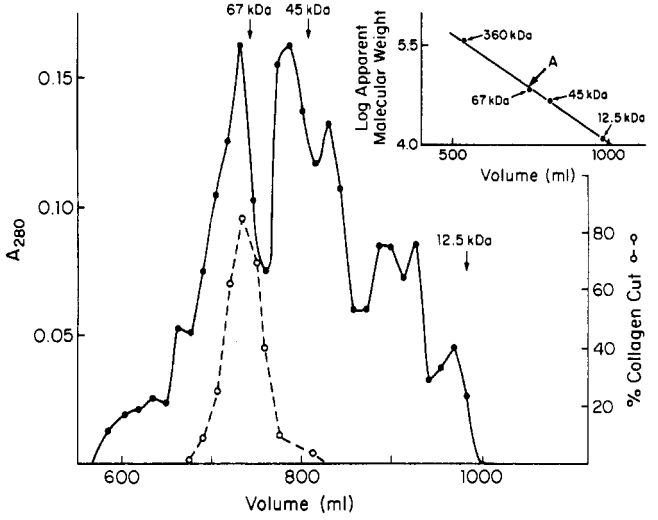

FIGURE 2: Sephacryl S-200 elution profile of granule lysate prepared from human PMNLs. The absorbance (●) and activity (○) profiles are from the chromatography of five leukophoresis concentrates following granule isolation and activation with NEM. The protein standards [phosphorylase *a* (360 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), and cytochrome (12.5 kDa)] were chromatographed prior to the addition of the crude collagenase material. See Methods for details.

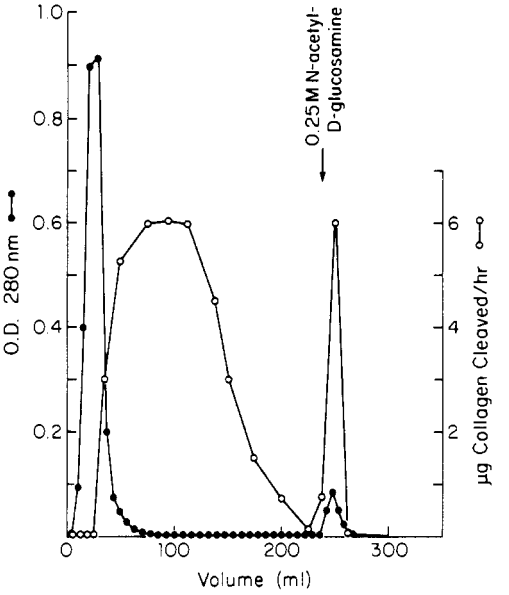

FIGURE 3: Wheat germ agglutinin–agarose elution profile of the active fractions collected from S-200 chromatography. The absorbance (●) and collagenase activity (○) were determined for each 16-mL fraction. The material applied had been activated by incubation with NEM. The bold arrow indicates the appearance of sugar eluant in the collected fractions as detected by a change in the index of refraction. See Methods for details.

cleavage products characteristic of mammalian collagenase (Figure 1C). When PMSF and NEM were omitted from the granule lysate and endogenous proteases were allowed to act, no cleavage was detected (Figure 1B). When a larger aliquot was assayed, a small amount of collagenase activity was detected, but it represented only 2% of the activity found in the NEM-activated fractions.

S-200 Chromatography. Granules isolated from human PMNLs were lysed, and the released soluble proteins were chromatographed on a Sephacryl S-200 column (Figure 2). When NEM-activated collagenase was chromatographed, activity was detected as a peak which eluted with an apparent molecular mass in the region of 65–75 kDa (see inset). Analysis of latent collagenase resulted in a slightly more rapid elution, with an apparent molecular mass larger by approximately 10 kDa (data not shown). Activated material was chromatographed 4 separate times with reproducible elution profiles. The activated collagenase which eluted between 690 and 780 mL was concentrated and dialyzed prior to lectin chromatography.

