Biochemistry

 Volume 19, Number 14 July 8, 1980

Kinetics of Activation of Prekallikrein by Prekallikrein Activator†

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ABSTRACT: A 28 000 molecular weight activator of prekallikrein was isolated from human plasma, and the kinetics of its enzymic activity toward prekallikrein was investigated. The activation follows Michaelis–Menten kinetics with a k_{cat} of ~ 3 s^{-1}; K_m is strongly dependent upon the ionic strength. Under suitable conditions the activation obeys first-order kinetics, and the first-order rate constant may be used to quantitate prekallikrein activator activity. Thus a two-stage assay, in which the first step involves the activation of prekallikrein by the activator and the second step quantitates the kallikrein generated, was developed to allow the measurement of prekallikrein activator in biologic samples. The prekallikrein activator content of therapeutic protein solutions, as determined by means of this assay, correlated well with the hypotensive activity of these solutions as determined in an animal model.

Factor XII (Hageman factor) is the zymogen of a serine protease, factor XIIa, which initiates a sequence of enzymic reactions leading to blood coagulation [for a review, see Kaplan (1978)]. It is also the first component of the plasma kinin forming system. In plasma, factor XIIa converts PK[1] to kallikrein which in turn liberates the vasoactive peptide bradykinin from high molecular weight kininogen. High molecular weight kininogen circulates in plasma as a complex with PK (Mandle et al., 1976).

Human factor XIIa consists of two polypeptide chains joined by one or more disulfide bridges; it has a M_r of $\sim 80\,000$, similar to that of the single-chain factor XII molecule (Revak et al., 1977). Other active forms of factor XII readily activate PK but have a diminished capacity to activate factor XI and, thereby, to initiate the intrinsic coagulation pathway (Kaplan & Austen, 1970; Radcliffe & Nemerson, 1976; Bagdasarian et al., 1973a). One such form, PKA, has been characterized as a 28 000 M_r fragment resulting from proteolytic cleavage by kallikrein, trypsin, factor XIa, or plasmin (Kaplan & Austen, 1971; Revak et al., 1974; Griffin, 1978). It has been variously designated factor XIIf (Movat & Özge-Anwar, 1974), Hageman factor fragments (McMillin et al., 1974), or prealbumin activator (Kaplan et al., 1972).

Our interest in PKA stemmed from sporadic reports of hypotension following the rapid infusion of a therapeutic fraction of human plasma, PPF, which is used primarily as a plasma volume expander. The hypotensive activity of certain lots of PPF was shown to be related to the occurrence of PKA in those lots (Alving et al., 1978). PKA apparently catalyzes the activation of PK in vivo with subsequent generation of bradykinin. Attempts to quantitate PKA in PPF solutions by means of existing biochemical methods (Ulevitch et al., 1974; Bagdasarian et al., 1973b; Laake & Venneröd, 1973) were unsuccessful. Accordingly, we have purified PKA, PK, and kallikrein for the purpose of elucidating the detailed kinetics of their enzymic operation as manifested with a variety of substrates. A knowledge of the kinetics of enzymic activation of PK to kallikrein has allowed the development of sensitive methods for the quantitation of low levels of PKA in therapeutic plasma fractions as well as in other biologic solutions.

Materials and Methods

Preparation of PK. Fresh human plasma (6.5 L) was collected in 0.38% sodium citrate dihydrate and diafiltered with 3 volume exchanges of 0.05 M Tris-HCl buffer (pH 8.0) utilizing a DC-30 hollow-fiber ultrafiltration unit (Amicon Corp., Lexington, MA). It was then passed through a 37 × 15 cm column of DEAE-Sephadex A-50 equilibrated and eluted with 0.05 M Tris-HCl buffer (pH 8.0), collecting 1-L fractions. PK was found in fractions 11–30, which were pooled, adjusted by addition of 6 M HCl and solid NaCl to 0.25 M NaCl and pH 7.4, and passed through a 5 × 17 cm column of Con A–Sepharose (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) equilibrated with 0.05 M Tris-HCl and 0.25 M NaCl (pH 7.4). The column was washed with 1.5 L of the same buffer, and then the PK was eluted with 1 L of 0.5 M α-methyl D-mannoside (Sigma Chemical Co., St. Louis, MO), 0.05 M Tris-HCl and 0.25 M NaCl (pH 7.4). All procedures up to this point were carried out at room temperature (22–25 °C) in plastic, stainless steel, or siliconized glass apparatus within 20 h of plasma collection. The α-methyl D-mannoside

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[1] Abbreviations used: PK, prekallikrein; PKA, prekallikrein activator; M_r, molecular weight; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; TAME, N^{α}-(*p*-toluenesulfonyl)-L-arginine methyl ester; AGLME, acetylglycyl-L-lysine methyl ester; $NaDodSO_4$, sodium dodecyl sulfate; Con A, concanavalin A; PPF, plasma protein fraction (USP).

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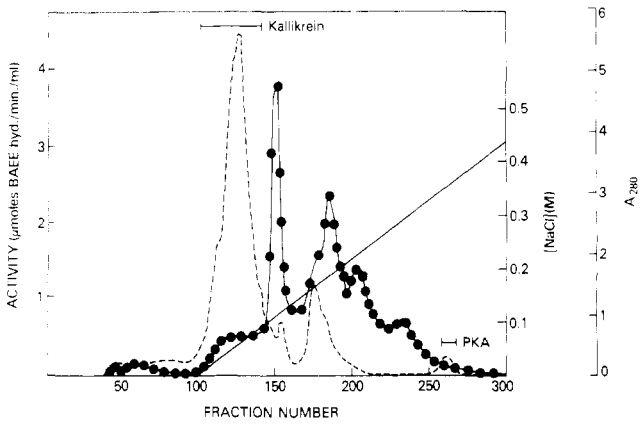

FIGURE 1: QAE-Sephadex A-50 chromatography of the kallikrein and PKA activity recovered from dye–Sephadex G-100 chromatography. The sample and the QAE-Sephadex were equilibrated with 0.05 M Tris-HCl buffer (pH 8.0). The column was developed with a linear NaCl gradient (0–0.5 M, 2 L), and fractions of 10 mL were collected. Fractions 100–140, containing kallikrein, and fractions 258–267, containing PKA, were separately pooled and concentrated as described in the text. BAEE esterase activity (- - -); NaCl concentration (—); A_{280} (●).

