117–120, Plenum, New York.
Leblanc, J.-P., & Laval, J. (1982) *Biochimie 64*, 735–738.
Leblanc, J.-P., Martin, B., Cadet, J., & Laval, J. (1982) *J. Biol. Chem. 257*, 3477–3483.
Lindahl, T. (1979) *Prog. Nucleic Acid Res. Mol. Biol. 22*, 135–192.
Lindahl, T., & Nyberg, B. (1972) *Biochemistry 11*, 3610–3618.
Lindahl, T., Ljungquist, S., Siegert, W., Nyberg, B., & Sperens, B. (1977) *J. Biol. Chem. 252*, 3286–3294.
Makino, F., & Munakata, N. (1978) *J. Bacteriol. 134*, 24–29.
Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372–1379.
Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–559.
Mosbaugh, D. W. (1988) *Rev. Biochem. Toxicol. 9*, 69–130.
Pedersen, P. L., Greenawalt, J. W., Reynafarje, B., Hullihen, J., Decker, G. L., Soper, J. W., & Bustamen, E. (1978) *Methods Cell Biol. 20*, 411–481.
Schnaitman, C., & Greenawalt, J. W. (1968) *J. Cell Biol. 38*, 158–175.
Seal, G., & Sirover, M. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7608–7612.
Seal, G., Arenaz, P., & Sirover, M. A. (1987) *Biochim. Biophys. Acta 925*, 226–233.
Talpaert-Borle, M., & Liuzzi, M. (1982) *Eur. J. Biochem. 124*, 435–440.
Talpaert-Borle, M., Campagnari, F., & Creissen, D. M. (1982) *J. Biol. Chem. 257*, 1208–1214.
Vollberg, T. M., Cool, B. L., & Sirover, M. A. (1987) *Cancer Res. 47*, 123–128.
Wittwer, C. U., & Krokan, H. (1985) *Biochim. Biophys. Acta 832*, 308–318.

Monoclonal Antibodies to Human Fibroblast Procollagenase. Inhibition of Enzymatic Activity, Affinity Purification of the Enzyme, and Evidence for Clustering of Epitopes in the NH_2-Terminal End of the Activated Enzyme†

Bente Birkedal-Hansen,‡,§ William G. I. Moore,‡,§ Robert E. Taylor,‡,§ Ajit S. Bhown,‖ and Henning Birkedal-Hansen*,‡,§

Department of Oral Biology, Institute of Dental Research, and Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294

Received February 22, 1988; Revised Manuscript Received May 2, 1988

ABSTRACT: This study describes 11 monoclonal antibodies (Mabs) against human fibroblast collagenase that (i) inhibit the specific catalytic activity of the enzyme and/or (ii) react with one or more forms of the enzyme on Western blots. Each of the Mabs specifically immunoprecipitated the M_r 57 000/52 000 procollagenase from [^{35}S]methionine-labeled culture medium. Five Mabs, designated VI-3, VI-4, 2C5, 4A2, and 7C2, inhibited the activity of fibroblast-type collagenase against soluble monomeric collagen and against reconstituted collagen fibrils but did not inhibit the genetically distinct human PMN leukocyte collagenase. The interstitial collagenase produced by human mucosal keratinocytes (SCC-25) was also inhibited, whereas the corresponding enzyme from rat was not. Assignment of epitopes to structural domains within the molecule based on immunoperoxidase staining of Western blots of collagenase and its autocatalytic fragments revealed that 9 of 11 epitopes, including those recognized by 4 inhibitory Mabs, were clustered in a 169-residue domain, which constitutes the NH_2-terminal part of the M_r 46 000/42 000 active enzyme. One Mab (X-2a) specifically recognized the M_r 57 000/52 000 zymogen species and failed to react with the active M_r 46 000/42 000 form. The inhibitory Mab VI-3 was used for immunoaffinity purification of procollagenase from culture media with a recovery better than 80% and a yield of ~1.4 mg of enzyme/L of medium.

Fibroblast-type collagenase, a neutral secretory metalloproteinase capable of cleaving interstitial types I–III collagens at a specific site in the helical domain, is expressed either constitutively or after TPA-induction by a number of different cell types including fibroblasts (Birkedal-Hansen et al., 1976, Aggeler et al., 1984), macrophages (Welgus et al., 1985, Wahl et al., 1977), osteoblasts (Otsuka et al., 1984), and keratinocytes (Lin et al., 1987). The enzyme is secreted as a major M_r 52 000 unglycosylated and a minor M_r 52 000 glycosylated form (Wilhelm et al., 1987). Both of these are inactive collagenase precursors that are subsequently converted to catalytic form by a poorly understood mechanism. Activation may be achieved either by preincubation with proteolytic enzymes such as trypsin (Stricklin et al., 1983), plasmin (Eekhout & Vaes, 1977), and kallikrein (Nagase et al., 1982) or by exposure to dissociative agents such as KSCN (Abe & Nagai, 1972), NaI (Abe et al., 1973), and SDS (Birkedal-Hansen & Taylor, 1982). Activation is often, but not necessarily, associated with loss of an 81-residue propeptide (Stricklin et al., 1977, 1983; Grant et al., 1987), which results in formation of a single-chain

† This work was supported by grants from the National Institute of Dental Research (DE 05817, DE 06028, DE 02670, DE 08228, and AR 20614) and by a gift from Procter and Gamble Co.
* Address correspondence to this author at the Department of Oral Biology.
‡ Department of Oral Biology.
§ Institute of Dental Research.
‖ Department of Medicine.

0006-2960/88/0427-6751$01.50/0

M_r 46000/42000 catalytic enzyme (Birkedal-Hansen & Taylor, 1982, Grant et al., 1987). Thus, M_r 57000/52000 collagenase may exist in active as well as latent form, whereas the M_r 46000/42000 form is invariably active. In addition, tryptic and organomercurial activation of M_r 52000 procollagenase gives rise to one or more (M_r 46000, M_r 44000) short-lived intermediates (Grant et al., 1987). Each of these molecular species also form "spontaneously", although slowly, during purification of the enzyme and during storage. We have observed yet another proteolytic cleavage of M_r 57000/52000 procollagenase during storage that gives rise to an NH_2-terminal M_r 33000 fragment including the intact 81-residue propeptide and a COOH-terminal M_r 22000 fragment. Our data suggest that the same catalytic site is responsible for this internal (autocatalytic) cleavage of collagenase and for the cleavage of native triple-helical collagen (G. Fields, H. Van Wart, and H. Birkedal-Hansen, unpublished results).

In this study we describe the reactivities of a complement of 11 monoclonal antibodies raised against human fibroblast M_r 57000/52000 procollagenase/collagenase. We have used the autocatalytic fragments of procollagenase to show that the epitopes are clustered in a 169-residue domain, which constitutes the NH_2-terminal region of the M_r 46000/42000 activated enzyme. In addition, we have identified five antibodies that block the specific catalytic activity of the enzyme and one that reacts exclusively with the M_r 57000/52000 (zymogen) form.

