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Characterization of Sarcoplasmic Reticulum Adenosinetriphosphatase Purified by Selective Column Adsorption†

Ari Gafni‡ and Paul D. Boyer*

ABSTRACT: Preparations of sarcoplasmic reticulum ATPase made by conventional procedures, with over 85% of the protein consisting of one band in sodium dodecyl sulfate gel electrophoresis, were solubilized in Triton X-100 and separated on an Affi-Gel blue column. All the ATPase activity was eluted in a single fraction containing about 60% of the applied protein. This purified fraction required combination with about 1 mol of fluoresceinyl 5-isothiocyanate (FITC) for inactivation, whereas the original preparation was inactivated by reaction with about 0.6 mol of FITC/mol. The inactive protein retained on the column had an amino acid composition like that of the active protein. The separation on the Affi-Gel blue column provides a convenient procedure for preparation of more active ATPase. The rate of inactivation of the ATPase solubilized in detergent-containing solutions was measured at different protein concentrations. The $t_{1/2}$ for inactivation was proportional to the square root of the protein concentration. Results are consistent with inactivation proceeding through a small fraction of monomeric ATPase present.

The Ca^{2+}- and Mg^{2+}-dependent sarcoplasmic reticulum (SR)[1] is composed of a single polypeptide chain with a M_r in the range of 100 000–120 000 (Martonosi & Halpin, 1971; Louis & Shooter, 1972; Thorley-Lawson & Green, 1973; Rizzolo et al., 1976; Dean & Tanford, 1978; MacLennan et al., 1978). The enzyme couples ATP hydrolysis to active transport of Ca^{2+} into the SR luminal space and is reported to comprise up to 90% of the membrane protein of the SR (Meissner, 1975; Fleischer & McIntyre, 1982). Numerous studies, utilizing various approaches, have shown that in the SR membrane an oligomeric form of the ATPase is the prevailing species [de Meis (1981) and references cited therein] although the monomeric enzyme is known to possess ATPase activity (Dean & Tanford, 1978; Kosk-Kosicka et al., 1983; Moller et al., 1980). The functional significance of the native ATPase oligomer is still a matter of controversy.

Recently, it was found that the SR ATPase is completely inactivated by binding 4.2 nmol of FITC/mg of protein (Pick & Karlish, 1980; Pick & Bassilian, 1980; Pick, 1981a,b). From these studies, it was suggested that the SR ATPase shows half-sites reactivity, i.e., that, due to strong interactions between the two subunits in an ATPase dimer, only one subunit is labeled with FITC at any given time. This conclusion was recently criticized by Mitchinson et al. (1982), who found that 0.89 mol of FITC was needed for complete inactivation of 1 mol of detergent-purified ATPase and concluded that a 1:1 stoichiometry exists between active ATPase and the inactivating agent.

Other studies from this laboratory indicated a possible functional role of enzyme dimers (McIntosh & Boyer, 1983). For further investigations, it appeared advantageous to attempt to obtain fully active enzyme through the aid of affinity chromatography. Suitability for affinity chromatography of enzyme dissolved by nonionic detergent was explored. We found, unexpectedly, that an inactive portion of the ATPase preparation could be selectively retained on a column of Affi-Gel blue. Such columns have been found to retain proteins with nucleotide binding sites (Stellwegen, 1977). This paper describes a simple procedure for the removal of inactive protein from SR ATPase preparation and presents studies with this preparation on the FITC inactivation and on the concentration dependency of inactivation of the preparation that give information about the dimeric state of the enzyme dissolved in detergent.

Materials and Methods

SR vesicles were isolated from rabbit skeletal muscle according to Champeil et al. (1978) and were stored at liquid nitrogen temperature in a medium containing 0.3 M sucrose, 0.1 M KCl, and 10 mM Hepes, pH 7.4. The specific ATPase activity of the SR vesicles was 1.3–1.5 μmol of P_i (mg of protein)$^{-1}$ min^{-1} and was determined at 25 °C in a medium containing 0.1 M KCl, 5 mM Mg^{2+}, 5 mM oxalate, 1 mM ATP, and 0.1 mM Ca^{2+} in 20 mM Mops buffer, pH 7.0. The basal ATPase activity (Ca^{2+} independent) was 25–40 times smaller than the Ca^{2+}-dependent activity.