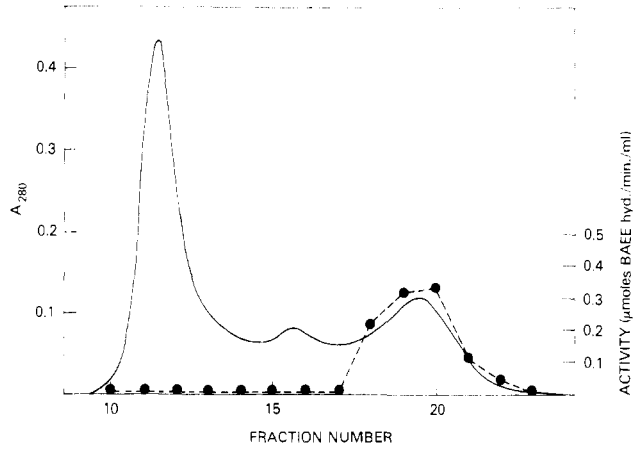

FIGURE 2: Sephadex G-100 chromatography of PKA recovered from the QAE-Sephadex A-50 column. The column was equilibrated with 0.05 M sodium phosphate and 0.05 M NaCl (pH 7.5) at a flow rate of 50 mL/h, and 10-mL fractions were collected. Fractions 18–22, containing PKA, were pooled and concentrated as described in the text. BAEE activity (●); A_{280} (—).

eluate was exhaustively dialyzed against 0.05 M sodium phosphate and 0.05 M NaCl (pH 7.5) at 5 °C. The resulting solution (920 mL) contained 2.6 PK units/mL with a specific activity of 3.3 units/mg. (One PK unit is the amount which, upon complete conversion to kallikrein, catalyzes the hydrolysis of 0.5 mM BAEE at an initial rate of 1 μmol/min at 37 °C, pH 8.0.) This solution in 1-mL aliquots was stored at −20 °C until used.

Preparation of PKA. The starting material for this preparation was supernate II + III from the Cohn fractionation (method 6) of human plasmapheresis plasma at Cutter Laboratories, Inc., Berkeley, CA (Cohn et al., 1946). This fraction contained primarily albumin (~75%), α-globulins, and β-globulins. To 40 L of supernate II + III (containing 20% ethanol and 30 mg of protein per mL) was added 40 g of Super-Cel (Johns Manville, Port Credit, Ontario), and the suspension was stirred for 14 h at −6 °C. The Super-Cel was collected by centrifugation, resuspended in 0.5 L of the supernatant liquid, and stirred for 16 h at room temperature (~23 °C). This step was found to be essential for recovery of PKA in solution; prior to incubation at room temperature the PK-activating activity was much lower and tightly bound to the surface of the Super-Cel. The suspension was filtered through a sintered-glass funnel and the filtrate dialyzed for 24 h at ~23 °C against 0.15 M NaCl to remove the ethanol. Albumin was removed by passing the dialyzed solution through a 5 × 29 cm column of a dye–Sephadex G-100, i.e., 1-amino-4-[[5-[(5-chloro-2,4-difluoropyrimidin-6-yl)amino]-2,4-disulfophenyl]amino]-9,10-dihydro-9,10-dioxo-2-anthracenesulfonic acid coupled to Sephadex G-100 [described as RD-2-Sephadex G-100 in Schroeder (1977)], and eluting the column with 0.05 M Tris-HCl and 0.15 M NaCl (pH 8.0). The eluate from this column (0.6 L) was free of albumin but contained substantially all of the PKA activity applied. It was freeze-dried and subsequently reconstituted in 55 mL of water. The resulting solution was dialyzed against 0.05 M Tris-HCl (pH 8.0) to give 62 mL of a solution containing 53 PKA units/mL with a specific activity of 1.08 units/mg. (The PKA unit is defined under Results.) This was applied to a 2.6 × 93 cm column of QAE-Sephadex A-50 equilibrated with 0.05 M Tris-HCl (pH 8.0) and developed with a linear gradient of NaCl (0–0.5 M, 2 L) in this buffer. The PKA was eluted at a salt concentration of 0.35 M. PKA-containing fractions (258–267, Figure 1) were pooled, concentrated by ultrafiltration (Amicon PM-10 membrane), and dialyzed against 0.05 M sodium phosphate and 0.05 M NaCl (pH 7.5) to give 10 mL of a solution containing 247 PKA units/mL with a specific activity of 217 units/mg. This was applied to a 2.5 × 72 cm column of Sephadex G-100 which was equilibrated with 0.05 M sodium phosphate and 0.05 M NaCl (pH 7.5) and eluted with the same buffer at a flow rate of 50 mL/h. The PKA activity was found only in the final protein peak, whose elution volume corresponded to a M_r of 30000 (Figure 2). Fractions 18–22 were pooled and concentrated by ultrafiltration (Amicon PM-10 membrane) to a volume of 2.3 mL containing 746 PKA units/mL with a specific activity of 1020 units/mg.

