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Evidence for an Intrazymogen Mechanism in the Conversion of Proacrosin into Acrosin†

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ABSTRACT: The mechanism responsible for the spontaneous initiation of proacrosin conversion into acrosin in vitro was studied and characterized by using the highly effective inhibitor leupeptin. In the presence of excess leupeptin [$(10^2–10^3)K_I$ to acrosin], proacrosin spontaneously and completely converted into acrosin at pH 8. However, only the initial enzyme product, m_α-acrosin, was produced, and the rate of conversion was not affected when exogenous m_α-acrosin was added to the reaction mixture. These results demonstrate that excess leupeptin eliminated all conversion and degradative reactions requiring active acrosin. Kinetically, the conversion of proacrosin into m_α-acrosin in the presence of excess leupeptin appeared first order. The observed half-life ($t_{1/2}$ = 1.4 h) did not vary over a 10-fold range of leupeptin or initial proacrosin concentrations. These data demonstrate that proacrosin can self-catalyze its own conversion into m_α-acrosin by an intrazymogen mechanism.

Acrosin is a serine proteinase of trypsin-like substrate specificity associated with the sperm cell acrosome. The majority of potential acrosin present in mammalian epididymal or ejaculated spermatozoa, however, is a zymogen form, proacrosin (Meizel, 1972; Meizel & Mukerji, 1975; Tobias & Schumacher, 1977; Polakoski & Parrish, 1977; Harrison & Brown, 1979). Since acrosin activity is essential for fertility [reviewed by Zaneveld et al. (1975)], it is therefore important to understand the mechanisms and regulation of the conversion of proacrosin into its catalytically active enzyme form(s).

In vitro, purified proacrosin spontaneously and rapidly converts into acrosin at neutral pH. The reaction occurs by limited proteolysis of the zymogen and appears "autocatalyzed", i.e., accelerated by exogenously added and/or endogenously generated acrosin (Meizel & Mukerji, 1975; Polakoski & Parrish, 1977). A minimum mechanism describing the conversion of proacrosin can thus be written

$$Z + E \rightarrow 2E \quad (1)$$

where Z and E are the zymogen and enzyme, respectively.

Equation 1, however, cannot satisfactorily explain how the first molecules of acrosin evolve, especially from highly purified preparations of proacrosin. Although it is possible that the reaction may be initiated by other proteinases, this explanation is unlikely for purified proacrosin as has been considered in detail elsewhere (Polakoski & Parrish, 1977; Parrish et al., 1978) and further shown in this report. Rather, alternative mechanisms for the initiation of proacrosin conversion into acrosin need to be considered.

Besides catalysis by the generated enzyme or some contaminating proteinase, only two other limited proteolytic mechanisms for zymogen conversion into enzyme are currently thought possible: inter- and intrazymogen-catalyzed conversions [reviewed by Kassel & Kay (1974) and Neurath & Walsh (1976)]. Trypsinogen can convert into trypsin via an interzymogen mechanism (Kay & Kassel, 1971) as is represented by

$$Z + Z \rightarrow Z + E \quad (2)$$

Alternatively, pepsinogen can convert into pepsin via an intrazymogen mechanism (Bustin & Conway-Jacobs, 1971)

$$Z \rightarrow E \quad (3)$$

These latter mechanisms involving zymogen self-catalyzed conversions (eq 2 and 3) may be kinetically differentiated from the enzyme-catalyzed reaction (eq 1). However, McPhie (1972) has cautioned that direct kinetic analyses of the zymogen conversion profiles are highly subject to misinterpretation if the enzyme-catalyzed mechanism (eq 1) predominates even at low initial zymogen concentrations and if degradation of the enzyme products occurs simultaneous with zymogen activation. Both of these events are known to occur in the conversion of proacrosin into acrosin (Polakoski & Parrish, 1977; Parrish & Polakoski, 1978). These difficulties may be circumvented by the use of an inhibitor which suppresses the enzyme-catalyzed conversion or degradation reactions but does not prevent the conversion mechanism catalyzed by the zymogen (eq 2 or 3). In this work, leupeptin, a highly effective acrosin inhibitor, was found to satisfy this latter requirement. Kinetic analyses of the spontaneous conversion of proacrosin into acrosin in the presence of excess leupeptin demonstrated that the reaction can occur by the intrazymogen mechanism shown in eq 3.