Wheat Germ Agglutinin–Agarose Chromatography. Activated collagenase was chromatographed on WGA–agarose following Sephacryl S-200 chromatography. The absorbance profile (Figure 3) shows that the majority of the protein did not interact with the lectin matrix, while the collagenase activity was retarded significantly. Greater than 70% of the activity was eluted with column buffer prior to the addition of *N*-acetylglucosamine. This displaced elution profile has been observed for several well-characterized glycopeptides chromatographed on WGA columns (Yamamato et al., 1981) and is indicative of a specific lectin–carbohydrate interaction. Because collagenase activity was not bound tightly but was displaced relative to unbound protein, a small volume of material was applied relative to the total column volume. The resolution of collagenase activity from unbound protein was optimal when the column volume was at least twice the volume of the sample applied. Virtually all the collagenase activity applied to the lectin column was recovered, but for the purification, only the activity clearly resolved from unbound protein was collected (fractions with $A_{280} < 0.02$). The fractions that were contaminated with the trailing edge of the unbound material could be rechromatographed on WGA–agarose to recover collagenase activity of comparable purity to the material that was fully resolved in the first pass. Latent collagenase had an identical behavior on WGA–agarose (data not shown) to that of activated collagenase. This material did not become activated upon chromatography but could be activated by NEM to a similar extent as the unfractionated material.

Table I: Enrichment of NEM-Activated PMNL Collagenase

	protein[a] (mg)	units[b]	sp act.	*x*-fold purification	yield (%)
whole cell lysate	3100	33000	10.6		
granule lysate	50	6300	126	12	19
S-200	10	1600	160	15	5
WGA-agarose	0.115[c]	1000	8700	820	3

[a] Protein levels determined by Bio-Rad protein assay (Bradford, 1976). [b] Units defined as micrograms of collagen cleaved per hour of incubation at 22 °C in 50 mM Tris-HCl and 1 mM $CaCl_2$, pH 7.8. [c] Protein level determined by amino acid analysis.

Purification. The results of the purification of activated collagenase are presented in Table I. The initial step involves the isolation of the collagenase-containing granules by differential sedimentation. This removes most of the soluble proteins and results in a 12-fold purification. The recovery of activity is calculated to be 19%, with the remainder of the activity being released into the soluble fraction due to premature granule lysis. The collagenase activity in the soluble fraction was rapidly lost upon storage, probably due to nonspecific proteolysis. Analysis of the proteins present in the granule lysate (Figure 4, lanes A and B) shows that the major soluble proteins were removed from the collagenase-containing granules. Gel exclusion resulted in very little change in the protein composition of the active material (Figure 4, lanes B and C), but the material was much less turbid and viscous upon concentration. This resulted in more reproducible chromatography on WGA–agarose than was obtained with unfrac-

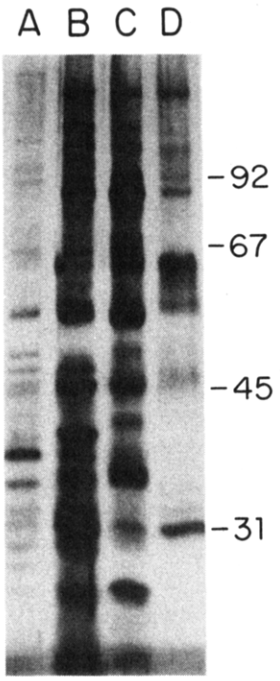

FIGURE 4: SDS–polyacrylamide gel of samples from each stage of the purification of NEM-activated collagenase from human PMNLs. Lane A represents the soluble proteins removed by preparation of the granules. The proteins released upon lysis of the granules are presented in lane B. The proteins in the pool of fractions containing collagenase activity following S-200 chromatography of activated collagenase is presented in lane C, and the proteins in the pool of active fractions from WGA–agarose chromatography are shown in lane D. The molecular weight markers are phosphorylase *a* (92 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa). The protein bands were visualized by using the Bio-Rad silver-staining kit. See Methods for details.

tionated granule lysate material. The low fold purification reported for the S-200 chromatography step resulted from a low yield (below 30%). Much of the loss occurred during the concentration and clarification of the sample prior to application onto the column.