Preparation of Kallikrein. The initial peak of esterase activity which was eluted from the QAE-Sephadex A-50 column during purification of PKA was found to be due to kallikrein (Figure 1). Fractions 100–140 were pooled, concentrated by ultrafiltration (Amicon XM-50 membrane), and dialyzed against 0.05 M sodium phosphate and 0.05 M NaCl (pH 7.5) to give 15 mL of solution containing 70 kallikrein units/mL with a specific activity of 10 units/mg.

Kinetic Methods. Rates of hydrolysis of BAEE were determined by using a Gilford 240 spectrophotometer equipped with a thermostated (37 °C) cuvette compartment and chart recorder. The sample (1–100 μL) was added to 2.0 mL of 0.5 mM BAEE, 0.05 M sodium phosphate, and 0.15 M NaCl (pH 8.0) in a quartz cuvette, and the absorbance at 253 nm was recorded for 5–10 min. The rate of change of absorbance, ΔA_{253}/min, was divided by 0.57 to determine the rate of BAEE hydrolysis in μmol/min. [The ϵ_{253} for BAEE hydrolysis is 1140; see Schwert & Takenaka (1955).]

Rates of hydrolysis of TAME and AGLME were determined titrimetrically with a Radiometer titrator (TTT-1c) and autoburet assembly (ABU-12). These hydrolysis reactions were carried out at 37 °C under N_2. The desired pH was maintained by continuous titration with 10 mM NaOH, and corrections were made for spontaneous hydrolysis of the substrates. Hydrolysis rates of BAEE were also determined titrimetrically when high substrate concentrations precluded the use of the spectrophotometric technique.

Kinetic data were analyzed by the method of Lineweaver & Burk (1934) to determine K_m and k_{cat}. Straight lines were fitted to data points by the method of least squares. The molecular weights of kallikrein and PKA were taken to be

85 000 and 28 000, respectively, in all calculations.

Assays for PKA. (*a*) *Two-Stage Assay.* PK [50 μL, 2–3 PK units/mL in 0.05 M sodium phosphate and 0.05 M NaCl (pH 7.5)] was warmed to 37 °C whereupon 25 μL of the PKA-containing sample was added and the mixture incubated at 37 °C for a measured time (2–100 min). A 25 μL aliquot of the mixture was then withdrawn, and the amount of kallikrein generated was determined, usually by the spectrophotometric (BAEE) procedure. The initial concentration of PK ($[PK]_0$) in the incubation mixture was determined by substituting a concentrated PKA solution (3–10 PKA units/mL) for the sample and incubating for a time sufficient to convert substantially all of the PK to kallikrein (10–20 min). The PKA concentration of the sample was calculated from the integrated first-order rate equation (see Results).

(*b*) *One-Stage Assay.* PK (50 μL, 2–3 PK units/mL) was added to 2.0 mL of 0.5 mM BAEE, 0.05 M sodium phosphate, and 0.05 M NaCl (pH 7.5) contained in a cuvette. This was allowed to equilibrate at 37 °C, the PKA-containing sample (1–50 μL) was added, and the absorbance at 253 nm was recorded for 10–15 min. A parabolic curve was generated, from which the PKA content of the sample was determined (see Results). Alternatively, titrimetric one-stage assays for PKA were carried out by adding 100 μL of PK to 6 mL of 0.0625 M TAME and 0.20 M NaCl in the titration cell and adjusting to pH 7.5 with titrant (10 mM NaOH), followed by the addition of water to a final volume of 7.4 mL. The PKA-containing sample (100 μL) was added, and the delivery of the titrant was continuously recorded with time. The PKA content of the sample was determined from the generated curve in a manner analogous to that of the spectrophotometric one-stage assay.

(*c*) *Direct Assay.* In addition to its ability to catalyze the activation of PK, PKA is capable of hydrolyzing the esters BAEE, TAME, and AGLME. Thus it was possible to follow PKA in the course of its purification by means of its direct activity toward these substrates.

Other Materials and Methods. Electrophoresis in 5% polyacrylamide gels containing $NaDodSO_4$ was performed by the method described in Bertolini et al. (1976). Immunoelectrophoresis in 1% agarose (Bio-Rad Laboratories, Inc., Richmond, CA) and 0.017 M barbital buffer (pH 8.6) was carried out at 10 V/cm for 45 min. Goat antisera to human factor XII and human PK were generously supplied by Dr. John Griffin (Scripps Clinic and Research Foundation, La Jolla, CA). Other antisera were purchased from Hyland Laboratories, Costa Mesa, CA. Purified human plasma kallikrein, isolated from Cohn fraction IV-1 (Sampaio et al., 1974), was kindly provided by Dr. Elliott Shaw (Brookhaven National Laboratory, Upton, NY). This material had a specific activity of 35 kallikrein units/mg in our assay system and appeared to be ~95% pure by $NaDodSO_4$–polyacrylamide gel electrophoresis. Coagulation assays for factor XI were performed by a modification of the activated partial thromboplastin time (Langdell et al., 1953), with plasma from patients congenitally deficient in factor XI (George King Biomedical, Inc., Overland Park, KN). One unit of factor XI is defined as the amount contained in 1 mL of normal human plasma.

Protein concentrations were determined by measuring the absorbance at 280 nm and employing an arbitrary absorption coefficient of 1. Protein solutions were concentrated by positive-pressure (N_2) ultrafiltration in Amicon stirred cells using the membranes described. Vacuum dialysis in collodion bags (No. 100, Schleicher & Schuell) was employed to further concentrate solutions of PK used for K_m determinations.