Experimental Procedures

Materials. BzArgOEt,[1] BzArgpNA, and leupeptin were purchased from the Sigma Chemical Co. and used without

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[1] Abbreviations used: leupeptin, *N*-acetyl-L-leucyl-L-leucyl-L-arginal; BzArgOEt, *N*-benzoyl-L-arginine ethyl ester hydrochloride; BzArgpNA, *N*-benzoyl-DL-arginine *p*-nitroanilide hydrochloride; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; M_r, relative molecular mass; LBTI, lima bean trypsin inhibitor.

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further purification. All other chemicals were reagent grade or better as obtained from commercial sources.

Boar sperm proacrosin was extracted and purified by the methods of Polakoski & Parrish (1977) with the following modifications: precipitation of the partially purified proacrosin with sodium chloride was not performed and 6 M guanidine hydrochloride was substituted for the 8 M urea previously used in the denaturation step prior to gel chromatography. This proacrosin preparation consisted of the previously described two forms with relative molecular weights (M_r) = 55 000 and 53 000 (Polakoski & Parrish, 1977) which were otherwise homogeneous as judged by $NaDodSO_4$–polyacrylamide disc gel electrophoresis (see methods below).

Purified boar m_α-acrosin was prepared by methods using leupeptin (unpublished experiments) and was also judged homogeneous by $NaDodSO_4$–polyacrylamide disc gel electrophoresis. The preparation had a specific activity of 277 U/mg where 1 international unit (U) is defined as the amount of enzyme required to catalyze the hydrolysis of 1 μmol of BzArgOEt/min when assayed by general procedures and the protein content was calculated from the solution absorbance at 280 nm (Polakoski & McRorie, 1973).

Spectrophotometric assays were routinely performed on a Gilford Model 240 single-beam spectrophotometer equipped with a Gilford Model 410 absorbance meter, an automatic data printer (Gilford Model 4009), and a thermostated (30 °C) cuvette holder.

Analytical Disc Gel Electrophoresis. $NaDodSO_4$–polyacrylamide disc gel electrophoresis was performed according to the general methods of Laemmli (1970) with some modifications. Typically, 10–30 μg of the sample protein in 100 μL of buffer was denatured and reduced in the presence of 1% $NaDodSO_4$ and 1% 2-mercaptoethanol by boiling in a water bath for 2 min. After the mixture was cooled, 5 μL of a 0.05% bromphenol blue tracking dye and a drop of glycerol were added to the sample which was then applied on a 10% polyacrylamide disc gel and subjected to electrophoresis at pH 8.2 (1.5% glycine and 0.3% Tris buffer containing 0.1% $NaDodSO_4$) at a constant current of 5 mA/gel. After the tracking dye had migrated 5 cm, the gels were removed from their casings and severed at the dye front. Proteins were visualized by staining with Coomassie brilliant blue R-250 (0.25% in 50% methanol–10% acetic acid) for 30 min at 70 °C and destained in 10% acetic acid at 70 °C. Relative migrations were determined from spectrophotometric scans of the gels at 560 nm by using a Gilford Model 2410-S linear transport attachment. Apparent molecular weights were calculated by standard methods (Weber & Osborn, 1969) against reference proteins (bovine serum albumin, 68 000; ovalbumin, 45 000; trypsin, 23 300; lysozyme, 14 300) which were subjected to electrophoresis separately but simultaneously with the samples.

BzArgOEt Assay for Acrosin Activity. Acrosin activity generated in the proacrosin conversion experiments was measured spectrophotometrically by following the rates of BzArgOEt hydrolysis (Schwert & Takenaka, 1955) at 253 nm. In a typical experiment, 3.0 mL of the test solution containing enzyme at pH 3 was placed in a 1-cm cuvette and to it added BzArgOEt (100 μL of stock solution in water) and a Tris–$CaCl_2$ buffer (100 μL). Final solution conditions were 0.5 mM BzArgOEt, 0.05 M Tris, and 0.05 M $CaCl_2$ at pH 8.0 and 30 °C. The change in absorbance was followed at 253 nm for 1 min and converted to micromoles of BzArgOEt hydrolyzed per minute by using a molar absorbance difference of 1150 M^{-1} cm^{-1} (Whitaker & Bender, 1965).