Experimental Procedures

Preparation of Procollagenase. Human fibroblast procollagenase was produced in Nunc cell factories maintained on alternating serum-free and serum-supplemented feeding regimens each of 6–8-days duration as previously described (Birkedal-Hansen et al., 1976). Procollagenase was purified by heparin–Sepharose, Zn^{2+}-chelate–Sepharose, and molecular sieve (Ultrogel AcA 44) chromatography as described (Birkedal-Hansen, 1987). Collagenase protein concentration was determined spectrophotometrically at 280 nm, using a factor of $E^{1\%}_{280nm}$ = 12.3 determined by Lowry protein assay (Lowry et al., 1951). Collagenase isolated in this manner consisted of a characteristic M_r 57000/52000 double band (Figure 1). The enzyme was more than 95% latent and could be converted to catalytic form either by trypsin or by organomercurials to yield a maximal activity of 900–1000 units/mg as determined by [^{3}H]collagen fibril assay (Birkedal-Hansen & Dano, 1981), where one unit (U) of collagenase activity cleaved 1 μg of collagen fibrils/min at 35 °C. Collagenase prepared in this fashion was free from gelatinase, which failed to bind to the Zn^{2+}-chelate column, from stromelysin, which emerged unabsorbed from the heparin–Sepharose column. M_r 46000/42000 activated collagenase was prepared by preincubation with 1 mM 4-APMA for 90 min at 37 °C or with 20 μg/mL trypsin for 10 min at 22 °C. Crude PMN leukocyte collagenase was obtained from human peripheral PMN leukocytes purified by ficoll–hypaque gradient centrifugation (Bøyum, 1974). The cells were suspended in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS–) at a density of 10^6 cells/mL and exposed to 10^{-6} M 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 1 h. After removal of cells by low-speed centrifugation, the medium protein was concentrated by precipitation with 60% saturated ammonium sulfate and dialyzed against assay buffer. The enzyme was fully activated by exposure to 1 mM 4-APMA. Keratinocyte collagenases were obtained from conditioned culture media of serially propagated rat mucosal keratinocytes as described (Lin et al., 1987) and from a TPA-induced human tongue carcinoma line (SCC-25) (H.-Y. Lin, B. Birkedal-Hansen, R. E. Taylor, and H. Birkedal-Hansen, unpublished data). Both enzymes were activated by brief exposure to trypsin as described (Lin et al., 1987).

Production of Monoclonal Antibodies to Fibroblast Procollagenase. Aliquots of 80 μg of procollagenase emulsified in Freund's complete adjuvant were injected in the rear foot pads and inguinal regions of Balb/C mice as outlined by Kearney (1984). Boosters in Freund's incomplete adjuvant or in phosphate-buffered saline were given at 3-day intervals. This schedule resulted in visible enlargement of the regional lymph nodes, which was taken as evidence of successful immunization. One day after the sixth injection, draining lymph nodes were excised and the cells obtained by teasing [(40–60) × 10^6 per mouse] were fused at a 1:1 ratio with murine myeloma cells (PX63-Ag8.653) by exposure to 36% poly(ethylene glycol) at 37 °C for 1 min as described by Kearney (1984). Aliquots of 5 × 10^5 cells per well were seeded in 24-well cluster dishes in RPMI 1640, 20% fetal calf serum supplemented with hypoxanthine, aminopterin, and thymidine (HAT). Enrichment with feeder cells obtained by peritoneal washing was done as needed. Hybridomas identified by phase-contrast microscopy were gradually transferred to HAT-free medium over a 2-week period. Wells with growth were tested for antibody production by ELISA, and positive hybridomas were cloned and recloned by limiting dilution. To screen for inhibitory activity, each clone was also grown in medium supplemented with Nu-serum, which does not inhibit collagenase activity. Immunoglobulin was isolated from culture media by passage over a protein A–Sepharose column. Ig was eluted with 0.1 M HOAc–0.2 M NaCl, pH 4.2, and immediately neutralized by addition of a 2 M Tris-HCl buffer, pH 8.1. Ig concentration was determined spectrophotometrically, using $E^{1\%}_{280nm}$ = 14.0. Alternatively, ascites were produced by intraperitoneal injection of 10^7 cells in Balb/C mice primed with 1 mL of pristane oil 7–14 days earlier. Ascites were harvested by peritoneal drainage. IgG antibodies were isolated by protein A–Sepharose affinity chromatography. IgM (and some IgG) antibodies that were not retained by protein A-Sepharose were processed by ammonium sulfate precipitation (45% saturated), followed by chromatography on a 2.5 × 100 cm column of Ultrogel AcA 44 equilibrated with Ca^{2+}/Mg^{2+}-free PBS (PBS–). Final purification was by HPLC using a MonoQ column eluted with a linear salt gradient (0.0–0.45 M NaCl). Ig class and subclass was determined by ELISA with commercially available (Southern Biotech, Birmingham, AL) chain-specific antibodies.

Conventional polyclonal antibodies were prepared in rabbits and used for reference purposes in this study as described elsewhere (Birkedal-Hansen, 1987). Briefly, human fibroblast procollagenase in PBS– was labeled by addition of a 1:10 volume of 1 mg/mL fluorescamine in dry acetone and resolved in the native state by SDS–PAGE using a 11% polyacrylamide gel as described by Neville (1971). The procollagenase doublet was excised under the guidance of UV light, homogenized in a Polytron homogenizer, and strained through decreasing size needles. The resultant paste was emulsified with Freund's complete or incomplete adjuvant and injected at multiple sites into the back skin of 3–4-kg white rabbits after collection of preimmune serum. Approximately 75 μg of procollagenase/kg of body weight was used per injection. Boosters were given every 2 weeks over a period of several months, and serum was harvested 1 week after each injection. Preimmune and immune Ig's were isolated by protein A–Sepharose chromatography as described.

Assays. ELISA was performed by the protocol described by Kearney (1984). EIA microtest plates (Flow Laboratories, McLean, VA) were coated with a 1–3 μg/mL solution of human fibroblast procollagenase diluted with borate-buffered saline (BBS, 0.12 M borate–0.15 M NaCl, pH 8.4) and kept overnight at 4 °C. After the wells were blocked with 1% bovine serum albumin (BSA), they were incubated with first antibody (hybridoma culture media) overnight at 4 °C. Incubation with second antibody, affinity-purified, alkaline phosphatase conjugated goat anti-mouse Ig (Southern Biotech, Birmingham, AL) (0.2 μg/mL) diluted in BBS–BSA was for 4 h at 22 °C. The substrate was 1 mg/mL *O*-phenylphosphate in 0.1 M diethanolamine buffer, pH 9.5, containing 0.24 mM $MgCl_2$. Plates were screened by visual inspection or read by a Titertek Multiscan MC equipped with a 405-nm filter.