A 55-fold purification was achieved by the chromatography of the S-200-purified collagenase on a WGA–agarose column. The proteins present in the pooled fractions containing the highest specific activity collagenase were clearly distinct from the material applied to the column (Figure 4, lanes C and D). The protein band in the region of 31 kDa was found throughout the WGA–agarose profile and may be wheat germ agglutinin that eluted from the resin. All other major proteins in this pool bound in a specific manner, showing a displaced elution relative to bulk protein similar to the elution of collagenase activity. The granule enrichment, followed by the two-column procedure presented here, resulted in an 820-fold enrichment with a final specific activity of 8700 units/mg. The final protein determination was made by amino acid analysis because of the interference of Brij-35 (included in the WGA–agarose column buffer) in our protein assays. The SDS–PAGE analysis of the WGA–agarose-enriched material (Figure 4, lane D) shows that no bands are detected in the apparent molecular mass range of 65–75 kDa, the predicted size of collagenase from S-200 chromatography. The correlation of apparent molecular weight between gel exclusion and SDS–PAGE, however, may not be reliable. The elution of PMNL collagenase may be affected by the presence of other macromolecules in the crude material; in addition, the relative migration of the enzyme on SDS–PAGE may be altered due to the presence of carbohydrate residues. SDS–PAGE analysis of the WGA–agarose-enriched material shows that no single protein band constitutes more than 10% of the protein. This suggests that collagenase is less than 10% of the protein in this enriched fraction.

Table II: Interaction of NEM-Activated PMNL Collagenase with Lectin Matrices[a]

lectin	binding[b] of collagenase
wheat germ agglutinin	+
erythroagglutinating phytohemagglutinin	+
leukoagglutinating phytohemagglutinin	–
pea	–
lentil	–

[a] All lectins obtained from Sigma. [b] Binding defined as the displacement of activity relative to the unbound protein (as observed in Figure 3 for WGA–agarose). Conditions of chromatography as described under Methods.

Lectin Chromatography. The chromatography of activated collagenase on various other lectins was explored to probe the specificity of the collagenase–lectin interaction. The data presented in Table II show that only WGA–agarose and E-PHA–agarose retarded collagenase activity with respect to bulk protein. The interaction of collagenase with E-PHA–agarose resulted in a similar displacement of collagenase activity relative to bulk protein as seen with WGA–agarose (Figure 3). The failure of collagenase to interact with L-PHA, pea, and lentil agglutinin demonstrates that a specific interaction is occurring between collagenase carbohydrate residues and the carbohydrate binding sites of WGA and E-PHA.

DISCUSSION

Purification of collagenase from human PMNLs has been difficult because collagenase is present in low amounts and because it is stored in secretory granules that copurify with protease-rich vesicles (lysosomes). In addition, a critical factor in purification of collagenase from PMNLs is the method used to activate the latent enzyme. Previous attempts to purify PMNL collagenase (Christner et al., 1982; Murphy et al., 1982) after allowing endogenous proteases to activate the enzyme through proteolysis have resulted in low specific activities. Activation of collagenase by endogenous proteases in difficult to control; low levels of collagenase activity are obtained from either poor conversion of the enzyme to an active form or degradation of active enzyme. In our purification, we use NEM to activate latent collagenase. This approach also made it possible to lyse granules in the presence of PMSF and NEM which are both effective protease inhibitors. This reduces the possibility of producing heterogeneity in the collagenase polypeptide as a result of proteolysis. This method produced 50-fold more collagenase activity than was obtained by allowing endogenous protease to activate the latent enzyme. The mechanism of NEM activation is unclear; the modified thiol may reside on collagenase or an associated inhibitor. NEM is preferable to other sulfhydryl reagents because of its small size and lack of charge, minimizing the possible effect of modification on the physical properties of the enzyme. It has been suggested that oxidation of a thiol is the mechanism of activation in vivo (Weiss et al., 1985); NEM modification might mimic this process.

The chromatographic behavior of PMNL collagenase on various lectin matrices provided information about the structure of the carbohydrate attached to the enzyme. Chromatography of glycopeptides of known carbohydrate structure on lectin affinity columns has resulted in correlation between the oligosaccharide structure of these molecules and their binding to lectin matrices. The binding of collagenase to E-PHA–agarose and WGA–agarose demonstrates that the

enzyme contains a complex type of asparagine-linked carbohydrate (Kornfeld & Kornfeld, 1970). This binding also suggests that the oligosaccharide contains at least one outer galactosyl residue and an *N*-acetylglucosamine residue linked to the β-linked mannose of the core structure (Cummings & Kornfeld, 1982a,b; Yamamoto et al., 1981; Yamashita et al., 1983). The absence of interaction of collagenase with pea and lentil agarose implies that the enzyme does not contain a fucosyl moiety bound to the asparagine-linked *N*-acetylglucosamine residue (Kornfeld et al., 1981). Collagenase enriched from conditioned media from a monoblast-like cell line (U937) induced with phorbol ester exhibited different lectin binding properties than the PMNL collagenase (Hersh et al., 1986). This indicates differences in the oligosaccharide structures of the collagenase produced by these two cell types.