Inhibition of m_α-Acrosin by Leupeptin. The equilibrium dissociation constant (K_I) for the inhibition of m_α-acrosin by leupeptin was determined by using standard kinetic methods (Dixon & Webb, 1964) against the substrate BzArgpNA as run under first-order conditions ([S] $\ll K_m$). An aliquot (100 μL) of a leupeptin stock solution (in 1 mM HCl) or 1 mM HCl alone and 3.0 mL of the substrate (0.05 mM of the L enantiomer) in a 0.05 M Tris and 0.05 M $CaCl_2$ buffer containing 1% dimethyl sulfoxide (Sigma) at pH 8.0 were admixed and incubated in a 1-cm cuvette to 30 °C. The reaction was initiated by addition of a 20-μL aliquot of m_α-acrosin (2 μg = 554 mU), and the initial rate of *p*-nitroaniline release was followed spectrophotometrically at 405 nm for ~1 min. Assays were performed in triplicate at each of the concentrations of leupeptin ([I]) = 0, $(3–5)K_I$ and $(^1/_3–^1/_5)K_I$, and the individual inhibition constants calculated according to the general equation (Dixon & Webb, 1964)

$$K_I = [I]/(v_u/v_i - 1) \quad (4)$$

where v_u and v_i are the initial rates of BzArgpNA hydrolysis in the absence and presence of the inhibitor, respectively.

Rate of Leupeptin Dissociation from Acrosin. In the majority of experiments performed in this work, a large excess of leupeptin was added to the proacrosin conversion solutions. Substantial dilutions were therefore required in order to assay for the amount of generated acrosin activity. Since leupeptin is a specific substrate aldehyde analogue (Umezawa & Aoyagi, 1977), it was expected to rapidly associate but only slowly dissociate from its complex with acrosin. Accordingly, to accurately assess acrosin activity after dilution, a minimum incubation time required to reestablish the leupeptin–enzyme equilibrium had to be determined.

Aliquots (50 μL) of m_α-acrosin (5 μg = 1.4 U) containing 0.2 mM leupeptin at pH 3 were adjusted to pH 8.0 and 0.05 M Tris and 0.15 M NaCl by addition of the concentrated buffer (5 μL). After 1 min at room temperature, the mixture was added to 10 mL of prewarmed (30 °C) 1 mM HCl and incubated at 30 °C. At timed intervals, 3.0 mL of the solution was withdrawn and assayed for BzArgOEt hydrolase activity.

Products and Rates of Proacrosin Activation in the Presence of Leupeptin. Analytical disc gel electrophoresis ($NaDodSO_4$–polyacrylamide) was used to identify the products of proacrosin activation in the presence of leupeptin. Simultaneously, the BzArgOEt hydrolysis assay was used to follow the time course of the reaction under these conditions. Proacrosin (0.9 mL = 0.21 mg in 1 mM HCl) and leupeptin (100 μL of a 1.1 mM stock in 1 mM HCl) were preincubated at room temperature for 5 min and then adjusted to pH 8.0 and 0.05 M Tris by addition of 100 μL of concentrated buffer. Duplicate 50-μL aliquots were withdrawn at sequential time points and treated in the following manner: the first aliquot was diluted 1:1 with a 2% $NaDodSO_4$ and 2% 2-mercaptoethanol solution (pH 3) and immediately boiled for 2 min in a water bath; the second aliquot was diluted into 10 mL of prewarmed (30 °C) 1 mM HCl and incubated for a minimum of 20 min. The first series of samples were stored and later in the day subjected to analytical disc gel electrophoresis as described above. The second series of samples were assayed for BzArgOEt hydrolase activity.

Kinetics of Proacrosin Activation in the Presence of Leupeptin. The kinetics of proacrosin activation in the presence of leupeptin have been studied as a function of leupeptin and initial zymogen concentrations and in the presence of added m_α-acrosin. In the typical experiment, 250 μL of 1 mM HCl containing the proacrosin was mixed with 50 μL of a leupeptin stock solution (in 1 mM HCl) or 25 μL of the leupeptin and

25 μL of the m_α-acrosin, and the activation reaction initiated by addition of buffer (30 μL of a 0.55 M Tris and 1.65 M sodium chloride solution) to pH 8.0. Solutions containing a constant proacrosin concentration (180 μg/mL) were activated in the presence of 0.2, 0.1, 0.04, or 0.02 mM leupeptin or 0.1 mM leupeptin and 2.5 μg (693 mU) of m_α-acrosin. Alternatively, the initial proacrosin concentration was adjusted to 90 or 18 μg/mL and activated in the presence of 0.04 mM leupeptin. Aliquots of the reaction mixtures (50 μL) were removed at sequential time intervals, added to 10 mL of 1 mM HCl, and incubated at 30 °C for a minimum of 20 min prior to assaying for evolved BzArgOEt hydrolase activity. All reactions were performed in triplicate, and aliquots were assayed until zymogen conversion was complete as determined by no apparent increase in BzArgOEt hydrolase activity.