Collagenase activity was measured as previously described (Birkedal-Hansen & Dano, 1981; Birkedal-Hansen, 1987) with reconstituted fibril gels formed by heat gelation of 20 μL of a 1.5 mg/mL solution of type I [^{3}H]collagen isolated from rat tail tendons and labeled by reaction with [^{3}H]acetic anhydride. Incubation was for 16 h at 35 °C. Collagenolytic activity was measured by the release of radioactivity to the medium by liquid scintillation spectrometry. Latent enzyme was activated as described (Birkedal-Hansen et al., 1976) either by preincubation with 20 μg/mL trypsin-TPCK for 10 min at 22 °C followed by addition of a 15-fold molar excess of soybean trypsin inhibitor or by inclusion in the assay buffer of 1 mM 4-APMA. Inhibitory activity was measured by preincubation (1 h, 22 °C) of hybridoma medium (RPMI, 10% Nu-serum) or purified Ig with a known reference standard of human fibroblast collagenase. The resultant activity was measured by radiofibril assay or by the rate of formation of $^3/_4$–$^1/_4$ fragments from monomeric [^{3}H]collagen in solution.

Electrophoretic Techniques. Electrophoresis was conducted under either denaturing or nondenaturing conditions in 11% polyacrylamide gels according to the method of Neville (1971). Under nondenaturing conditions, samples were incubated at 22 °C for 1 h in sample buffer adjusted to 2% SDS. Denatured samples were prepared in the same buffer by heating (95 °C, 3 min) in the presence of 0.5% 2-mercaptoethanol. Electrophoresis was for 2 $^1/_2$ h at 30 mA per 1.5-mm gel slab. Samples were transferred to nitrocellulose paper by the method of Towbin et al. (1979) as modified by Burnette (1981). Zymography to detect gelatinolytic activity during purification of collagenase was performed by the method of Heussen and Dowdle (1980). Immunoprecipitation was performed essentially as described by Rosenfeld et al. (1982). Subconfluent or early confluent cultures were labeled for 4–24 h with 50 μCi/mL [^{35}S]methionine, and 250-μL aliquots were incubated overnight at 4 °C with 50 μg/mL Mab or control nonimmune murine Ig or with 50 μg/mL Pab (immune or preimmune IgG). Protein A–Sepharose (3 mg) was added and incubation continued for 2 h at 22 °C. The antigen–antibody–protein A–Sepharose complex was collected by centrifugation, washed three times with 50 mM Tris-HCl buffer containing 0.19 M NaCl, 6 mM EDTA, 2.5% Triton X-100, 0.2% SDS, and 100 U/mL trasylol. Antigen–antibody complexes were dissociated by boiling in electrophoretic sample buffer containing 2.0% SDS and 5% 2-mercaptoethanol. The reaction mixtures were resolved by SDS–PAGE and processed for fluorography by PPO–DMSO as described by Bonner and Laskey (1974). Total secreted protein was precipitated by quinine sulfate as described by Murphy et al. (1985).

Western Blot Staining. Electrotransfers were stained by the method of Larsson et al. (1984). After being blocked with BBS–BSA (1 h at 37 °C), the blots were incubated with hybridoma medium or purified Ig in 50 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl (TBS) and 1% Triton X-100 (TBS–Triton) for 16 h at 4 °C. After being washed 3 × 20 min with TBS–Triton, the blots were incubated with horseradish peroxidase conjugated swine anti-rabbit or rabbit anti-mouse Ig (Dako, Copenhagen, Denmark) (1:200) in TBS–Triton for 1 h at 22 °C. After another washing cycle in TBS–Triton (2 × 20 min) and 50 mM Tris-HCl, pH 7.5 (1 × 20 min), color was developed by incubation with an aqueous solution of 3,3′-diaminobenzidine–H_2O_2.

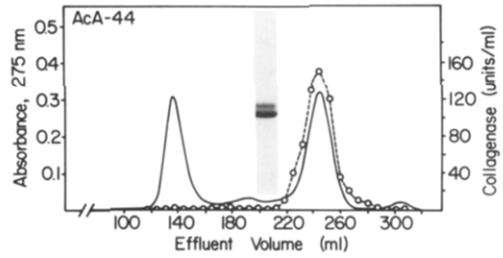

FIGURE 1: Final step in the purification of human fibroblast procollagenase. Partially purified procollagenase from 7 L of serum-free culture medium was eluted from heparin–Sepharose and Zn^{2+}–chelate–Sepharose columns and applied to a 2.5 × 95 cm Ultrogel AcA 44 column. The second peak contains M_r 57 000/52 000 procollagenase (inset). SDS–PAGE, stained with Coomassie blue. (—) Absorbance at 280 nm; (O–O) latent collagenase activity.

Immunoaffinity Chromatography. Mab Ig was coupled to CNBr-activated Sepharose 4B according to the manufacturers' instructions. The columns were equilibrated with 50 mM Tris-HCl–0.2 M NaCl, pH 7.5. After application of crude medium, the column was washed with 1 M NaCl in the same buffer. Bound protein was eluted either in active form with 3 M KSCN in 50 mM Tris-HCl, pH 7.5, or in latent form with 0.1 M HOAc–0.2 M NaCl–10 mM $CaCl_2$, pH 4.2. The eluate was immediately neutralized with 2 M Tris-HCl, pH 8.1.

Capture of Active Forms of Collagenase by α_2-Macroglobulin. Human α_2-macroglobulin (α2-M) was purified from outdated plasma by the method of Kurecki et al. (1979) as modified by Sottrup-Jensen et al. (1984) using poly(ethylene glycol) precipitation (12%) followed by Zn^{2+}-chelate and Sephacryl S-300 chromatography. The inhibitory capacity of the α2-M was determined by trypsin binding assay based on BAEE reactivity (Howard et al., 1980). The preparation used in this study blocked the action of 0.7 mol of active, pNGB titrable trypsin per mole of α2-M. Active collagenase present in procollagenase/collagenase preparations was complexed to α2-M by incubation with 900 μg/mL of inhibitor (15-fold molar excess) in assay buffer for 1 h at 22 °C. The samples were then resolved by SDS–PAGE under nondenaturing conditions as described above and transferred to nitrocellulose paper and immunoperoxidase stained with mono- or polyclonal antibodies to human fibroblast (pro)collagenase.

Miscellaneous. Stepwise Edman degradations were performed in a modified (Bhown et al., 1980) Beckman 890 C sequenator as described earlier (Bhown et al., 1981).