† From the Chemistry and Biochemistry Departments and Molecular Biology Institute, University of California at Los Angeles, Los Angeles, California 90024. *Received February 7, 1984.* This research was supported by a grant from the National Science Foundation (PCM 75-18884).

‡ Recipient of a Senior Investigatorship, American Heart Association Greater Los Angeles Affiliate. Present address: Institute of Gerontology and Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109.

[1] Abbreviations: FITC, fluoresceinyl 5-isothiocyanate (isomer I); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; SR, sarcoplasmic reticulum; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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Triton X-100 was purchased from Sigma while FITC (isomer I) was from Molecular Probes (Junction City, OR). The dye was dissolved in dry dimethylformamide to give a ~5 mM stock solution.

Affi-Gel blue (100–200 mesh) was a product of Bio-Rad Laboratories. The gel was repeatedly washed with 1 M KCl solution until the filtrate was free of blue color and was then washed with distilled water to eliminate the salt.

Protein concentration was determined by the Folin method with bovine serum albumin as the standard. The solutions contained 3% SDS to avoid interference of the Triton X-100 (Silva & Verjovski-Almeida, 1983).

Chromatography of SR Proteins. An Affi-Gel blue column (0.8 × 7.5 cm) was washed with 10 mL of 1 M KCl solution followed by 10 mL of a mixture containing 10% Triton X-100, 7.5 M urea, and 0.5 M NaOH. The column was subsequently equilibrated with a 20 mM Mops buffer, pH 7.0, containing 1% Triton X-100, 5 mM $MgCl_2$, and 0.4 mM $CaCl_2$ (elution buffer). A suspension of SR vesicles containing ~30 mg of protein/mL was dissolved by mixing it with 3 volumes of a 10% Triton X-100 solution in elution buffer, so as to yield 10 mg of detergent/mg of protein. Traces of undissolved material were centrifuged off (2 min, 15600*g* in an Eppendorf Model 5414 microcentrifuge) and discarded. A 400-μL aliquot of the clear solution was applied to the Affi-Gel blue column. The column was initially developed with the elution buffer, then with elution buffer containing increasing concentrations of KCl, and finally with the urea–Triton–NaOH mixture mentioned above. The elution rate was 7–8 mL/h, and 0.5-mL fractions were collected. In some of the experiments, these fractions were mixed with an equal volume of 60% sucrose solution in the elution buffer to stabilize the ATPase against inactivation. Several portions of the dissolved SR proteins not applied to the column were diluted 4–30-fold in the elution buffer, while another aliquot was mixed (within a few min after dissolving the vesicles) with an equal volume of 60% sucrose. These solutions served as references for ATPase activity.

Time- and Concentration-Dependent Inactivation of SR ATPase. In these experiments, a clear solution of SR protein, prepared as described above, was brought to a concentration of 7 mg/mL. A series of dilutions was then made from this solution into aliquots of the elution buffer to yield the desired protein concentrations. These samples were incubated at room temperature (22–23 °C) and their ATPase activities determined at various times.

FITC Labeling Experiments. These were performed with both intact SR vesicles and dissolved SR proteins. In a typical experiment, the sample to be labeled (500 μL, protein concentration 0.5–3 mg/mL) was brought to pH 8.4 by centrifuging it through a 6-mL G-50 centrifuge column (Penefsky, 1977) that had been equilibrated with 20 mM Mops buffer, pH 8.4, containing 5 mM $MgCl_2$, 0.4 mM $CaCl_2$, and 1% Triton X-100. The desired amount of FITC was added, from a 5×10^{-4} M aqueous solution (prepared from the stock solution of the dye in dimethylformamide and used immediately), and the reaction mixture was incubated at room temperature for 30 min. Unreacted dye was removed by passing the samples through 6-mL G-50 centrifuge columns equilibrated with elution buffer. Protein concentrations, ATPase activity, and FITC concentration were then determined, the latter from the value of A_{498} after the solution was made 0.1 N in NaOH. A value of 80000 M^{-1} cm^{-1} was used for E_{498} of the bound FITC (Pick & Karlish, 1980; Mitchinson et al., 1982). In some of the early experiments, a pH 10 buffer was used as the labeling medium, and the incubation time with FITC was 10–15 min.