Pertinent Kinetic Calculations. It has been shown that 1 mol of boar proacrosin (Z) converts into 1 mol of active acrosin (E) (Polakoski & Parrish, 1977). Thus, the concentration of Z at time = 0 ($[Z]_0$) is equivalent to the concentration of E after the conversion is complete ($[E]_\infty$) so that at any time = t

$$[Z]_t/[Z]_0 + [E]_t/[Z]_0 = [Z]_t/[E]_\infty + [E]_t/[E]_\infty = 1 \quad (5)$$

Since $[E]_t$ is directly proportional to the change in absorbance (ΔA) occurring in 1 min when using the BzArgOEt assay, it can be derived from eq 5 that

$$[Z]_t = (\Delta A_\infty - \Delta A_t)C \quad (6)$$

where C is the appropriate conversion constant.

The original equations of Herriott (1938) and McPhie (1972) were used to analyze the kinetics of proacrosin conversion into acrosin in the presence of excess leupeptin.

If Z converts into E by either of the intermolecular (second-order) mechanisms given in eq 1 and 2, then

$$-d[Z]/dt = k_2[Z][E] \text{ or } k_2[Z]^2 \quad (7)$$

where k_2 is a second-order rate constant. That rate constant can be calculated from the slope of the linear transformation of eq 7:

$$\log [\Delta A_\infty/\Delta A_t - 1] = -k_{obsd}t \quad (8)$$

where the respective concentrations of Z and E are expressed in terms of BzArgOEt hydrolysis and

$$k_{obsd} = 2.303k_2[Z]_0 \quad (9)$$

If Z converts into E by the intrazymogen (first-order) mechanism given in eq 3, then

$$-d[Z]/dt = k_1[Z] \quad (10)$$

where k_1 is a first-order rate constant. The rate constant can also be calculated from the slope of the line:

$$\log [1 - \Delta A_t/\Delta A_\infty] = -k_{obsd}t \quad (11)$$

where the concentration of Z is expressed in terms of Bz-Arg-O-Et hydrolysis, and

$$k_{obsd} = 2.303k_1 \quad (12)$$

Results

Inhibition of m_α-Acrosin by Leupeptin. The general class of substrate aldehyde analogues reversibly inhibit their respective serine proteinase by forming a covalent, hemiacetal adduct with the enzyme's active site serine hydroxl side chain (Kennedy & Schultz, 1979). The rate of hemiacetal formation is typically fast but decomposes slowly. This behavior is also evident in the binding of leupeptin to acrosin.

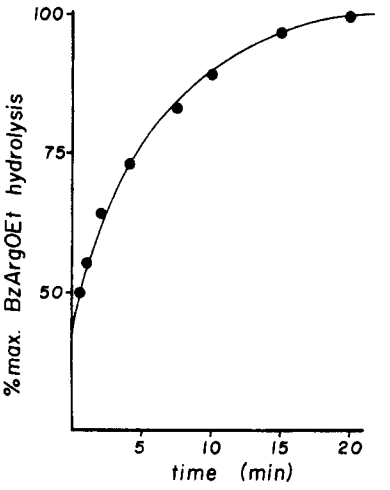

FIGURE 1: Time course of the dissociation of leupeptin from m_α-acrosin. Solutions initially containing 100 μg/mL m_α-acrosin and 0.2 mM leupeptin at pH 8 were diluted 1:200 in 1 mM HCl and subsequently assayed for BzArgOEt hydrolase activity after the indicated incubation times. Each data point (●) represents the mean of triplicate determinations.

After a 1-min incubation, m_α-acrosin was strongly inhibited by dilute concentrations of leupeptin. The respective inhibition constant found in this work was $K_{I,\alpha} = 0.64 \pm 0.02$ μM, as determined against the substrate BzArgpNA. It should be noted that the observable inhibition of acrosin by leupeptin proceeds in both rapid and slow progressive ($t_{1/2} \simeq 10$ min) (Fritz et al., 1973) stages, leading to an overall K_I which is some 10-fold better than the binding constant reported here. However, for the purposes of this investigation, knowledge of the apparent K_I coincident with the rapid phase was necessary since, in later experiments, the acrosin evolving from proacrosin activation had to be completely and rapidly inhibited.

The slow dissociation of leupeptin from m_α-acrosin is shown in the Figure 1. Note that a 20-min incubation period was required to achieve constant activity from diluted aliquots of the enzyme which had been preequilibrated with saturating concentrations of leupeptin. Accordingly, care was taken to incubate subsequent assay samples after the required dilution for a minimum of 20 min so that acrosin activities could be consistently and reproducibly assessed.