RESULTS

Construction and Selection of Hybridomas That Express Anti-Human Procollagenase Antibodies. Hybridomas were constructed by fusion of murine myeloma cells (line PX63-Ag 8.653) with lymph node cells immunized against purified M_r 57 000/52 000 human fibroblast (pro)collagenase (Figure 1) by a brief immunization schedule using relatively large doses of antigen (50–80 μg per injection). From 7 fusions, each

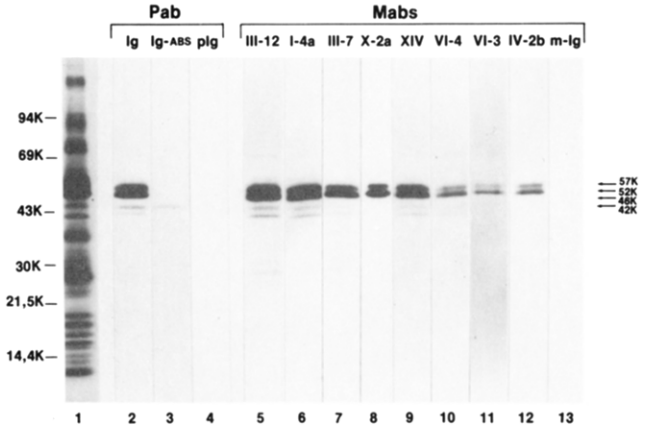

FIGURE 2: Immunoprecipitation of procollagenase from [^{35}S]-methionine-labeled fibroblast culture medium by Pabs and Mabs. Lane 1, total medium protein; lane 2, rabbit Pab, 400 μg/mL IgG; lane 3, Pab absorbed with procollagenase by passage over procollagenase–Sepharose column, 400 μg/mL IgG; lane 4, preimmune Ig, 400 μg/mL IgG; lanes 5–13, murine Mabs, 10 μg/mL Ig. Lane 5, Mab III-12; lane 6, Mab I-4a; lane 7, Mab III-7; lane 8, Mab X-2a; lane 9, Mab XIV; lane 10, Mab VI-4; lane 11, Mab VI-3; lane 12, Mab IV-2b; lane 13, nonimmune murine Ig. SDS–PAGE fluorogram.

Table I: Inhibition of Interstitial Collagenases by Mabs and Pabs[a]

		% inhibition of collagenase activity from			
antibody	[Ig] (μg/mL)	h-Fib	h-PMNL	h-Ker	r-Ker
polyclonal Ig	1000	80	3	74	0
preimmune Ig	1000	3	0	0	0
Mab					
VI-3	50	80	18	92	14
VI-4	50	43	0	76	0
2C5	50	88	13	64	9
III-7	50	0	0	16	5

[a] Aliquots (20–25 mU) of collagenase were mixed with Ig at the concentration indicated and assayed for resultant activity for 16 h at 35 °C by the radiofibril assay (Birkedal-Hansen & Dano, 1981). Preimmune (rabbit) Ig, and noninhibitory Mab III-7 were used as controls. Collagenases were prepared from human fibroblasts (h-Fib), human PMN leukocytes (h-PMNL), human mucosal carcinoma keratinocytes (SCC-25) (h-Ker), and normal rat mucosal keratinocytes (r-Ker).

resulting in between 50 and 200 hybridomas, 11 Mabs that reacted with Western blots and/or inhibited the catalytic activity of the enzyme were studied in more detail. Each of these antibodies specifically immunoprecipitated the characteristic M_r 57 000/52 000 procollagenase doublet from [^{35}S]methionine-labeled human skin fibroblast culture medium, although with somewhat varying efficacy (Figure 2).

Inhibition of Collagenase Activity. Less than 5% of all ELISA-positive hybridomas inhibited the catalytic activity of the enzyme. Four initial fusions, after immunization with native or heat-denatured M_r 57 000/52 000 procollagenase, yielded only two inhibiting antibodies (VI-3, VI-4). Three subsequent fusions, after immunization with 4-APMA-activated M_r 46 000/42 000 collagenase, resulted in recovery of three additional inhibitory Mabs (2C5, 7C2, 4A2). The six remaining Mabs immunoprecipitated procollagenase from solution and reacted with the antigen on Western blots but did not block the catalytic activity. Human PMN leukocyte collagenase, which appears to be a distinct gene product (Hasty et al., 1984), was not inhibited to any significant extent (Table I). We have also examined the ability of the Mabs to inhibit fibroblast-type collagenases elaborated by epithelial cells, including human oral carcinoma cells (SCC 25) and normal rat oral mucosal epithelial cells. The epithelial collagenase from man was inhibited, but that from rat was not (Table I).

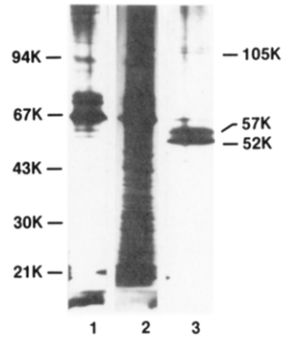

FIGURE 3: Purification of human fibroblast procollagenase by immunoaffinity chromatography. A 200-mL sample of serum-free fibroblast culture medium was passed over an affinity column prepared by coupling 10 mg of Mab VI-3 to 2 mL of CNBr-activated Sepharose 4B. The column was washed in succession with 1 M NaCl and distilled H_2O, and bound protein was eluted with 0.1 M HOAc, pH 4.2, containing 0.2 M NaCl and 10 mM $CaCl_2$. Lane 1, crude medium protein; lane 2, unbound material (10× concentrated); lane 3, adsorbed material eluted with 0.1 M acetate buffer. In addition to the M_r 57 000/52 000 doublet, a small amount of dimer (M_r 105 000) is also eluted. SDS–PAGE; silver stained.

Affinity Purification of Human Fibroblast Procollagenase by Mab Affinity Chromatography. Three Mabs (III-7, X-2a, VI-3) were initially coupled to CNBr–Sepharose for construction of affinity matrices to isolate human fibroblast procollagenase. Mab X-2a did not retain the enzyme, whereas Mabs III-7 and VI-3 did. The enzyme could subsequently be eluted essentially in pure form either with 3 M KSCN or with 0.1 M HOAc, pH 4.2. Figure 3 shows the result of a single passage of 200 mL of serum-free human fibroblast culture medium over a 2-mL column constructed by coupling of 10 mg of VI-3 IgG_1 to 6 g of CNBr-Sepharose. On the basis of activity assays, 80% of the enzyme was retained on the column. After a single wash with 1 M NaCl, the enzyme was eluted with 0.1 M HOAc–0.2 M NaCl–10 mM $CaCl_2$, pH 4.2. A total of 290 μg of procollagenase was recovered. The purity judged from SDS–PAGE was 90% and the recovery better than 80%.

Assignment of Mab Epitopes to Different Structural Domains of the (Pro)collagenase Molecule. Cleavage of the enzyme with CNBr resulted in complete loss of Mab immunoreactivity. This approach, therefore, could not be used for assignment of epitopes to distinct structural domains within the molecule. Instead, we took advantage of two spontaneous and apparently autocatalytic fragmentation reactions that occur during storage of the enzyme, namely, (i) conversion of the M_r 57 000/52 000 zymogen to the M_r 46 000/42 000 activated form (Figure 4) and (ii) cleavage of the zymogen into two major identifiable, M_r 33 000 and M_r 22 000, fragments.