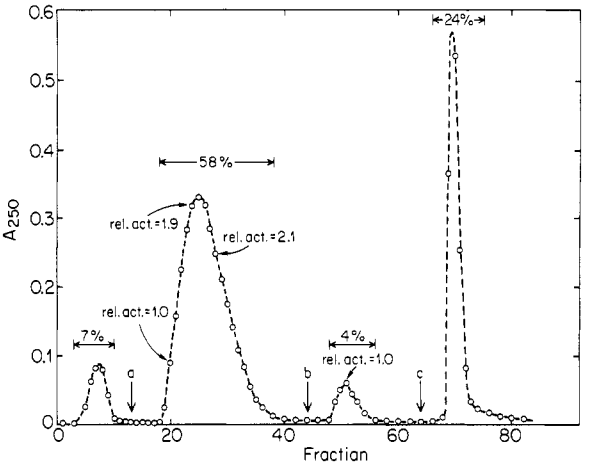

FIGURE 1: Chromatography of SR proteins on an Affi-Gel blue column. The column was equilibrated with the low ionic strength elution buffer (see text for details), and the dissolved SR proteins were applied. The column was initially washed with the elution buffer until no more protein could be recovered. The arrows indicate points where the buffer was changed: (a) elution buffer + 70 mM KCl; (b) elution buffer + 400 mM KCl; (c) mixture consisting of 10% Triton X-100, 7.5 M urea, and 0.5 N NaOH. Numbers above the peaks indicate percent protein in corresponding band, relative to total amount of protein applied. The relative activities given for several fractions are compared with the activity of a reference sample of dissolved SR proteins not applied to the column. The concentration of protein in a given fraction is proportional to the A_{750} of its reaction product with the Folin reagent.

Polyacrylamide Gel Electrophoresis. Electrophoresis was done on 10% acrylamide according to Laemmli (1970). For electrophoresis, the ATPase was dissolved in a buffer containing 0.063 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 2% β-mercaptoethanol, and 0.001% bromphenol blue and then run on a slab gel containing 10% acrylamide. To evaluate the relative amount of protein in the ~10^5-dalton band, this band was cut out and the Coomassie blue extracted by incubation in 0.05 M Tris, 0.1% SDS, and 0.4 M glycine overnight. The optical density of this solution was then compared with the one obtained for a combined extract made from all other protein bands on the gel.

Amino Acid Analyses. These were done on a Beckman 120C amino acid analyzer, after the protein samples had been hydrolyzed for 24 h in constant-boiling HCl at 106 °C.

Results

Column Separations. A typical column separation of SR proteins by the Affi-Gel blue column is depicted in Figure 1. A protein band, containing ~7% of the total applied protein and showing no ATPase activity, was eluted after the void volume. No more protein was recovered when the column was washed further with the low ionic strength elution buffer. More protein bands may, however, be eluted by increasing the ionic strength of the running buffer. Thus, the use of 70 mM KCl induced the elution of a protein band that in various experiments was found to contain 55–62% of the total protein applied and that showed high ATPase activity. Only very small amounts of protein were obtained by further increasing the ionic strength, about 4% of the applied protein coming off with 400 mM KCl and practically none with an additional increase of KCl to 1 M. A 10% Triton X-100 solution, 0.5 N NaOH, or 7.5 M urea all failed to elute any more protein. However, when a mixture of these three reagents was used, a sharp band of protein was washed off the column that contained ~24% of the applied protein (obviously with no ATPase activity). The total recovery of protein is thus ~93%.