Products of Proacrosin Conversion in the Presence of Leupeptin. The products of proacrosin conversion in the presence of leupeptin were identified by using $NaDodSO_4$–polyacrylamide disc gel electrophoresis. As is shown in Figure 2, leupeptin, included at a concentration (0.1 mM) sufficient to inhibit >99.4% of any acrosin activity present in the reaction mixture, did not prevent the conversion of proacrosin into acrosin. The reaction, however, only proceeded to the formation of the initial enzyme product, m_α-acrosin ($M_r \simeq 49\,000$) which, in the absence of leupeptin, rapidly degrades to m_β-acrosin ($M_r \simeq 34\,000$) (Polakoski & Parrish, 1977; Parrish & Polakoski, 1978). Since degradation of m_α- to m_β-acrosin is an intermolecular reaction similar to the enzyme–zymogen conversion mechanism given in eq 1 (Parrish & Polakoski, 1978), these results are evidence that leupeptin effectively inhibited all intermolecular conversion or degradation reactions involving acrosin. Accordingly, the ability of proacrosin to convert into m_α-acrosin by either an inter- (eq 2) or an intrazymogen mechanism (eq 3) is indicated.

Kinetics of Proacrosin Conversion in the Presence of Leupeptin. The evolution of acrosin esterase activity from the identical proacrosin conversion solution used above was followed over time. In the presence of 0.1 mM leupeptin, the

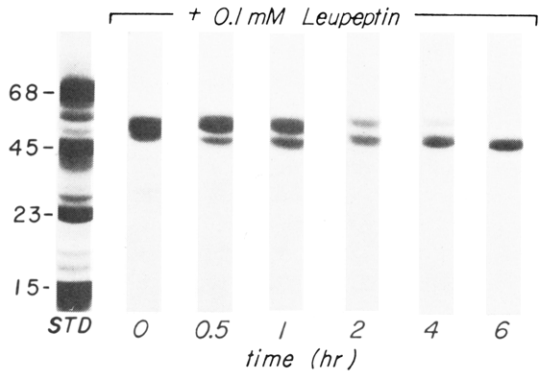

FIGURE 2: $NaDodSO_4$–polyacrylamide gels following the generation of boar proacrosin conversion products with time in the presence of leupeptin. Timed, sequential aliquots of a proacrosin conversion mixture containing 0.1 mM leupeptin were treated and electrophoresed at pH 8.2 on 10% polyacrylamide disc gels (1% $NaDodSO_4$), and proteins were stained with Coomassie brilliant blue R-250 as is described in the text. Standard proteins were prepared similarly and electrophoresed simultaneously with the respective samples. Their respective molecular weights are indicated in the abscissa (=×1000).

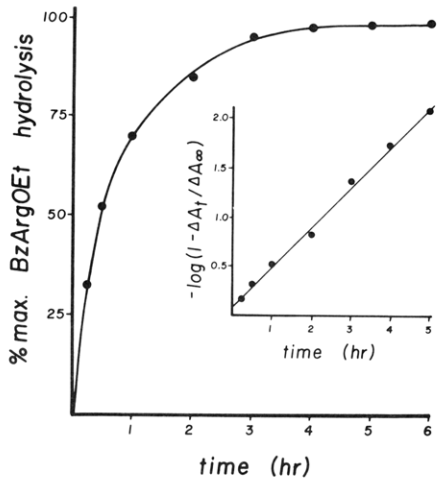

FIGURE 3: Time course for the evolution of acrosin esterase activity from boar proacrosin converting in the presence of leupeptin. BzArgOEt hydrolase activities were determined for sequentially withdrawn, diluted (1:200 at pH 3), and incubated (20 min at 30 °C) aliquots of the reaction solution which initially contained 180 μg of proacrosin at pH 8 (0.05 M Tris buffer) and 0.1 mM leupeptin. Data points (●) represent the means of three determinations. Inset: data analyzed according to the first-order relationship given in eq 11 in the text. Respective correlation was 99.9%.

reaction was considerably slower and appeared first order in contrast to the rapid rates and second-order kinetics observed in the absence of leupeptin (Meizel & Mukerji, 1975; Polakoski & Parrish, 1977). These data are summarized in the composite Figure 3 where the observed evolution of acrosin BzArgOEt hydrolase activity is shown to correlate well with a first-order analysis. Similarly, the time courses for the evolution of BzArgOEt hydrolase activity from proacrosin solutions containing a 10-fold range of leupeptin concentrations (0.2–0.02 mM), in the presence of added m_α-acrosin (with 0.1 mM leupeptin), and over a 10-fold range of initial proacrosin concentrations (18–180 μg/mL with 0.04 mM leupeptin) all appeared to follow first-order kinetics. Representative data are plotted in Figure 4. More importantly, the respective first-order rate constants were each found to be identical within experimental error. These are summarized in Table I and represent the means and standard deviations for each experiment run in triplicate and individually analyzed according to eq 8. Least-squares linear regression correlations were