The purification of latent collagenase will be necessary to determine whether the enzyme exists as an inactive proenzyme (zymogen) or is associated with an inhibitor as it resides in the secretory granules of human PMNLs. Purification of latent and active collagenase has been reported (Macartney & Tschesche, 1983a), but no purification table or specific activities were presented. When we chromatographed NEM-activated collagenase on DEAE-Sephacel, elution with a shallow NaCl gradient resulted in a yield of greater than 50% of the initial activity. But when latent collagenase was processed through an identical protocol, less than 3% of the initial collagenase activity could be detected by thiol modifying agents. Attempts to activate by proteolysis or to recombine the DEAE fractions recovered no additional activity. This agrees with an earlier observation (Murphy et al., 1982) and demonstrates the difficulty in isolating latent collagenase which can be activated. The steps presented in this report provide a method for isolation of latent collagenase in a form that can subsequently be activated with NEM. However, active collagenase can be detected in these preparations after storage of latent enzyme at 4 °C for several weeks. This procedure will be useful in determining the mechanism by which collagenase is maintained in a latent form in the secretory granules of human PMNLs.

Previous attempts to enrich for collagenase from PMNLs have used affinity columns designed to take advantage of unique properties of the enzyme. Pancreatic trypsin inhibitor bound to Sepharose (Christner et al., 1982) and the use of a zinc-chelating matrix (Murphy et al., 1982) resulted in only a 5–7-fold enrichment of collagenase. Chromatography using collagen coupled to a column support in our laboratory and others (Eisen et al., 1975; Werb & Reynolds, 1975) resulted in very low recovery of collagenase activity and little increase in specific activity. In contrast, by capitalizing on the properties of the oligosaccharide structure found on this form of the enzyme, lectin affinity chromatography of an enriched collagenase fraction resulted in a 55-fold purification of the enzyme with almost complete recovery of activity.

The purification procedure presented in this paper produces an 820-fold enrichment of the enzyme from human PMNLs. The specific activity achieved is higher than those previously reported for the enzyme (Christner et al., 1982; Murphy et al., 1982). The specific activity reported for the purified human skin fibroblast collagenase (Stricklin et al., 1977) is approximately 6 times greater than the activity we obtain with our enriched PMNL collagenase (using a different assay procedure). This agrees with our estimate of having purified PMNL collagenase to about 10% of the remaining protein. These results suggest that collagenase constitutes 0.01% of the protein in human PMNLs. This demonstrates the need for affinity chromatography procedures such as lectin chromatography in future attempts to purify the enzyme from this source.

ACKNOWLEDGMENTS

We are grateful to Drs. William Wickner and Jouni Uitto for their guidance and encouragement and to S. Jennifer Stary for her fine technical assistance. We also express our appreciation to Elizabeth Walters for her assistance in the preparation of the manuscript.

Registry No. NEM, 128-53-0; collagenase, 9001-12-1.

REFERENCES

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Cawston, T. E., Murphy, G., Mercer, E., Galloway, A., Hazleman, B. L., & Reynolds, J. J. (1983) *Biochem. J. 211*, 313–318.

Christner, P., Damato, D., Reinhart, M., & Abrams, W. (1982) *Biochemistry 21*, 6005–6011.

Cummings, R. D., & Kornfeld, S. (1982a) *J. Biol. Chem. 257*, 11230–11234.

Cummings, R. D., & Kornfeld, S. (1982b) *J. Biol. Chem. 257*, 11235–11240.

Eisen, A. Z., Bauer, E. A., Stricklin, G. P., & Jeffrey, J. J. (1974) *Methods Enzymol. 34B*, 420–424.

English, D., & Anderson, B. R. (1974) *J. Immunol. Methods 5*, 249–252.

Harper, E. (1980) *Annu. Rev. Biochem. 49*, 1063–1078.

Harris, E. D., & Vater, C. A. (1982) *Methods Enzymol. 82*, 423–452.

Hasty, K. A., Hibbs, M. S., Kang, A. H., & Mainardi, C. L. (1984) *J. Exp. Med. 159*, 1455–1463.