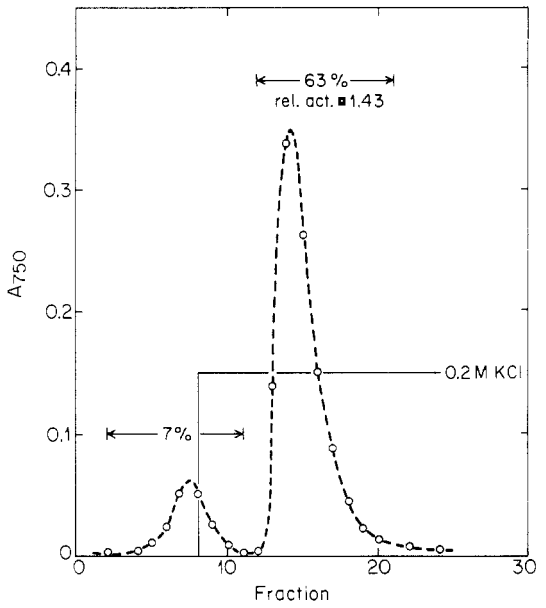

FIGURE 2: Elution profile of protein bands from the Affi-Gel blue column under conditions aimed at minimizing the time needed to recover the active protein band. The column was washed at an increased rate; a relatively high ionic strength (0.2 M KCl) buffer was used that was added so as to yield the minimal complete separation between the two protein peaks.

An evaluation of the recovery of ATPase activity is somewhat more complex since the activities of both the reference sample and of the fractions collected off the column decrease with time and, as will be discussed below, the rate of inactivation depends on protein concentration and thus varies among the various fractions. To minimize the effects of inactivation, the experiment presented in Figure 2 was done. The column was washed at an increased rate (12 mL/h), a KCl concentration of 0.2 M was used, and the salt was applied at such a time so as to give the minimal complete separation between the two protein peaks. As a result, the active protein was completely recovered from the column in about 1 h in a relatively narrow band that contained 63% of the applied proteins. This band contains the proteins corresponding to bands 2 and 3 shown in Figure 1. The sample that served as a reference in this experiment was mixed, within a few minutes after the SR vesicles were dissolved, with a 60% sucrose solution in a 1:1 ratio. Each of the chromatography fractions were also mixed with the same sucrose solution immediately after being eluted from the column. This treatment eliminates the loss of activity in the reference solution as well as in the chromatography fractions after coming off the column; the presence of 30% sucrose greatly increases the stability of the ATPase (Murphy et al., 1982). The activity of the pooled fractions of the active band was found to be 1.43 times that of the reference sample, thus amounting to 90% of the total activity applied to the column. This is a lower limit for the activity associated with the active protein band because some activity was likely lost before the various fractions were eluted and stabilized by sucrose. An attempt to avoid inactivation during the chromatography by including 30% sucrose in the washing buffers resulted in poor separation of proteins on the column, and this approach is therefore not useful. On the basis of the results given earlier, we conclude that practically all the ATPase activity is contained in about 62% of the SR proteins. The SDS gel electrophoresis experiments (data not presented) showed 85% of the SR proteins to be in the 10^5-dalton band. Thus, a significant fraction of the protein in this band (27%) has no ATPase activity.

Table I: Amino Acid Composition of the SR ATPase Preparations[a]

amino acid	active ATPase band (this paper)	SR ATPase Thorley-Lawson & Green (1975)	SR ATPase Meissner et al. (1973)
lysine	51	55	56
histidine	12	13.5	14
arginine	47	42	53
aspartic acid	86	91.5	91
threonine	71	60.5	61
serine	70	76.5	52
glutamic acid	107	109	111
proline	47	50	57
glycine	78	80	73
alanine	94	87.5	89
half-cystine	ND[b]	26.5	24
valine	80	80	80
methionine	36	34.5	36
isoleucine	64	62.5	65
leucine	97	99	101
tyrosine	24	23	24
phenylalanine	41	41	55
tryptophan	ND	20	ND

[a] The M_r used is 115 000. [b] Not determined. The values for half-cystine and tryptophan given by Thorley-Lawson and Green were used in calculating M_r 115 000.