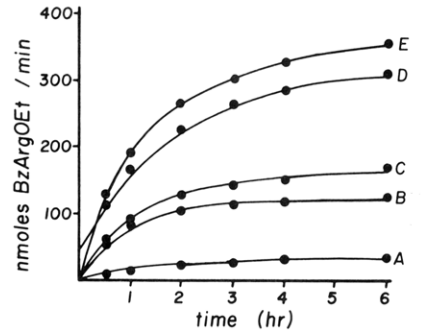

FIGURE 4: Representative conversion time courses for boar proacrosin in the presence of leupeptin under various conditions. BzArgOEt hydrolase activities were determined by methods similar to those described in Figure 3. The reaction solutions initially contained (A) 0.04 mM leupeptin and 18 μg/mL proacrosin, (B) 0.2 mM leupeptin and 180 μg/mL proacrosin, (C) 0.1 mM leupeptin and 180 μg/mL proacrosin, (D) 0.1 mM leupeptin, 180 μg/mL proacrosin, and 693 mU of m_α-acrosin, and (E) 0.02 mM leupeptin and 180 μg/mL proacrosin. Each data point (●) represents the mean of three determinations. Reaction solution conditions were as follows: 0.15 M sodium chloride and 0.05 M Tris at pH 8.0 and 23 °C.

Table I: Observed First-Order Rate Constants for the Conversion of Boar Proacrosin in the Presence of Leupeptin under Various Conditions

condition[a]	k_{obsd} (h^{-1})[b]
[leupeptin] = 0.2 mM[c]	0.494 ± 0.049
[leupeptin] = 0.1 mM[c]	0.523 ± 0.017
[leupeptin] = 0.04 mM[c]	0.509 ± 0.095
[leupeptin] = 0.02 mM[c]	0.475 ± 0.048
$[proacrosin]_0$ = 90 μg/mL[d]	0.527 ± 0.050
$[proacrosin]_0$ = 18 μg/mL[d]	0.492 ± 0.037
+ 693 mU of m_α-acrosin[e]	0.505 ± 0.048

[a] Standard solution conditions were as follows: 0.15 M sodium chloride and 0.05 M Tris at pH 8 and 23 °C. [b] Observed rate constants represent the means and standard deviations in triplicate experiments. [c] Initially contained 180 μg/mL proacrosin. [d] Contained 0.04 mM leupeptin. [e] Initially contained 180 μg/mL proacrosin and 0.04 mM leupeptin.

>99.8% in all of these experiments.

Discussion

Previous studies have shown that the in vitro conversion of proacrosin into acrosin is a limited proteolytic event that can be catalyzed by trypsin and/or acrosin (Meizel & Mukerji, 1975; Polakoski & Parrish, 1977). The results presented in this report demonstrate that the conversion can also occur by an intraproacrosin self-catalyzed mechanism. This conclusion is principally based on a kinetic evaluation of the conversion reaction as is described in the following text.

The spontaneous and complete conversion of highly purified boar proacrosin into acrosin was not prevented in the presence of a large excess [$(10^2$–$10^3)K_I$ to acrosin] of the effective acrosin inhibitor, leupeptin (Figures 2 and 3). The reaction proceeded, however, only to the formation of the initial enzyme product, m_α-acrosin (Figure 2). In the absence of inhibitors, m_α-acrosin is highly unstable at neutral pH, readily degrading to m_β-acrosin (Polakoski & Parrish, 1977; Parrish & Polakoski, 1978) by an intermolecular mechanism similar to eq 1. In addition, the observed half-life ($t_{1/2}$ = 1.4 h) for the conversion of proacrosin in the presence of excess leupeptin was not perturbed by the exogenous addition of m_α-acrosin or by alterations in the concentration of leupeptin (Table I). Accordingly, at the concentrations studied, leupeptin effectively prevented all acrosin activity exogenously added or endogenously generated in the reaction but did not significantly interact with proacrosin so as to inhibit or prevent its conversion

into acrosin. Thus, leupeptin acted to isolate the putative zymogen-catalyzed conversion mechanism (eq 2 or 3) from the enzyme-catalyzed mechanism (eq 1).