Results

Preparation and Characterization of PK. Chromatography of human plasma on DEAE-Sephadex at low ionic strength produced an eluate rich in factor XI, PK, and immunoglobulin (IgG) and substantially free of α- and β-globulins and albumin. This eluate, after suitable concentration, could be used as a substrate for PKA without further purification. Removal of the bulk of the IgG by means of Con A–Sepharose chromatography, however, afforded a 20- to 50-fold purification while providing efficient concentration. Following Con A–Sepharose chromatography, immunoelectrophoresis of the PK with antiserum to normal human serum or antiserum to human IgG produced identical cathodal precipitin arcs; no arcs were seen with anti-IgA or anti-IgM. This demonstrates that even after the Con A–Sepharose step, IgG is a major contaminant of the PK. Antiserum to human PK produced a precipitin arc slightly more cathodal than that produced by anti-IgG; the position and intensity of the arc was unchanged when the PK was activated (with PKA) prior to immunoelectrophoresis.

The PK was devoid of esterolytic activity toward BAEE or TAME, and no such activity developed upon incubation at 37 °C for 16 h with or without added Super-Cel. When kallikrein (10 μL, 70 kallikrein units/mL) was admixed with PK (350 μL, 2.6 PK units/mL), the resulting solution was found to have the same BAEE esterolytic activity as that observed when the kallikrein was added to a buffer control. Such a mixture of kallikrein and PK could be incubated for 4 h at 37 °C without a change in BAEE esterolytic activity. These results indicate that the PK preparation was free of kallikrein, factor XII or XIIa, and kallikrein inhibitors and demonstrate that PK is not activated by kallikrein. Absence of kininogen was demonstrated by incubating the PK with trypsin and testing the incubate for kinin by means of a rat-uterus assay.

PK prepared in the manner described contained 2.4 units of factor XI/mL. In some preparations, factor XI was removed by employing an additional purification step involving chromatography on SP-Sephadex C-50 (Kaplan et al., 1972). Following such purification the specific activity of the PK was increased to 15.2 PK units/mg, and the only trace quantities of factor XI could be detected. Nevertheless, activation kinetics (with PKA) of this purified PK were identical with those observed when PK preparations containing substantial amounts of factor XI were employed. Thus, the contamination of PK preparations by factor XI does not appear to be detrimental to the use of such preparations as a substrate for PKA.

Preparation and Characterization of PKA. Cohn supernate II + III, though it contains only a small proportion of factor XII in plasma, was found to be a convenient source of PKA. Stirring this supernate with Super-Cel at −6 °C in the presence of 20% ethanol (conditions under which plasma protease inhibitors are practically inactive) resulted in adsorption of virtually all of factor XII and at least partial activation to factor XIIa, which remains bound to the surface. This could be demonstrated by the ability of the centrifuged Super-Cel pellet to activate PK; however, the supernatant solution lacked this capacity. The supernate did, however, contain active kallikrein. When the Super-Cel was collected and suspended in a small volume of this kallikrein-containing supernate at room temperature, a slow release of PKA into the solution occurred and was substantially complete in 16 h. The formation of PKA was apparently the result of proteolysis by kallikrein of the surface-bound factor XII/XIIa (Griffin, 1978). After removal of the Super-Cel, the PKA was purified by the procedures described under Materials and Methods.

Table I: Kinetic Constants for TAME and BAEE Hydrolysis by Kallikrein at 37 °C, pH 8.0

substrate	K_m (mM)	k_{cat} (s^{-1})
TAME in H_2O	6.6	98
TAME in 0.15 M NaCl	4.2	115
BAEE in H_2O	0.36	54
BAEE in 0.15 M NaCl	0.17	65

Electrophoresis of the PKA preparation in polyacrylamide gels containing $NaDodSO_4$ revealed a major band (91%) migrating at a rate corresponding to a M_r of 28 000 and a minor band (9%) with a M_r of 40 000. Immunoelectrophoresis with antiserum to human factor XII revealed a single precipitin arc with prealbumin mobility. No arcs were observed on immunoelectrophoresis with antiserum to whole human plasma.

Sterile solutions of PKA at concentrations greater than 10 μg/mL were found to be quite stable at room temperature, showing no loss in activity after 1 year. Dilute solutions (1 ng–1 μg/mL) exhibited a rapid loss of activity through surface adsorption (to plastic as well as glass) which could be prevented by the addition of 0.1% gelatin or 0.1% albumin. Heating a solution of PKA in 5% albumin (pH 7) at 60 °C resulted in a first-order decay of activity with a $t_{1/2}$ of 17 h; at 56 °C a $t_{1/2}$ of 55 h was observed. The calculated activation energy for thermal denaturation of PKA is 2.7×10^5 J/mol.

Hydrolysis of TAME and BAEE by Kallikrein. The kallikrein obtained during the isolation of PKA (see Materials and Methods) was used for these studies. Comparison of the specific activity of this material (10 kallikrein units/mg) to that of the highly purified enzyme (35 kallikrein units/mg) established a purity of 29%. The kinetics were studied titrimetrically at 37 °C, pH 8.0, in water and in 0.15 M NaCl. TAME concentrations of 5, 10, 20, and 50 mM and BAEE concentrations of 0.2, 0.5, 2, and 5 mM were employed. The spontaneous hydrolysis rate at each concentration of substrate was subtracted from the rate observed in the presence of kallikrein (2.67 μg/mL final concentration). Calculated values for K_m and k_{cat} are given in Table I.

On the basis of this information, an assay for kallikrein was developed. In this assay the sample to be tested was admixed with BAEE (initial concentration 0.5 mM) in 0.05 M sodium phosphate and 0.15 M NaCl (pH 8.0), and the reaction was followed spectrophotometrically. Under these conditions the initial rate of BAEE hydrolysis was a linear function of kallikrein concentration, and spontaneous hydrolysis of BAEE was negligible.