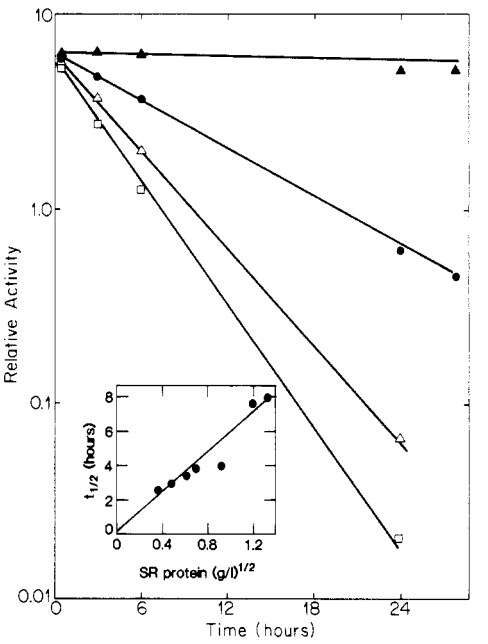

FIGURE 3: Time-dependent inactivation of SR proteins dissolved in Triton X-100 for various concentrations of (total) protein, at room temperature (22–23 °C). Details of the experimental procedures used are given in the text. Protein concentrations are (●) 1.88 mg/mL, (△) 0.50 mg/mL, (□) 0.25 mg/mL, and (▲) 0.50 mg/mL in presence of 30% sucrose. The inset describes the dependence of the half-life of the inactivation reaction, $t_{1/2}$, on the square root of the total concentration of dissolved SR proteins. The value of $t_{1/2}$ for the various protein concentrations was evaluated from activity vs. time plots of the type shown.

Amino Acid Analyses. Analysis of the proteins in the active band (band 2) in Figure 1 is summarized in Table I. Published amino acid compositions of SR ATPase from several laboratories show a significant degree of variation (Martonosi & Halpin, 1971; Louis & Shooter, 1972; Meissner et al., 1973; MacLennan et al., 1971; Thorley-Lawson & Green, 1975). Our results for the active ATPase band are in good agreement with those of Thorley-Lawson & Green (1975) and of Meissner et al. (1973).

ATPase Inactivation Studies. Figure 3 presents the specific ATPase activity of SR proteins dissolved in Triton X-100 as

Table II: Summary of FITC Labeling Experiments

sample	protein in 10^5-dalton band (μM)[a]	FITC (μM)	pH	incubation time (min)	FITC/protein in conjugate[b]	activity loss (%)
SR vesicles	32	15	10	10	0.15	25
	32	60	10	15	0.24	47
	40	100	8.4	30	0.47	89
dissolved SR	40	70	8.4	30	0.55	95
proteins[c]	40	200	8.4	30	0.65	>99
ATPase[d]	6.5	3.2	8.4	30	0.30	32
	6.5	6.5	8.4	30	0.70	65
	6.5	13	8.4	30	0.97	99

[a] Based on our finding that 85% of SR proteins are in this band and assuming a M_r of 115000. [b] Total protein content assumed to be ATPase and a M_r of 115000. [c] SR vesicles were dissolved in 10-fold excess Triton X-100 as described under Materials and Methods. [d] Purified by separation on an Affi-Gel blue column.

a function of the incubation time at room temperature, for several protein concentrations. The inactivation kinetics are clearly first order for all protein concentrations studied, and the specific activities extrapolate to a common value at zero time. A remarkable stabilization of the ATPase in the presence of 30% sucrose is apparent, as noted before by Murphy et al. (1982). The stability of the enzyme under these conditions was used in the present study to compare the activity of column-separated SR proteins with the reference sample as described earlier. When the half-life values for inactivation ($t_{1/2}$) are plotted against the square root of the (total) concentration of SR proteins in solution, a linear relationship, also shown in Figure 3, is obtained. Extrapolation of the straight line to zero concentration of protein yields a $t_{1/2}$ of about 10 min. The significance of these observations is dealt with under Discussion.

FITC Labeling Experiments. In agreement with previous reports (Pick & Karlish, 1981; Pick & Bassilian, 1981; Mitchinson et al. 1982), we found that binding of FITC to the SR ATPase, both before and after solubilization in Triton X-100, proceeded rapidly with a concomitant reduction in ATPase activity. The labeling experiments are summarized in Table II. For SR proteins before column separation, we found the extent of activity loss to be about twice the fraction of enzyme labeled so that complete inactivation was obtained upon apparent labeling of 50% of the ATPase molecules. The protein dissolved in detergent was found to be more accessible to the FITC since a more efficient labeling was obtained under the same concentration of dye (see Table II). However, even when solubilized protein and a 4.5-fold excess of FITC were used, only 65% of the 10^5-dalton protein could be labeled. In contrast, when the interaction of FITC with ATPase that had been purified by chromatography on the Affi-Gel blue column (band 2) was studied, a binding of one FITC molecule per polypeptide chain (accompanied by a complete loss of activity) was observed even when only a 2-fold excess of dye over protein was used. An incomplete labeling was obtained when the concentrations of FITC used were comparable or smaller than the concentration of ATPase. Moreover, a direct relationship between the amount of labeling and the loss of ATPase activity is evident from the data in Table II.