Hersh, C. L., Yeh, R. K., Callaway, J. E., Garcia, J. A., Jr., & Gilmore-Hebert, M. (1986) *Biochemistry* (preceding paper in this issue).

Kornfeld, R., & Kornfeld, S. (1970) *J. Biol. Chem. 245*, 2536–2545.

Kornfeld, K., Reitman, M. L., & Kornfeld, R. (1981) *J. Biol. Chem. 256*, 6633–6640.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lazarus, G. S., Brown, R. S., Daniels, J. R., & Fullmer, H. M. (1968) *Science (Washington, D.C.) 159*, 1483–1485.

Macartney, H. W., & Tschesche, H. (1983a) *Eur. J. Biochem. 130*, 71–78.

Macartney, H. W., & Tschesche, H. (1983b) *Eur. J. Biochem. 130*, 79–83.

Macartney, H. W., & Tschesche, H. (1983c) *Eur. J. Biochem. 130*, 85–92.

Maniatis, T., Frisch, E. F., & Sambrook, J. (1982) in *Molecular Cloning: A Laboratory Manual*, p 348, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Murphy, G., & Sellers, A. (1980) *The Extracellular Regulation of Collagenase Activity. Collagenase in Normal and Pathological Connective Tissue* (Wooley, D. E., & Evanson, J. M., Eds.) pp 65–81, Wiley, London.

Murphy, G., Bretz, U., Baggiolini, M., & Reynolds, J. J. (1980) *Biochem. J. 192*, 517–525.

Murphy, G., Reynolds, J. J., Bretz, U., & Baggiolini, M. (1982) *Biochem. J. 203*, 209–221.

Roswit, W. T., Halme, J., & Jeffrey, J. J. (1983) *Arch. Biochem. Biophys. 225*, 285–295.

Sakamoto, S., Sakamoto, M., Goldhaber, P., & Glimcher, M. J. (1978) *Arch. Biochem. Biophys. 188*, 438–449.

Stricklin, G. P., & Welgus, H. G. (1983) *J. Biol. Chem. 258*, 12252–12258.

Stricklin, G. P., Bauer, E. A., Jeffrey, J. J., & Eisen, A. Z. (1977) *Biochemistry 16*, 1607–1615.
Stricklin, G. P., Eisen, A. Z., Bauer, E. A., & Jeffrey, J. J. (1978) *Biochemistry 17*, 2331–2337.
Stricklin, G. P., Jeffrey, J. J., Roswit, W. T., & Eisen, A. Z. (1983) *Biochemistry 22*, 61–68.
Uitto, V.-J., Turto, H., Huttunen, A., Lindy, S., & Uitto, J. (1980) *Biochim. Biophys. Acta 613*, 168–177.
Vater, C. A., Hahn, J. L., & Harris, E. D., Jr. (1981) *Collagen Relat. Res. 1*, 527–542.
Vater, C. A., Nagase, H., & Harris, E. D., Jr. (1983) *J. Biol. Chem. 258*, 9374–9382.
Weiss, S. J., Peppin, G., Ortiz, X., Ragsdale, C., & Test, S. T. (1985) *Science (Washington, D.C.) 227*, 747–749.
Welgus, H. G., & Stricklin, G. P. (1983) *J. Biol. Chem. 258*, 12259–12264.
Werb, Z., & Reynolds, J. J. (1975) *Biochem. J. 151*, 645–653.
Wilhelm, S. M., Javed, T., & Miller, R. L. (1984) *Collagen Relat. Res. 4*, 129–152.
Yamamoto, K., Tsuji, T., Matsumoto, I., & Osawa, T. (1981) *Biochemistry 20*, 5894–5899.
Yamashita, K., Hitoi, A., & Kobata, A. (1983) *J. Biol. Chem. 258*, 14753–14755.

Pyrimidodiazepine, a Ring-Strained Cofactor for Phenylalanine Hydroxylase†

David C. Pike, Mary T. Hora, Steven W. Bailey, and June E. Ayling*
Department of Pharmacology, College of Medicine, University of South Alabama, Mobile, Alabama 36688
Received December 20, 1985; Revised Manuscript Received April 2, 1986