Hydrolysis of AGLME, TAME, and BAEE by PKA. The hydrolysis of AGLME by PKA was studied titrimetrically at 37 °C, pH 7.0, at PKA concentrations of 1.9×10^{-8} and 3.8×10^{-8} M (0.6 and 1.2 PKA units/mL) and AGLME concentrations from 3 to 80 mM. Double reciprocal plots gave values of 3.6 mM for K_m and 10.1 s^{-1} for k_{cat}. No substrate inhibition was observed, even at 80 mM AGLME. The hydrolysis of TAME (50 mM) and BAEE (0.5 mM) by PKA was investigated titrimetrically at pH 8.0 and 37 °C. The k_{cat} for TAME hydrolysis was found to be 4.8 s^{-1}, and for BAEE a k_{cat} of 2.7 s^{-1} was estimated. K_m values were not determined.

These experiments demonstrated that accurate measurement of PKA by direct hydrolysis of synthetic substrates would require that the sample contain at least 1 PKA unit/mL. Since this is 10–1000 times the level typically found in samples of interest, it was necessary to develop more sensitive assays for low levels of PKA. The introduction of PK into the assay system in a manner such that the rate of kallikrein generation is a function of PKA concentration provides the basis for such assays (vide infra).

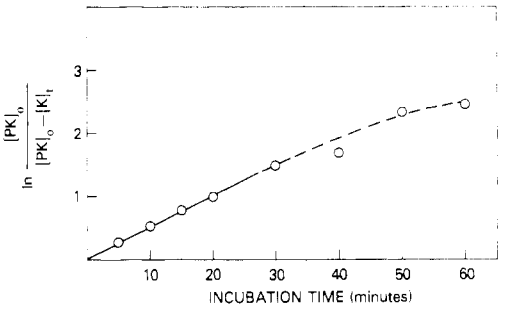

FIGURE 3: First-order reaction plot for the activation of PK by PKA at pH 7.5. PKA (0.25 mL of a solution containing 150 ng of PKA/mL in 0.1% albumin) was incubated with 0.50 mL of PK [2.6 PK units/mL in 0.05 M sodium phosphate and 0.05 M NaCl (pH 7.5)] at 37 °C. At the times indicated on the abscissa, 50 μL of the incubate was added to 2.0 mL of 0.5 mM BAEE, 0.05 M sodium phosphate, and 0.05 M NaCl (pH 8.0), and the rate of hydrolysis was determined spectrophotometrically. This rate of hydrolysis corresponds to the concentration of kallikrein generated in t minutes, i.e., $[K]_t$. The initial concentration of PK, i.e., $[PK]_0$, was determined by substituting a concentrated PKA solution (7 PKA units/mL) for the sample, incubating for 10 min (a time sufficient to convert all of the PK to kallikrein), and then transferring 50 μL of the incubate to the BAEE solution and determining the rate of hydrolysis spectrophotometrically. The quantity $[PK]_0 - [K]_t$, then, equals the concentration of PK remaining at time t, and the ordinate represents the natural logarithm of the reciprocal of the fraction of the initial PK concentration present at that time. Linearity in such a plot is indicative of first-order kinetics (Elkins-Kaufman & Neurath, 1948).

Table II: Kinetic Constants for the Activation of PK by PKA at 37 °C, pH 7.5[a]

buffer	I	K_m PK units/mL	K_m μM	k_{cat} (s^{-1})
0.05 M Tris-HCl	0.034	1.4	0.47	3.3
0.05 M Tris-HCl, 0.05 M NaCl	0.084	4.0	1.34	3.1
0.05 M Tris-HCl, 0.10 M NaCl	0.134	6.7	2.25	2.7
0.05 M Tris-HCl, 0.15 M NaCl	0.184	14.8	5.0	2.3
0.05 M Na_2HPO_4, 0.15 M NaCl	0.270	38	12.8	2.3

[a] K_m was initially determined in PK units/mL. After the purity (i.e., specific activity) of the PK preparation was determined, K_m was recalculated as micromolar concentration of prekallikrein. (One PK unit corresponds to 336 pmol of prekallikrein.) Both values are reported here, and the latter was used to compute k_{cat}.

Kinetics of Activation of PK by PKA. Initial experiments of the time course of kallikrein formation in incubates of PK with PKA suggested that, under the conditions employed, the rate of activation was first order in PK (Figure 3). This observation was in apparent conflict with earlier work (Laake & Venneröd, 1973) in which the activation appeared to follow zero-order kinetics at a substrate concentration of 2.2 PK units/mL.

Activation kinetics were determined at 37 °C, pH 7.5, at ionic strengths from 0.034 to 0.27. Substrate concentrations from 2.6 to 26 PK units/mL were employed. These data were analyzed by double reciprocal plots, which demonstrated a marked dependence of K_m upon the ionic strength; k_{cat} appeared to be only slightly influenced (Table II). Additional rate studies were performed at ionic strengths from 0.05 to 0.40. When the logarithm of k_{cat}/K_m was plotted against the square root of ionic strength, a straight line was obtained (Figure 4). This result is in accord with the Brønsted theory