"Spontaneous" activation of procollagenase results in conversion of the M_r 57 000/52 000 bands to a M_r 46 000/42 000 doublet (Figure 4A, lane 1) much in the same manner as activation by brief trypsin pretreatment (Figure 4A, lane 4). The sample shown in Figure 4, parts A and B, contained approximately equal amounts of each of the four major components and little or no intermediate (lane 1). To determine whether any or all of these bands possessed catalytic activity, we used an inhibitor-capture technique based on the observation that α2-M forms stable, SDS-resistant complexes with active collagenase but not with latent forms of the enzyme. Capture by the inhibitor can therefore be used to distinguish between these forms and, when combined with SDS–PAGE, to assign catalytic activity to individual electrophoretic components. Figure 4A shows that the M_r 46 000/42 000 bands

Table II: Mab and Pab Reactivity with (Pro)collagenase and (Pro)collagenase Fragments on Western Blots

			procollagenase/collagenase fragments				
antibody	class	inhibn	M_r 57 000/ 52 000	M_r 46 000/ 42 000	M_r 33 000	M_r 22 000	CNBr
VI-3	IgG_{2a}	+	+	+	+	0	0
2C5	IgG_{2a}	+	0	0	0	0	0
4A2	IgM	(+)	+	+	+	0	0
7C2	IgG_{2a}	+	+	+	+	0	0
VI-4	IgM	+	+	+	+	0	0
III-7	IgG_1	0	+	+	+	0	0
III-12	IgG_1	0	+	+	+	0	0
X-2a	IgG_1	0	+	+	+	0	0
XIV	IgG_{2b}	0	+	+	+	0	0
IV-2b	IgM	0	+	+	+	0	0
I-4a	IgM	0	+	+	+	0	0
Pab		+	+	+	+	(+)	(+)

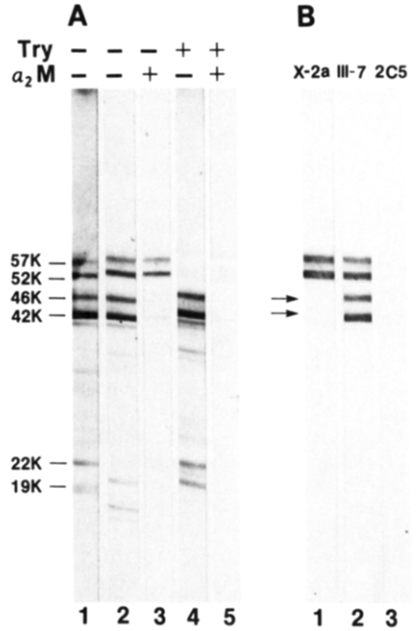

FIGURE 4: Western blot staining of M_r 57 000/52 000 procollagenase and M_r 46 000/42 000 (active) collagenase with Mabs and Pabs. Each lane contained ~30 ng of total collagenase. (A) Lanes 1–5 were stained with rabbit Pab, 7.0 μg/mL Ig, to identify latent and active components by α2-M capture technique. Lane 1, starting sample containing approximately equal amounts of the M_r 57 000/52 000 procollagenase and spontaneously activated M_r 46 000/42 000 collagenase; lanes 2 and 3, same sample preincubated (1 h, 22 °C) either with assay buffer (lane 2) or with 900 μg/mL α2-M (1.25×10^{-6} M) (lane 3) to complex catalytically active collagenase (the inhibitor selectively removed the M_r 46 000/42 000 bands); lane 4, same sample activated by trypsin (20 μg/mL, 10 min, 22 °C) (the reaction was stopped by a 15-fold molar excess of soybean trypsin inhibitor; the M_r 57 000/52 000 procollagenase bands were quantitatively converted to the M_r 46 000/42 000 form); lane 5, trypsin-activated sample preincubated with α2-M (the inhibitor removed the M_r 46 000/42 000 bands and the minor, doublet component in the M_r 19 000–22 000 range). (B) Lanes 1–3 were stained with Mabs (5 μg/mL Ig). Lane 1, Mab X-2a; lane 2, Mab III-7; lane 3, Mab 2C5.

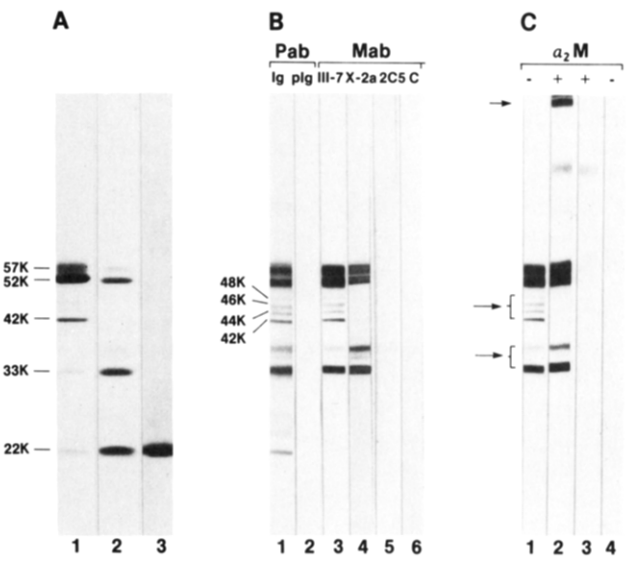

FIGURE 5: Reaction of Mabs and Pabs with procollagenase M_r 33 000 and M_r 22 000 fragments. (A) Spontaneous fragmentation of procollagenase upon storage. Lanes 1 and 2 show increasing conversion of M_r 57 000/52 000 procollagenase to fragments migrating at M_r 33 000 and M_r 22 000. Lane 3, HPLC-purified M_r 22 000 fragment. SDS–PAGE; stained with Coomassie blue. (B) Western blot staining of sample in Figure 6A, lane 1. Each lane contained 0.3 μg total of enzyme protein. Lanes 1 and 2 were stained with 7 μg/mL rabbit Ig isolated from immune (Ig, lane 1) and preimmune sera (p-Ig, lane 2). Lanes 3–6 were stained with 5 μg/mL murine Mab Ig. Lane 3, Mab III-7; lane 4, Mab X-2a; lane 5, Mab 2C5; lane 6, nonimmune murine Ig. (C) α2-M capture of procollagenase/collagenase fragments. The sample used in Figure 6B was preincubated with buffer alone (lane 1) or with 900 μg/mL α2-M (1 h, 22 °C) (lanes 2–4). Staining was with 5 μg/mL Mab III-7. The inhibitor removed the four bands in the M_r 48 000–42 000 range that represent activated collagenase and intermediates (middle arrow) but did not remove the M_r 33 000 doublet (lower arrow). Note reactivity of α2-M–collagenase complex at the top of gel (upper arrow). Lane 3, 900 μg/mL α2-M incubated in the absence of collagenase. Lane 4, same as lane 1, stained with 5 μg/mL nonimmune murine Ig.

generated spontaneously (lanes 2, 3) or by trypsin activation (lanes 4, 5) were removed by the inhibitor, whereas the M_r 57 000/52 000 bands were not. It was therefore concluded that the M_r 46 000/42 000 species were catalytically active and that the M_r 57 000/52 000 species remained latent.