Discussion

Our present results show that while about 85% of the total protein content of SR is in a $\sim 10^5$-dalton protein band apparent in SDS gel electrophoresis, all of the ATPase activity is associated with only about 60% of SR proteins. Thus, clearly a significant fraction of the 10^5-dalton band (27%) possesses no ATPase activity. The active ATPase may be separated from this inactive protein by selective adsorption on Affi-Gel blue with the procedure described in this work. This constitutes a convenient as well as an efficient procedure for preparing the ATPase in a purified form of high activity.

The FITC labeling experiments are in accord with the noted purifications. A complete loss of activity of the native (unpurified) ATPase is observed when about 0.65 mol of FITC bind per mol of protein (see Table II) while for the purified ATPase a 1:1 ratio between bound dye and protein is needed for complete inactivation. We therefore conclude that one FITC binding site exists in each ATPase polypeptide chain and that the lower numbers obtained with unpurified ATPase are due to the presence of inactive proteins that do not bind FITC under the conditions used. This conclusion is in line with the suggestion made by Murphy et al. (1982) that roughly half of the 10^5-dalton protein is active ATPase. Mitchinson et al. (1982) also interpreted their FITC labeling results in a similar way.

The nature of the $\sim 10^5$-dalton impurity is of interest. Thorley-Lawson & Green (1973) studied the cleavage of the proteins in this band by trypsin and concluded that over 95% of the protein content was ATPase. Yamaguchi & Post (1983) performed isoelectric focusing on SR ATPase and found that the catalytic activity appeared with a single, though broad, band, indicating that proteins other than the ATPase protein are not present in the 10^5-dalton band to any significant extent. Our amino acids analysis results support this conclusion. The composition of the active ATPase band, purified with Affi-Gel blue, agrees well with the amino acid composition of the total protein content of the 10^5-dalton band even though 27% of the protein has been removed by the purification procedure. This observation indicates that the protein being removed has the same composition as that of the active ATPase. It seems, therefore, that the 10^5-dalton protein in SR is a mixture of active and inactive ATPase molecules. The ratio between these two components may vary among different preparations depending on the experimental procedures used, the length of storage of the vesicles, etc.

The degree of association of the SR ATPase in solution under the conditions of our experiments is of importance since it may affect the efficiency of separation between active and inactive ATPase molecules if these tend to form mixed aggregates. The state of aggregation of the SR ATPase when the membrane is dissolved by nonionic detergents has been addressed in numerous studies and is strongly dependent on the concentrations both of the protein and of the detergent as well as on other experimental conditions (Kosk-Kosicka et al., 1983; Ludi & Hasselbach, 1983; Murphy et al., 1982; Silva & Verjovski-Almeida, 1983). Our ATPase inactivation experiments (Figure 3) show that while the loss of activity at all protein concentrations used follows first-order kinetics the

rate constant strongly depends on the total concentration of protein and a linear relationship exists between the half-life for inactivation and the square root of protein concentration. Such concentration dependency points to an aggregation process of the type presented by the following scheme:

$$D \overset{K_d}{\rightleftharpoons} 2M$$

$$M \xrightarrow{k_p} M'$$

$$D \xrightarrow{k_p'} 2M'$$

Here, D, M, and M′ are the ATPase dimer, monomer, and inactive monomer, respectively. The total activity is the sum of the activities of dimer and monomer:

$$\text{Act} = \text{Act}_D + \text{Act}_M = a[D] + b[M]$$

where a and b are constants. The inactivation is given by

$$\frac{d\text{Act}}{dt} = a\frac{d[D]}{dt} + b\frac{d[M]}{dt}$$

As the protein concentration tends to zero, the monomer–dimer equilibrium is shifted toward the former species. Extrapolation of our data to zero concentration of protein (Figure 3, inset) yields a value for $t_{1/2}$ of the order of 10 min. Thus, if the protein in our experiments consisted of a mixture of monomer and dimer, one would anticipate a biphasic inactivation pattern, the amplitude of the fast phase being proportional to the relative amount of monomer in solution. The inactivation kinetics (Figure 3) show no such fast step; hence, we conclude that at the protein concentrations used in the present study the concentration of monomeric ATPase is small and the protein is predominantly in an aggregated form. A direct decomposition of ATPase dimers to inactive product (with the rate controlled by k_p') should be independent of enzyme concentration and, hence, can also not contribute significantly to the inactivation process.