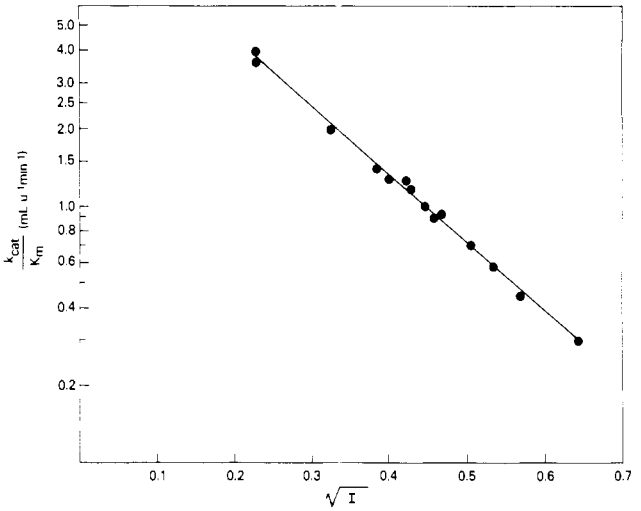

FIGURE 4: Effect of ionic strength (I) on the efficiency of activation of PK by PKA. The first-order rate constant for activation of PK (2.0 PK units/mL final concentration) by PKA (0.020 PKA unit/mL) was determined in 0.05 M Tris-HCl (pH 7.5) containing various concentrations of NaCl (0–0.4 M). k_{cat}/K_m was calculated by dividing the first-order rate constant (min^{-1}) by the PKA concentration (PKA units/mL).

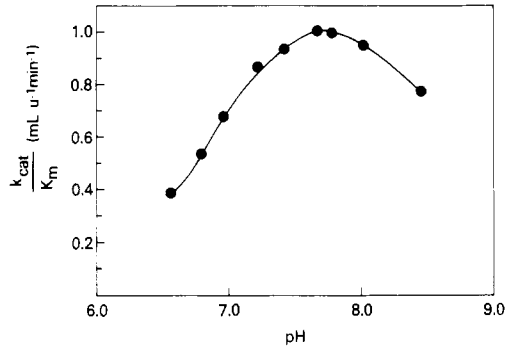

FIGURE 5: pH–rate profile for activation of PK by PKA. PK (3.3 units/mL in 0.05 M Na_2HPO_4 and 0.05 M NaCl) was adjusted with HCl to the pH values indicated. 100 μL of the PK solution was incubated with 50 μL of PKA (150 ng/mL in 0.2 M NaCl and 0.1% albumin) at 37 °C. A 50-μL aliquot of the mixture was assayed for kallikrein at 10–15 min by means of the spectrophotometric assay with BAEE as substrate, and the first-order rate constant was calculated as described in the text. k_{cat}/K_m was obtained by dividing the rate constant (in min^{-1}) by the PKA concentration in the incubation mixture (0.048 PKA unit/mL).

for a primary kinetic salt effect (Moore, 1955).[2]

The pH dependence of the activation was studied over the pH range 6.56–8.45 in 0.05 M sodium phosphate and 0.05 M NaCl [ionic strength (I) = 0.2]. These experiments were carried out at a single PK concentration (2.2 PK units/mL); consequently, only k_{cat}/K_m was determined (Figure 5). Maximal activity was seen at a pH of 7.5–8.0; the pK_a value for the acidic limb was estimated to be 6.7.

Assays for PKA. From a consideration of the kinetics of activation of PK by PKA, the sensitive two-stage assay for PKA described under Materials and Methods was developed. In the first stage of the assay, PKA catalyzes the activation of PK under conditions of ionic strength and substrate concentration whereby a first-order reaction obtains. The kallikrein produced in a given time is then determined in the second stage of the assay.

The integrated first-order rate expression for the activation is

$$k = \frac{1}{t} \ln \frac{[PK]_0}{[PK]_0 - [K]_t}$$

in which $[PK]_0$ is the initial prekallikrein concentration, $[K]_t$ is the concentration of kallikrein generated after t minutes of incubation (see the legend to Figure 3), and k is the first-order rate constant.

One PKA unit was defined as the amount of enzyme which, in 1 mL, activates PK with a first-order rate constant of 1 min^{-1} at pH 7.5, 37 °C, and I = 0.20. Since the rate constant for an enzyme-catalyzed reaction is equal to the product of the enzyme concentration and an intrinsic constant,[3] it follows that the prekallikrein activator concentration, [PKA], expressed in PKA units/mL, can be evaluated by the equation:

$$[PKA] = \frac{1}{t} \ln \frac{[PK]_0}{[PK]_0 - [K]_t}$$

Note that $[PK]_0$ and $[K]_t$ may be expressed in any convenient units provided only that the same units are used for both. When $[PK]_0$ and $[K]_t$ are determined by means of the spectrophotometric assay with BAEE as substrate, it is convenient to use the observed ΔA_{253}/min as a measure of kallikrein concentration.

Concentrations of PKA as low as 0.001 unit/mL may be reproducibly quantified by means of this assay. This concentration results in the conversion, during 100 min, of ~10% of the PK to kallikrein—a level which can be easily measured. The contribution by PKA to the total esterolytic activity of the incubate is negligible; for $[PK]_0$ = 2 PK units/mL and [PKA] = 0.1 PKA unit/mL, the BAEE-esterolytic activity due to PKA is ~0.3% of that due to the kallikrein generated in 1 min.

Some deviation from first-order behavior is observed when the conversion to kallikrein exceeds about 50% (see Figure 3). In addition, errors in the calculated PKA value (arising from inaccuracies in the measurement of the amount of kallikrein formed) are magnified at high percentages of conversion. Therefore, the percentage conversion is preferably kept below 50% by suitably diluting the sample or shortening the incubation time. In most samples encountered, the levels of PKA were such that a first-stage incubation time of 15–60 min was satisfactory. Generally, the first-stage incubation mixture was sampled at two time points (i.e., at 20–30 min and again at 50–60 min), and the resulting PKA values were calculated for each time.