ABSTRACT: Homologues of 6-methyl-7,8-dihydropterin (6-Me-7,8-PH_2) and 6-methyl-5,6,7,8-tetrahydropterin (6-Me-PH_4), expanded in the pyrazine ring, were synthesized to determine the effect of increased strain on the chemical and enzymatic properties of the pyrimidodiazepine series. 2-Amino-4-keto-6-methyl-7,8-dihydro-3*H*,9*H*-pyrimido[4,5-*b*][1,4]diazepine (6-Me-7,8-PDH_2) was found to be more unstable in neutral solution than 6-Me-7,8-PH_2. Its decomposition appears to proceed by hydrolytic ring opening of the 5,6-imine bond, followed by autooxidation. 6-Me-7,8-PDH_2 can be reduced, either chemically or by dihydrofolate reductase (K_m = 0.16 mM), to the 5,6,7,8-tetrahydro form (6-Me-PDH_4). This can be oxidized with halogen to quinoid dihydropyrimidodiazepine (quinoid 6-Me-PDH_2), which is a substrate for dihydropteridine reductase (K_m = 33 μM). Whereas quinoid 6-methyldihydropterin was found to tautomerize to 6-Me-7,8-PH_2 in 95% yield in 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, quinoid 6-Me-PDH_2 gives only 53% 6-Me-7,8-PDH_2, the remainder decomposing via an initial opening of the diazepine ring. Additional evidence for the extra strain in the pyrimidodiazepine system is the cyclization of quinoid 6-*N*-(2′-aminopropyl)divicine to quinoid 6-Me-PH_2 in 57% yield in 0.1 M Tris-HCl, pH 7.4. By comparison, no quinoid 6-Me-PDH_2 is formed from the homologue quinoid 6-*N*-(3′-aminobutyl)divicine. A small (2%) yield of 6-Me-PDH_4 is found if the unstable C4a-carbinolamine intermediate is trapped by enzymatic dehydration and reduction. Although phenylalanine hydroxylase utilizes 6-Me-PDH_4 (K_m = 0.15 mM), the maximum velocity of tyrosine production is 20 times slower than that with 6-Me-PH_4, indicating that a ring opening reaction is not a rate-limiting step in the hydroxylase pathway. Further, the maximum velocities of 2,5,6-triamino-4(3*H*)-pyrimidinone, 2,6-diamino-5-(methylamino)-4(3*H*)-pyrimidinone, and 2,6-diamino-5-(benzylamino)-4(3*H*)-pyrimidinone span a 35-fold range. These cofactors would theoretically form the same oxide of quinoid divicine if oxygen activation involves a carbonyl oxide intermediate. Thus, the limiting step is also not transfer of oxygen from this hypothetical intermediate to the phenylalanine substrate.

Phenylalanine hydroxylase is a tetrahydrobiopterin-dependent aromatic amino acid monooxygenase that converts excess dietary phenylalanine to *p*-tyrosine. Isotopic labeling of pyrimidine cofactor analogues (Bailey et al., 1982) and detection of a C4a-hydroxypterin product (Kaufman, 1975; Lazarus et al., 1982) have demonstrated that activation of molecular oxygen in this reaction involves its covalent addition to cofactor. The initial adduct has not yet been observed. A C4a-hydroperoxide of the structurally related dihydroflavin has been shown to be an early intermediate of several enzymes (Ballou, 1984). Hamilton has proposed that a highly reactive carbonyl oxide can be generated from the C4a-hydroperoxide of either cofactor by cleavage of their respective C4a–N5 bonds (Hamilton, 1974). The direct utilization of the hydroperoxide vs. this ring-opened mechanism is outlined in Figure 1. Although model studies have shown that flavin C4a-hydroperoxides may be sufficiently potent to account in themselves for the oxidation of many flavoprotein substrates (Bruice, 1984), the possibility of ring-opened intermediates, especially of *p*-hydroxybenzoate and phenol hydroxylases, is still debated (Detmer & Massey, 1985). Beyond the involvement of position C4a of cofactor, little is known of the structure of the ultimate oxygen reagent formed by phenylalanine hydroxylase; for example, no evidence yet affirms or refutes a pyrazine ring cleavage mechanism.

The effect of ring size on inherent strain and ring opening and closure reactions has been studied in many systems. In most cases, seven-membered rings are more strained than their six-membered counterparts. A homologue of 6-methyltetra-

† This investigation was supported by Grant GM-30368 awarded by the National Institute of General Medical Sciences and Grant CA 31852 awarded by the National Cancer Institute. A preliminary report of part of this work has been given (Pike et al., 1984).

0006-2960/86/0425-4762$01.50/0