A mechanism for inactivation that satisfactorily agrees with the data in Figure 3 is

$$D \overset{K_D}{\rightleftharpoons} 2M \xrightarrow{k_p} 2M'$$

where $K_D = [M]^2/[D] \ll 1$, where the monomer–dimer equilibrium is fast compared with the rate of inactivation, and where mixed dimers composed of active and inactive monomers form, in which the active unit is protected in the same way as the subunits in a fully active dimer. The activity of the sample under study is assumed to be proportional to the concentration of active enzyme in the sample. Since the affinity of an enzyme protein toward an active and inactive second protein molecule is assumed to be the same, the following relation holds:

$$[D]/[D]_T = [M]/[M]_T$$

where D and M are active species while $[D]_T$ and $[M]_T$ are the total concentrations (active plus inactive) of dimeric and monomeric species. The dissociation constant is $K_D = [M]_T^2/[D]_T$. Since the concentration of monomer in solution was shown before to be negligible

$$\frac{d\text{Act}}{dt} = a\frac{d[D]}{dt} = -\frac{ak_p}{2}[M] = -\frac{ak_p}{2}\frac{[M]_T}{[D]_T}[D] = -\left(\frac{ak_p}{2}K_D^{1/2}/[D]_T^{1/2}\right)[D]$$

Hence

$$\text{Act}_{(t)} = \text{Act}_{(0)}e^{-t/\tau}$$

where $\tau = 2[D]_T^{1/2}/(k_pK_D^{1/2})$.

The activity loss thus follows first-order kinetics; however, the half-life of this inactivation process depends on the square root of (total) enzyme concentration. The fact that our experimental data fit this pattern is a strong indication that the assumptions made in constructing the model for inactivation are indeed valid.

The possible significance of the formation of mixed dimers composed of active and inactive subunits to the efficiency of separation of these forms on the Affi-Gel blue column needs consideration. It is obvious from our experiments that the inactive form of the 10^5-dalton protein is more strongly attached to the column than the active enzyme. If the mixed dimers were also trapped on the column, then part of the active ATPase would be lost during chromatography. This loss is not significant because practically all the ATPase activity is recovered from the column in the active protein band. Moreover, the fraction of SR protein that has to be labeled by FITC to completely abolish ATPase activity (~64%) agrees very well with the amount of active ATPase recovered from the column (62% of total SR proteins). We thus conclude that there is no retardation in the elution of active subunits due to the formation of mixed dimers. This may be due to an enhanced dissociation of ATPase dimers induced by binding to the column (e.g., that the bound protein is monomeric) or, alternatively, to a dynamic process in which the active monomers produced as part of the fast monomer–dimer exchange at equilibrium are washed from the strongly attached inactive subunits.

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Registry No. FITC, 3326-32-7; ATPase, 9000-83-3.

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Isolation and Properties of a Single-Strand 5′→3′ Exoribonuclease from Ehrlich Ascites Tumor Cell Nucleoli†

Linda S. Lasater and Duane C. Eichler*

ABSTRACT: A single-strand-specific, nucleolar exoribonuclease from Ehrlich ascites tumor cells has been isolated and purified free from other nucleases. The exonuclease degraded single-stranded RNA processively from either a 5′-hydroxyl or a 5′-phosphorylated end and released 5′-mononucleotides. The enzyme digested single-strand poly(C), poly(U), and poly(A) equally well but did not degrade duplex poly(C)·poly(I) or poly(A)·poly(U). Less than 0.2% of duplex DNA or 1.5% of heat-denatured DNA was degraded under the conditions which resulted in greater than 26% degradation of RNA. The ribonuclease required Mg^{2+} (0.2 mM) for optimum activity and was inhibited by ethylenediaminetetraacetic acid but not by human placental RNase inhibitor. The native enzyme had a Stokes radius of 42 Å and a sedimentation coefficient ($s_{20,w}$) of 4.3 S. From these values, an apparent molecular weight of 76 000 was derived by using the Svedberg equation. The localization and unique mode of degradation suggest a role for the 5′→3′ exoribonuclease in ribosomal RNA processing.