Immunoperoxidase staining with each of the Mabs revealed three characteristic patterns of reactivity (Figure 4B, Table II). One Mab (X-2a) reacted with the M_r 57 000/52 000 zymogen bands, but failed to recognize the activated M_r 46 000/42 000 enzyme (Figure 4B, lane 1); nine Mabs, including Mab III-7 shown in Figure 4B (lane 2) as a representative of this group, reacted equally well with the M_r 57 000/52 000 and M_r 46 000/42 000 bands; one Mab (2C5) did not react with any of the bands (Figure 4B, lane 3). The finding that the epitope recognized by Mab X-2a was destroyed during activation suggests that the antibody reacted with an amino acid sequence located in the 81-residue NH_2-terminal propeptide (Goldberg et al., 1986) or with a conformation-dependent structure that was lost as a result of internal rearrangement. Since it was subsequently found that the epitope was preserved even after complete reduction and denaturation of the zymogen (data not shown), it was concluded that Mab X-2a recognized a specific amino acid sequence in the propeptide. The epitope for Mab 2C5 apparently was lost or masked on Western blots and could not be iden-

230 240 250 260 270 280 Residue

---AQDDIDGIQAIYGRSQNPVQPIGPQTPKACDSKLTFDAITTIRGEVMFFKDRFYMRT-- procollagenase

IGPQTPKACDSKLTFDAITTIRGEVMFFKDRFYMRT-- Mr 22,000 peptide

FIGURE 6: NH_2-terminal sequence of the M_r 22000 peptide. The peptide was purified from spontaneously degrading procollagenase by reversed-phase HPLC. A stretch of 40 amino acids was sequenced by stepwise Edman degradation and aligned with the amino acid sequence of human fibroblast procollagenases predicted from a cDNA clone (Goldberg et al., 1986). The numbering is from the predicted NH_2 terminus of the procollagenase molecule. The sequence showed that the M_r 22000 peptide was formed by cleavage of the Pro_{250}–Ile_{251} bond.

tified. The remaining nine Mabs recognized epitopes in the M_r 46000/42000 catalytic enzyme.

To further narrow down the location of these nine epitopes, we took advantage of the autocatalytic cleavage of M_r 57000/52000 procollagenase into two major fragments (Figure 5A). With conventional molecular mass standards, M_r's were 33000 and 22000 in the native state (Figure 5A, lane 2) and 30000 and 25000 after reduction and denaturation. This cleavage is responsible for the instability of the proenzyme upon storage and ultimately results in complete degradation of the enzyme protein (Figure 5A). The reactivity of Mabs with the M_r 33000 and M_r 22000 procollagenase fragments is shown in Figure 5B and Table II. With the exception of Mab 2C5 (which did not stain), each of the Mabs recognized the M_r 33000 fragment and none reacted with the M_r 22000 fragment. The failure of any of the Mabs to recognize the M_r 22000 fragment was subsequently verified by Western blots of the isolated peptide (data not shown). Even the polyclonal antibody reacted poorly with this fragment (Figure 5B, lane 1).

The M_r 33000 fragment was recognized by the procollagenase-specific Mab X-2a (Figure 5B, lane 4) and therefore constituted the NH_2-terminal part of the zymogen, which has been shown to contain the propeptide (Goldberg et al., 1986). Like native procollagenase, the "M_r 33000 fragment" consisted of a group of two/three peptides that migrate within a 3000–4000 M_r range and therefore in all likelihood still contain the two suspected N-glycosylation sites (Asn_{120}, Asn_{143}). To place the smaller M_r 22000 fragment within the molecule, we have isolated ~2 mg of this fragment by reversed-phase HPLC (Figure 5, lane 3) and sequenced a stretch of 45 amino acid residues from the NH_2 terminus. When aligned with the sequence of the intact procollagenase molecule (Goldberg et al., 1986), this partial sequence unequivocally established that the fragment was formed by cleavage of the Pro_{250}–Ile_{251} bond in the zymogen molecule (Figure 6). On the basis of an average residue weight of 115, we predict that the fragment accounts for all, or nearly all, of the 205-residue COOH-terminal portion of the enzyme that follows the Pro_{250}–Ile_{251} cleavage site. Together, the M_r 33000 and M_r 22000 fragments appear to account for all, or nearly all, of the intact M_r 57000/52000 procollagenase molecule.

The M_r 33000 fragment was not removed by α2-M and appears to be devoid of catalytic activity, at least before exposure to SDS (Figure 5C). However, the four minor bands that constitute active and intermediary forms of collagenase (Stricklin et al., 1983) were removed by the inhibitor (Figure 5C). The intermediary species at M_r 48000/44000 are probably identical with the partially converted molecules described by Grant et al. (1987), which have retained 18 residues of the propeptide proximal to the Phe_{81}–Val_{82} cleavage site. Although the M_r 33000 fragment appears to be devoid of catalytic activity, it can be "activated" in a manner similar to the intact M_r 57000/52000 collagenase precursor either

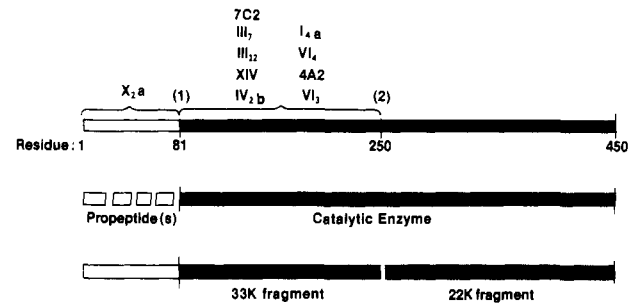

FIGURE 7: Assignment of epitopes to domains within the procollagenase molecule. Linear model of procollagenase constructed after the cDNA sequence published by Goldberg et al. (1986). The M_r 52000 proenzyme consists of an 81-residue propeptide followed by a 369-residue catalytic enzyme (M_r 42000). Activation results in loss of the 81-residue propeptide (1), which yields several small peptides and the 369-residue catalytic protein. Internal cleavage at the Pro_{250}–Ile_{251} bond (2) yields a M_r 33000 fragment, which contains the intact propeptide and a M_r 22000 COOH-terminal domain. Nine Mabs reacted with the 169-residue fragment between the propeptide and Pro_{250}; one (X-2a) reacted with the propeptide; none reacted with the M_r 22000 COOH-terminal fragment.

spontaneously or by exposure to trypsin, 4-APMA, or SDS (Birkedal-Hansen et al., unpublished data). The catalytic status of the M_r 22000 COOH-terminal fragment could not be determined because of its poor reactivity with both mono- and polyclonal antibodies.