The conversion of proacrosin into active enzyme by an intrazymogen mechanism (eq 3), once isolated from the acrosin-catalyzed mechanism (eq 1) through the use of leupeptin, was differentiated from the alternative interzymogen mechanism (eq 2) by studying the kinetics of the reaction. Like the enzyme-catalyzed mechanism, the interzymogen mechanism is kinetically second order (refer to eq 7–9) while the intrazymogen mechanism is first order (see eq 10–12). As shown in the Figures 3 and 4, the conversion of proacrosin into acrosin in the presence of excess leupeptin appeared first order with time, thereby suggesting that the reaction occurs via the intrazymogen mechanism. These results, however, are not definitive since second-order reactions can appear first order under certain conditions [see discussions of McPhie (1972)]. Rather, the differences between the intra- and interzymogen mechanisms, being first- and second-order reactions, respectively, have been resolved through an analysis of the effects of initial proacrosin concentration on the observed rate constant (k_{obsd}) for conversion. The derivations of Herriott (1938) and McPhie (1972) predict that a 10-fold dilution of the initial proacrosin concentration would cause at least a 10-fold decrease in k_{obsd} (increase in $t_{1/2}$) if the interzymogen (second-order) reaction occurs (see eq 9). Conversely, if the reaction occurs by the intrazymogen (first-order) mechanism, the k_{obsd} (or $t_{1/2}$) will be unaffected (see eq 12). As shown in Table I, the k_{obsd} for the conversion of proacrosin into acrosin in the presence of excess leupeptin was insensitive to alterations in the initial proacrosin concentration. These results also refute the possibility that proacrosin conversion under these conditions was catalyzed by some undetected neutral proteinase that was not inhibited by leupeptin. Otherwise, its dilution along with the proacrosin would also have caused a significant decrease in the observed $t_{1/2}$ of the reaction. Accordingly, the most reasonable explanation for these kinetic results is that proacrosin can self-catalyze its own conversion into m_α-acrosin by the intrazymogen mechanism represented by eq 3.

Other observations recorded in this laboratory also support the occurrence of an intrazymogen mechanism in the conversion of proacrosin into m_α-acrosin. Highly purified proacrosin spontaneously and completely converted into only m_α-acrosin when incubated at neutral pH in the presence of a some 1500-fold excess of lima bean trypsin inhibitor (LBTI; K. L. Polakoski, unpublished results). Since the evolution of acrosin could only be followed by $NaDodSO_4$–polyacrylamide disc gel electrophoresis, the technique using LBTI could not afford parameters necessary to differentiate between an inter- and intrazymogen conversion mechanism. However, these results clearly demonstrated that, like the experiments using leupeptin, the conversion of proacrosin into acrosin does not require acrosin activity. Moreover, the experiments using LBTI are strong evidence that the selective conversion of proacrosin into m_α-acrosin is not due to some peculiarity in the mechanism of acrosin inhibition by leupeptin. In additional experiments, highly purified proacrosin was found to spontaneously convert into active enzyme when covalently bound to an insoluble resin (Kavka & Polakoski, 1979). The acrosin so prepared apparently remained insolubilized since it could not be eluted from the resin and has retained enzymatic activity even after standing for over a year at neutral pH. Under similar conditions, solubilized acrosin loses its activity within hours due to autodegradation (Parrish & Polakoski, 1978). This latter finding effectively refutes criticisms of the foregoing conclusion in this report which may argue that, based on hypothetical mechanisms, the first-order process observed for the conversion of proacrosin into m_α-acrosin in the presence of excess leupeptin might represent some rate-limiting intramolecular event which occurs during an interzymogen-catalyzed conversion mechanism (similar to eq 2). Like autodegradation, such zymogen-catalyzed conversion mechanisms require intermolecular contacts which were most likely disallowed when the zymogen and enzyme product are attached to the insoluble resin. Accordingly, all of these combined results strongly indicate that the proacrosin self-catalyzed conversion into m_α-acrosin proceeds by an intrazymogen mechanism.

The physiological significance of an intrazymogen mechanism in the conversion of proacrosin into acrosin in vivo is not known. However, this mechanism does eliminate the absolute requirement that some additional sperm acrosomal proteinase act to convert proacrosin into acrosin, a process which is essential for sperm penetration of the ovum (Zaneveld et al., 1975). Therefore, contraceptive agents directed to inhibit proacrosin conversion will most likely have to also prevent the intrazymogen conversion mechanism. Moreover, a variety of nonproteolytic agents which are found in the female tract or in spermatozoa greatly stimulate the in vitro rates of proacrosin conversion into acrosin without affecting the catalytic efficiency of acrosin. These include anionic phospholipids (Parrish et al., 1978) and glycosaminoglycans which were obtained from uterine fluids (Wincek et al., 1979) or from commercial sources (Parrish et al., 1980). Since the use of leupeptin or leupeptin-like inhibitors isolates and, more importantly, quantitates the intrazymogen conversion mechanism, the effects of these and other potential activators and/or inhibitors of the conversion of proacrosin into acrosin may now be more readily assessed.