In mammalian cells, the precursor of ribosomal RNA has a molecular weight of about 4.5×10^6 and a sedimentation coefficient of 45 S. The 45S precursor RNA is sequentially cleaved to yield mature 18S, 5.8S, and 28S rRNAs in addition to spacer fragments (Perry, 1976; Abelson, 1979). The arrangement of the primary transcript is thought to have the following structure: (5′)-spacer-18S-spacer-5.8S-spacer-28S-(3′) (Perry, 1976; Nazar, 1982; Dawid & Wellauer, 1976). The transcribed spacer sequences are removed stepwise at each maturation step (Perry, 1976; Abelson, 1979; Busch et al., 1982). The precursor 45S RNA molecule is enzymatically cleaved into intermediate 20S and 32S molecules which are further cleaved and trimmed to the final 18S, 5.8S, and 28S mature ribosomal RNA molecules (Nazar, 1982; Busch et al., 1982; Bowman et al., 1983).

For elucidation of the enzymes involved in ribosomal RNA processing, research in our laboratory has been directed toward the identification of ribonucleases that are associated with purified nucleoli. Thus far we have identified three distinct single-strand-specific ribonucleases. Two of these enzymes are single-strand-specific endonucleases which we have designated nucleolar RNase I and nucleolar RNase II, respectively (Eichler & Tatar, 1980; Eichler & Eales, 1982). In this report, we described a third single-strand-specific activity, nucleolar RNase III, which has been identified as a 5′→3′ exoribonuclease. Its properties, which distinguish it from the other nucleolar ribonucleases and imply a possible role in processing of ribosomal RNA, are presented in this paper.

Materials and Methods

Materials. Unlabeled and labeled [^{3}H]poly(C) (22.8 mCi/mmol), [^{3}H]poly(A) (42 mCi/mmol), [^{3}H]poly(U) (525 mCi/mmol), [^{3}H]poly(C)·poly(I) (9.8 mCi/mmol), and [^{3}H]poly(U)·poly(A) (19.6 mCi/mmol) were obtained from Miles Chemicals Corp. Cytidine 3′,5′-[5′-^{32}P]diphosphate (2000 Ci/mmol) was purchased from ICN. [γ-^{32}P]ATP (1000–2000 Ci/mmol) was prepared as described by Maxam & Gilbert (1977). Cytidine 3′,5′-diphosphate was purchased from P-L Biochemicals. The dye-binding protein assay kit was obtained from Bio-Rad. Poly(ethylenimine)-impregnated cellulose thin-layer chromatography plates were purchased from Brinkmann.

Proteins and Enzymes. Bacterial alkaline phosphatase and T_4 polynucleotide kinase were purchased from Bethesda Research Laboratories. S_1 nuclease and T_4 RNA ligase were obtained from P-L Biochemicals. Bovine serum albumin, ovalbumin, and yeast alcohol dehydrogenase were obtained from Sigma. Human placental ribonuclease inhibitor was prepared by the method of Blackburn (1979).

RNAs. ^{32}P-Labeled f_2 RNA (4×10^4 cpm/nmol) was prepared essentially according to the procedure of Glitz (1968) for MS2 RNA, and at the same time, modifications of this procedure also permitted the preparation of ^{32}P-labeled *Escherichia coli* RNA (2.5×10^4 cpm/nmol). Yeast 5.8S rRNA was prepared according to the procedure of Rubin (1975) from frozen yeast cells (*Saccharomyces cerevisiae* X2180-1B) generously provided by Dr. Jeremy Thorner (University of California at Berkeley). The 5′-^{32}P labeling of yeast 5.8S rRNA was carried out according to the procedure of Donis-

† From the Department of Biochemistry, College of Medicine, University of South Florida, Tampa, Florida 33612. *Received November 9, 1983; revised manuscript received April 2, 1984.* This work was supported by National Institutes of Health Grant R01 GM 29162-02.

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