The findings summarized above permit us to assign 10 of the epitopes to domains within the procollagenase molecule in the following manner (Figure 7): The Mab X-2a epitope is located in the 81-residue propeptide; the remaining 9 epitopes are located in a 169-residue segment, which lies downstream from the propeptide and constitutes the NH_2-terminal portion of the activated enzyme; no epitope has yet been identified in the 205-residue COOH-terminal domain.

DISCUSSION

This study describes the properties of a complement of monoclonal antibodies to human fibroblast collagenase/procollagenase. Taking advantage of the fragmentation of the collagenase zymogen, which occurs during activation of the enzyme and during an apparently autocatalytic internal cleavage of the molecule, we were able to assign the epitopes to one of three major molecular domains. Nine of 11 epitopes were clustered within a 169-residue segment that constitutes the NH_2-terminal domain of the activated enzyme, one was located in the propeptide, and one could not be assigned. The clustering of epitopes in the 169-residue NH_2-terminal portion of the active enzyme and the failure to identify any epitopes in the larger 205-residue COOH-terminal domain suggest that the COOH-terminal half of the enzyme is poorly immunogenic and possibly lies buried inside the protein.

Each of 4 inhibitory antibodies recognized epitopes located in the 169-residue domain. Although it is possible that an antibody may bind at some distance from the active site and

still interfere measurably with the rate of catalysis of a macromolecular substrate, this observation is consistent with other evidence summarized below which suggests that the active site is located in this (169-residue) domain. The active site has not been identified with certainty, but Whitham et al. (1986) and McKerrow (1987) have noted that residues 213–228 constitute a potential Zn^{2+} binding site, which is probably also the active site. This domain is located in the 169-residue domain, approximately 22 residues upstream from its COOH terminus (see Figure 7). In addition, studies under way in our laboratory (Birkedal-Hansen, Fields, and Van Wart, unpublished) have shown that the M_r 33 000 fragment (which consists of the propeptide and the 169-residue domain) is a latent protease, which can be activated to generate two/three catalytically active fragments in the M_r 20 000–23 000 range. These fragments are distinct from the single (poorly immunogenic) M_r 22 000 COOH-terminal peptide generated by cleavage of the proenzyme at Pro_{250}–Ile_{251} (Figure 7). In our experience, catalytically active M_r 20 000–23 000 fragments can be generated from the latent M_r 33 000 fragment either autocatalytically or by trypsin. They are also formed as spontaneous degradation products of the M_r 46 000/42 000 active enzyme and, in Figure 4A (lanes 1, 4), emerge as two/three weak α2-M sensitive bands seen toward the bottom of the gel.

Mab X-2a recognized specifically the M_r 57 000/52 000 form of the enzyme. Conversion of M_r 57 000/52 000 to M_r 46 000/42 000 forms (autocatalytically or by limited proteolysis) resulted in generation of an α2-M reactive catalytic site and in loss of the X-2a epitope. Since this Mab reacted equally as well with the denatured and completely unfolded forms of the enzyme as with the intact, native proenzyme, we suspect that the antibody recognizes a specific amino acid sequence in the propeptide polypeptide chain rather than a conformational epitope. If so, the antibody would appear to discriminate between various molecular mass forms of collagenase and not necessarily between latent or active enzyme. The additional observation that Mab X-2a does not recognize any of the well-resolved 48 000/44 000 intermediates formed during activation (Figure 4B, lane 4) permits us to further narrow the location of this epitope. The M_r 48 000/44 000 bands apparently correspond to the smallest intermediates formed during the activation reaction, which have retained $\sim$18 residues of the propeptide (Grant et al., 1987). It is therefore most likely that the epitope is contained partially or completely within the first 63 amino acid residues of the propeptide. Once it had been determined that the Mab X-2a epitope was located in the propeptide, it became possible to assign the M_r 33 000 autocatalytic fragment to the NH_2-terminal portion of the zymogen and, ultimately, to assign the remaining epitopes to the 169-residue domain located downstream from the propeptide (Figure 7).

The inhibitory Mabs did not measurably block the action of human PMN leukocyte collagenase, nor did they block the action of interstitial collagenases from a more distant species such as rat. This finding is in agreement with the observation made by Hasty et al. (1984) that an inhibitory Mab raised against PMN leukocyte collagenase did not inhibit fibroblast-type collagenase. Together with evidence of differences between the two enzymes in terms of molecular size (Stricklin et al., 1977; Mccartney & Tschesche, 1983), substrate specificity (Horwitz et al., 1977), and storage/release (Hasty et al., 1986), these findings support the notion that fibroblast-type and PMN leukocyte-type collagenases are distinct gene products. The existence of two genetically distinct proteases with identical or highly overlapping catalytic functions, however, is not unique. It is well established that the metabolic activation of plasminogen may proceed along one of two pathways, mediated by either urokinase-type (u-Pa) or tissue-type (t-Pa) plasminogen activators (Dano et al., 1984). A body of evidence suggests that the two enzymes serve somewhat different biologic functions in that t-Pa is a vascular protease that dissolves fibrin clots whereas u-Pa mediates extravascular plasmin-dependent proteolysis in the tissues (Dano et al., 1984). Although PMN leukocytes and stromal cells (which produce fibroblast-type collagenase) are involved in different biologic functions, it is not yet clear whether the two enzymes perform distinct or identical catalytic functions in the dissolution of collagen fibrils. It also remains unclear why it is either necessary or advantageous to evolve two distinct proteases with highly overlapping catalytic functions.

ACKNOWLEDGMENTS

The expert technical and secretarial assistance of Lester Jack Windsor, Milton Pierson, Jennifer Stevens, Beverly Joseph, and Brenda Carroll is acknowledged. The monoclonal antibodies used in this study were prepared with the assistance of the Hybridoma Core Facility of the Multipurpose Arthritis Center at UAB.

Registry No. Procollagenase, 39287-99-5; collagenase, 9001-12-1.

REFERENCES

Abe, S., & Nagai, Y. (1972) *J. Biochem. (Tokyo) 71*, 919–922.

Abe, S., Shinmei, M., & Nagai, Y. (1973) *J. Biochem. (Tokyo) 73*, 1007–1011.

Aggeler, J., Frisch, S. M., & Werb, Z. (1984) *J. Cell Biol. 98*, 1656–1661.

Bhown, A. S., Cornelius, T. W., Mole, J. E., Lynn, J.-D., Tidwell, W. A., & Bennett, J.-C. (1980) *Anal. Biochem. 102*, 35–38.

Bhown, A. S., Mole, J. E., & Bennett, J.-C. (1981) *Anal. Biochem. 110*, 355–359.

Birkedal-Hansen, H. (1987) *Methods Enzymol. 144*, 140–171.