Samples containing >1 PKA unit/mL may be diluted prior to assay, or such samples may be analyzed by the one-stage assay. In this procedure, the activation of PK is allowed to proceed in the presence of kallikrein substrate (e.g., BAEE), and the hydrolysis of the latter is measured continuously for 10–15 min. The initial portion of the generated curve is a

[2] The Brønsted equation for the reaction between species a and b, having net charges of z_a and z_b, respectively, may be written (Moore, 1955) $\log k = 2Az_az_bI^{1/2} + \text{constant}$, where k is the observed rate constant at ionic strength I. A, the Debye–Hückel constant, is dependent only upon temperature and the dielectric constant of the solvent; it has a value of approximately 0.5 for water at 37 °C. Since for a first-order, enzyme-catalyzed reaction, k = [enzyme]k_{cat}/K_m, a plot of $\log k_{cat}/K_m$ against $I^{1/2}$ (at constant enzyme concentration) will yield a straight line with a slope of $2Az_az_b \simeq z_az_b$. The observed slope of such a plot for the activation of PK by PKA (Figure 4) is −2.6, indicating that PK and PKA have opposing net charges at pH 7.5 and that the product of their charges is −2.6.

[3] For an enzyme-catalyzed reaction obeying zero-order kinetics (i.e., with a saturating concentration of substrate) this intrinsic constant is k_{cat}. When first-order kinetics obtain, however, as in the present situation, the intrinsic constant is k_{cat}/K_m. Under the conditions chosen for defining the PKA unit (pH 7.5, 37 °C, and I = 0.20) it has a value of 1 mL (PKA unit)$^{-1}$ min^{-1}.

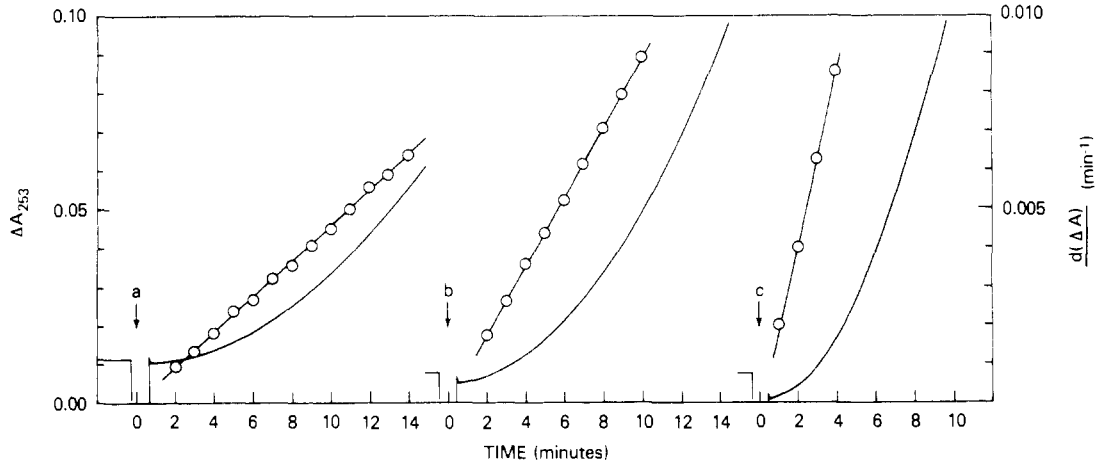

FIGURE 6: One-stage PKA assay. Cuvettes containing 0.13 unit of PK in 2.0 mL of 0.5 mM BAEE, 0.05 M sodium phosphate, and 0.05 M NaCl (pH 7.5) were kept at 37 °C in the spectrophotometer. A solution containing PKA (2.5 PKA units/mL) was added (arrows), and the change in absorbance was recorded for 10–15 min (—). $d(\Delta A)/dt$ was determined graphically and plotted against time (O). The slope $[d^2(\Delta A)/dt^2]$ of the line thus produced is proportional to the PKA concentration in the cuvette. (a) 10 μL of PKA, $d^2(\Delta A)/dt^2 = 4.56 \times 10^{-4}$ min^{-2}; (b) 20 μL of PKA, $d^2(\Delta A)/dt^2 = 8.88 \times 10^{-4}$ min^{-2}; (c) 50 μL of PKA, $d^2(\Delta A)/dt^2 = 2.22 \times 10^{-3}$ min^{-2}.

parabola; when the derivative of that curve with respect to time $[d(\Delta A)/dt]$ is plotted against time, the result is a straight line with the slope $d^2(\Delta A)/dt^2$. This slope is proportional to the rate of kallikrein generation (Figure 6). The concentration of PKA (in PKA units/mL) in the cuvette is calculated as

$$[\text{PKA}] = \frac{1}{0.57[\text{PK}]} \frac{d^2(\Delta A)}{dt^2}$$

where [PK] is the concentration (in PK units/mL) of PK in the cuvette.

Discussion

We have determined that the activation of PK by PKA obeys Michaelis–Menten kinetics with a K_m of ~10 PK units/mL at physiologic ionic strength. Other investigators have estimated a K_m of 0.25–0.5 PK unit/mL for the activation by surface-bound factor XIIa (Laake & Venneröd, 1973), indicating that the surface-bound enzyme displays a much higher affinity for PK than does PKA. PKA also differs substantially from surface-bound factor XIIa in its direct activity toward the substrate AGLME; we determined a K_m of 3.6 mM and a k_{cat} of 10.1 s^{-1} for the reaction of PKA with this substrate, whereas other workers have determined K_m = 9 mM and k_{cat} = 0.024 s^{-1} for its hydrolysis by surface-bound factor XIIa (Ulevich et al., 1974). These investigators also noted substrate inhibition at AGLME concentrations >40 mM, while we observed no inhibition of PKA at 80 mM AGLME.

The K_m for the activation of PK by PKA is strongly dependent upon ionic strength. The reaction between two species with opposite net charge would be expected to show a decrease in rate with increasing ionic strength. Such is observed for the interaction of PK and PKA, which have isoelectric points of 8.7 (Kaplan et al., 1972) and 4.2 (Venneröd & Laake, 1974), respectively.