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Interaction of Dinucleotides with Muscle Phosphofructokinase†

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ABSTRACT: The binding of adenosine diphosphoribose (ADPR), NADH, NADPH, NADP, and NAD by rabbit skeletal muscle phosphofructokinase was determined on the basis of their ability to competitively inhibit the binding of adenosine cyclic 3′,5′-phosphate (cAMP) and of their ability to induce a conformational change as indicated by thiol reactivity. The binding affinities of ADPR and the dinucleotides decreased in the order listed above. The dissociation constant for ADPR was about 5 μM as estimated from the inhibition of cAMP binding. Whereas ADPR and the dinucleotides were capable of blocking the reactivity of two thiol groups in a manner similar to that of other adenine nucleotides, ADPR had no effect on the rate of arylation of the most reactive thiol group of phosphofructokinase, a thiol group whose reactivity is thought to be an indicator of the active–inactive protein conformational transition. Furthermore, ADPR was capable of inhibiting the effect of cAMP on the arylation rate of the reactive thiol group. ADPR was not capable of activating the reaction catalyzed by phosphofructokinase, but instead prevented the activation of phosphofructokinase by cAMP in an apparent competitive manner. These results indicate that ADPR, and presumably the other nicotinamide dinucleotides, binds to the adenine nucleotide activating site of phosphofructokinase but is unable to produce the active conformation. On the basis of the relative affinities of phosphofructokinase for 8-azido-cAMP, 8-amino-cAMP, and cAMP, it was deduced that the adenine nucleotide site favors the anti-glycosidic conformation of nucleotide effectors over the syn-glycosidic form. Substitution at the N-6 position of the adenine ring prevents binding to the adenine nucleotides site in contrast to the known binding behavior of adenine nucleotides to the catalytic site.

Rabbit skeletal muscle phosphofructokinase displays a complex regulatory behavior that is the consequence of its interaction with a variety of metabolites at specific binding sites [see Uyeda (1979) for a review]. Three of the binding sites are capable of tightly binding adenine nucleotides: the catalytic site, an inhibitory site, and an activating site (Kemp & Krebs, 1967). The activating site, which shows greatest specificity for adenine mononucleotides, reacts with 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine, a derivative of adenosine extended from the 5 position of ribose that has been used for the modification of the dinucleotide site of several dehydrogenases (Pettigrew & Frieden, 1978). This observation, coupled with the known binding of phosphofructokinase to blue dextran, a probe for dinucleotide folds (Bohme et al., 1972), suggested a study of the interaction of nicotinamide adenine dinucleotides with phosphofructokinase.

The present study shows that dinucleotides apparently compete with mononucleotides at the activating site of muscle phosphofructokinase. This interaction does not activate the enzyme but instead inhibits the activating action of the mononucleotides.

Materials and Methods

Enzyme Preparation. Phosphofructokinase (twice crystallized) was prepared from fresh rabbit muscle as described by Kemp (1975). The second crystals were collected by centrifugation and dissolved in the indicated buffer. For removal of ammonium sulfate, the phosphofructokinase solution was dialyzed with one change against 100 volumes of the same buffer, at room temperature. When it was necessary to remove bound ATP, the enzyme solution was passed through a charcoal–cellulose (1:1 w/w) column (4.5 × 20 mm). A 280/260 absorbancy ratio greater than 1.6 was taken as an indication that ATP had been essentially removed from the protein (Parmeggiani et al., 1966).

Binding Experiments. The fast-flow equilibrium dialysis method of Colowick & Womack (1969) was used to determine the dissociation constant for adenosine cyclic 3′,5′-phosphate (cAMP)[1] and for competitive binding assays. The cylindrical

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[1] Abbreviations used: ADPR, adenosine diphosphoribose; NMN, nicotinamide mononucleotide; cAMP, adenosine cyclic 3′,5′-phosphate; DTT, dithiothreitol; Cl_3CCOOH, trichloroacetic acid; IMP, inosine 5′-phosphate; $(Nbs)_2$, 5,5′-dithiobis(2-nitrobenzoic acid).

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