Birkedal-Hansen, H., & Dano, K. (1981) *Anal. Biochem. 115*, 18–26.

Birkedal-Hansen, H., & Taylor, R. E. (1982) *Biochem. Biophys. Res. Commun. 107*, 1173–1178.

Birkedal-Hansen, H., Cobb, C. M., Taylor, R. E., & Fullmer, H. M. (1976) *J. Biol. Chem. 251*, 3162–3168.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Bøyum, A. (1974) *Tissue Antigens 4*, 269–274.

Burnette, W. N. (1981) *Anal. Biochem. 112*, 195–203.

Dano, K., Andreasen, P. A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L. S., & Skriver, L. (1984) *Adv. Cancer Res. 44*, 139–266.

Eekhout, Y., & Vaes, G. (1977) *Biochem. J. 166*, 21–31.

Goldberg, G. I., Wilhelm, S. M., Kronberger, A., Bauer, E. A., Grant, G. A., & Eisen, A. Z. (1986) *J. Biol. Chem. 261*, 6600–6605.

Grant, G. A., Eisen, A. Z., Marmer, B. L., Roswit, W. T., & Goldberg, G. I. (1987) *J. Biol. Chem. 262*, 5886–5889.

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

Hasty, K. A., Hibbs, M. S., Kang, A. H., & Mainardi, C. L. (1986) *J. Biol. Chem. 261*, 5645–5650.

Heussen, C., & Dowdle, E. B. (1980) *Anal. Biochem. 102*, 196–202.

Horwitz, A. L., Hance, A. J., & Crystal, R. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 897–901.

Howard, J. B., Vermeulen, M., & Swenson, R. (1980) *J. Biol. Chem. 255*, 3820-3823.
Kearney, J. F. (1984) in *Fundamental Immunology* (Paul, W. E., Ed.) pp 751-766, Raven, New York.
Kurecki, T., Kress, L. F., & Laskowski, M. (1979) *Anal. Biochem. 99*, 415-420.
Larsson, L. I., Skriver, L., Nielsen, L. S., Grondahl-Hansen, J., Kristensen, P., & Dano, K. (1984) *J. Cell Biol. 98*, 894-903.
Lin, H.-Y., Wells, B. R., Taylor, R. E., & Birkedal-Hansen, H. (1987) *J. Biol. Chem. 262*, 6823-6831.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Mccartney, H. W., & Tschesche, H. (1983) *Eur. J. Biochem. 130*, 71-78.
McKerrow, J. H. (1987) *J. Biol. Chem. 262*, 5943.
Murphy, G., Reynolds, J. J., & Werb, Z. (1985) *J. Biol. Chem. 260*, 3079-3083.
Nagase, H., Cawston, T. E., De Silva, M., & Barrett, A. J. (1982) *Biochim. Biophys. Acta 702*, 133-142.
Neville, D. M., Jr. (1971) *J. Biol. Chem. 246*, 6328-6334.
Otsuka, K., Sodek, J., & Limeback, H. (1984) *Eur. J. Biochem. 145*, 123-129.
Rosenfeld, M. G., Kreibich, G., Popor, D., Kato, K., & Sabatini, D. D. (1982) *J. Cell Biol. 93*, 135-143.
Sottrup-Jensen, L., Stepanick, T. M., Jonew, C. M., Lonblad, P. B., Kristensen, T., & Wierzbicki, D. M. (1984) *J. Biol. Chem. 259*, 8293-8303.
Stricklin, G. P., Bauer, E. A., Jeffrey, J. J., & Eisen, A. Z. (1977) *Biochemistry 16*, 1607-1615.
Stricklin, G. P., Jeffrey, J. J., Roswit, W. T., & Eisen, A. Z. (1983) *Biochemistry 22*, 61-68.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1173-1178.
Wahl, L. M., Olsen, C. E., Sandberg, A. L., & Mergenhagen, S. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4955-4958.
Welgus, H. G., Campbell, E. J., Bar-Shavit, Z., Senior, R. M., & Teitelbaum, S. L. (1985) *J. Clin. Invest. 76*, 219-224.
Whitham, S. E., Murphy, G., Angel, P., Rahmsdorf, H. J., Smith, B., Lyons, A., Harris, T. J. R., Reynolds, J. J., Herrlich, P., & Docherty, A. J. P. (1986) *Biochem. J. 240*, 913-916.
Wilhelm, S. M., Eisen, A. Z., Teter, M., Clark, S. D., Kronberger, A., & Goldberg, G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3756-3760.

Calculation of Rate and Equilibrium Constants for a Ping-Pong Mechanism from Steady-State Data†

Theodore S. Jardetzky*,‡ and Mark Seville*,§

Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138, and Department of Biochemistry, Biophysics and Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80262

Received February 5, 1988; Revised Manuscript Received April 26, 1988

ABSTRACT: A method for the determination of individual rate constants and equilibrium constants from simple steady-state kinetic data is presented for a simple ping-pong mechanism. This involves determining the Michaelis constants and catalytic constants for three pairs of amino acids and keto acids. The equations that relate these steady-state constants to the equilibrium and rate constants are derived. The usefulness and limitations of the treatment are discussed in terms of hypothetical case studies. The method is then used to derive the dissociation constants and rate constants for an experimental system consisting of *Escherichia coli* tyrosine aminotransferase and several of its substrates. This simple analysis will be of use in the study and comparison of enzymes generated by site-directed mutagenesis where multiple time-consuming studies become impractical.

The determination of microscopic rate constants for enzyme-catalyzed reactions provides valuable information in the detailed analysis and comparison of enzyme reaction mechanisms. It is a complicated and time-consuming process involving the use of specialized equipment, which is not available in most laboratories. Here we report a method by which individual rate constants can be estimated from simple steady-state data that can be obtained on a standard bench-top spectrophotometer. In addition, the method provides a way of calculating the enzyme–substrate equilibrium constants.

The ping-pong mechanism originally proposed by Cleland (1963) adequately describes the sequential two-substrate reaction catalyzed by aminotransferases. The transamination reaction is normally fully reversible and can be measured in either direction. Thus, the actual number of substrates that can be utilized for kinetic analysis is, in fact, four (two sets of two). For any four substrate system, which consists of two amino acids and the two corresponding keto acids, a minimum of eight rate constants are required to define the fully reversible pair of transaminations. The full complement of steady-state parameters consists of four Michaelis constants, two catalytic constants, and four inhibition constants.

Cleland (1963) has previously derived the relationship between these steady-state parameters and the individual rate constants. Unfortunately, determination of all the steady-state parameters, which can be obtained from a given four-substrate

† This work was initiated at the Department of Biophysical Chemistry, Biozentrum, Basel, Switzerland, and supported in part by Swiss National Science Foundation Grant 30.098-0.85, awarded to K. Kirschner.
* Correspondence should be addressed to either author.
‡ Harvard University.
§ University of Colorado.

0006-2960/88/0427-6758$01.50/0