Methods for quantitating PKA by its direct esterolytic activity toward TAME, BAEE, or AGLME were investigated; such methods were found to lack the degree of sensitivity required for the samples of interest. Attempts to measure PKA in these samples by the method of Bagdasarian et al. (1973b), in which normal plasma serves as the source of PK and the amount of kallikrein generated is measured by the hydrolysis of TAME, were unsuccessful owing to the low levels of PKA present.

By means of a two-stage assay in which PKA is allowed to activate PK and the resulting kallikrein is measured, a high degree of amplification is achieved which provides the sensitivity necessary to quantitate PKA in the 1–100 ng/mL range. A similar assay was described by Laake & Venneröd (1973) for the measurement of surface-bound factor XIIa; however, the 50-fold higher K_m for activation by PKA as compared to factor XIIa results in a quite different kinetics. At the level of PK used, the activation by factor XIIa is zero order, whereas activation by PKA is first order.

Enzyme assays based upon first-order kinetics require that the substrate concentration be much less than K_m, whereupon the combination of enzyme and substrate becomes rate limiting. When this condition is fulfilled, the first-order rate constant becomes equal to the product of the enzyme concentration and the efficiency, k_{cat}/K_m. Therefore, the first-order rate constant may be used as a measure of the enzyme concentration, provided that the efficiency is constant under the chosen activation conditions. [For a discussion of the limitations of first-order kinetic assays, see Elkins-Kaufman & Neurath (1948).]

We have found that first-order kinetics may be applied successfully for the quantitation of PKA under the conditions described. Deviation from first-order behavior is observed only when the extent of conversion of PK to kallikrein is greater than ~50% (see Figure 3). The direction of this deviation is opposite to that which would be expected to result from decreased saturation of the enzyme; it is possibly the result of competitive inhibition by product (kallikrein).

A variation of the PKA assay was developed in which activation of PK and hydrolysis of BAEE or TAME by the kallikrein formed proceeded simultaneously. In this method the extent of conversion of PK to kallikrein is quite small, allowing the application of pseudo-zero-order kinetics. This modification is less sensitive than the two-stage assay since the concentration of PK is generally quite low. It has been valuable, however, in investigating the inhibition of PKA by the plasma inhibitors C1-esterase inhibitor and antithrombin III. Both of these agents inhibit the esterolytic activity of kallikrein when the enzyme and inhibitor are incubated together prior to assay, whereas little or no inhibitory activity is observed in the absence of such preincubation. Therefore, it was possible to demonstrate unequivocally the inhibition of PKA by both of these substances by means of the one-stage assay, whereas in the two-stage assay it was impossible to distinguish between inhibition of the PKA or inhibition of any

kallikrein generated. The direct esterolytic activity of PKA toward BAEE was also found to be inhibited by antithrombin III and by C1-esterase inhibitor.

The two-stage assay has been used to measure PKA in therapeutic products where its presence has been associated with hypotensive reactions (Alving et al., 1978). Because this assay depends on the conversion of PK to kallikrein, it permits an accurate prediction of the activity in vivo, i.e., the generation of kallikrein with the subsequent liberation of bradykinin. PKA levels measured in this manner were found to correlate well with hypotensive activity in an animal model (M. Fournel, unpublished experiments).

Acknowledgments

We are grateful for the expert editorial assistance of Dr. John Finlayson (Bureau of Biologics) and the fine technical work of Timothy Zuffi and Paul Ng (Cutter Laboratories, Inc.).

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A Kinetic Method for Determining Dissociation Constants for Metal Complexes of Adenosine 5′-Triphosphate and Adenosine 5′-Diphosphate†

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ABSTRACT: A general kinetic method is described for determining the dissociation constants of metal–ATP complexes that act as inhibitory substrate analogues for any enzyme that utilizes $MgATP^{2-}$. The usefulness of the procedure is illustrated by the results obtained from studies of the inhibition of hexokinase by lanthanide–ATP (LnATP) complexes. At relatively low concentrations of Mg^{2+}, these complexes act as linear competitive inhibitors with respect to $MgATP^{2-}$. In the presence of higher, fixed concentrations of Mg^{2+}, however, double reciprocal plots of the inhibition by LnATP vs. MgATP are nonlinear, and the data can be used to determine the ratio of the dissociation constants for the LnATP and MgATP complexes. As values are available for the dissociation constant of MgATP under a variety of conditions, that for any LnATP complex can be calculated. The dissociation constant for EuATP at pH 8.0 is 0.16 μM, while that for GdATP is 0.91 μM at pH 6.0, 0.087 μM at pH 7.95, and 1 μM at pH 8.65. Between pH 6 and 8, the ratio of the dissociation constants for GdATP and $MgATP^{2-}$ remains constant, and thus, within this range of pH, the lanthanide species involved must be Gd^{3+} and $GdATP^{-}$. The method can also be applied to the determination of dissociation constants for inhibitory metal–ADP complexes if $MgADP^{-}$ is used as the variable substrate.

A number of methods have been used to evaluate the dissociation constants for the complexes formed by nucleotides and metal ions (O'Sullivan & Smithers, 1979). These procedures, which involve equilibrium measurements, have been used successfully to determine dissociation constants for the $MgATP^{2-}$ and $MgADP^{-}$ complexes where the values lie within a suitable range (Adolfsen & Moudrianakis, 1978). However, for complexes whose dissociation constants are less than 1 μM, most of these methods are no longer sensitive enough. For example, with the 8-hydroxyquinoline method, Ellis & Mor-

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0006-2960/80/0419-3127$01.00/0