Disruption of prosomes by some bivalent metal ions results in the loss of their multicatalytic proteinase activity and cancels the nuclease resistance of prosomal RNA

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Prosome are ribonucleoprotein particles constituted by a variable set of about 20 proteins found associated with untranslated mRNA. In addition, they contain a small RNA, the presence of which has been an issue of controversy for a long time. The intact particles have a multicatalytic proteinase (MCP) activity and are very stable; we have never observed autodigestion of the particle by its intrinsic proteinase activity. Surprisingly it was found that Zn^{2+} and Cu^{2+} ions at concentrations of 0.1–1 mM disrupt the prosome particles isolated from HeLa cells and duck erythroblasts and abolish instantaneously its MCP activity, without altering the two-dimensional electrophoretic pattern of the constituent proteins. Fe^{2+} , however, seems to induce autodegradation rather than dissociation of the prosome constituents. Most interestingly, protein or oligopeptide substrates protect the particle and its proteinase activity from disruption by Zn^{2+} or Cu^{2+} . Nuclease-digestion assays reveal that the prosomal RNA, which is largely resistant in the intact particle, becomes digestible after dissociation of prosomes by Zn^{2+} . These data give, for the first time, unambiguous proof of the presence of an RNA in the particle. Furthermore, they demonstrate a structure–function relationship between the complex and its enzyme activity, which seems to be based on the particle as an entity and not on the single constituent proteins.

INTRODUCTION

Prosome are small cylinder-shaped ubiquitous ribonucleoprotein particles, first observed as subcomplexes of free messenger ribonucleoprotein in HeLa cells [5] and later defined as a biochemical entity of its own in mouse and duck erythroblasts ([1,6] reviewed in [7]). The individual particle, sedimenting at 19S, is made up of about 20 characteristic proteins with M_r ranging from 19000 to 36000. According to our data [8] and those published recently [9,10], the genes coding for these proteins constitute a new family.

Small RNA (pRNA) with a length of 80–120 nucleotides has been isolated from purified prosomes of many types of cells and tissues, such as mouse or duck erythroblasts, rat liver, HeLa cells, calf liver, *Drosophila* and plants [6,11–14]. This RNA seemed to be an intrinsic part of prosomes, since nucleases can only digest it after the elimination by phenol extraction of the prosomal protein components [15]. However, often investigators have not found pRNA in particles that seemed to be identical with prosomes. The presence of RNA as an essential feature of the particle has therefore remained controversial.

Prosome are found widely distributed from archaebacteria to the human species [7,16]. They are localized in eukaryotic cells both in the nucleus and the cytoplasm [16–18]. In the latter they are partially associated with the intermediate filaments of the cytoskeleton [18]. Indirect immunofluorescence studies indicated that specific prosomal antigens are variably located in the nucleus or in the cytoplasm of cells according to the stage of development and differentiation [17,19,20].

Of the biological activities of prosomes (for a review see refs. [7,16]), the proteinase activity is currently the best characterized. It has been shown that prosomes and the multicatalytic proteinase complex (MCP or ‘proteasomes’), first described by Orlowski &

Wilk [3], are identical entities [4,21] and we will therefore refer to the proteolytic activity of the complex as the MCP activity of the prosome–MCP particle. An interesting feature of this enzymic property is the presence of multiple proteolytic activities in the complex. Recently, evidence has accumulated demonstrating that these 720000 M_r particles are also part of a 26S proteolytic complex which degrades ubiquitin-conjugated proteins [22,23].

Several investigators have attempted to split prosomes into subfractions bearing the individual proteolytic activities. Such attempts have not been successful, since the conditions necessary invariably led to denaturation of the secondary protein structure. Therefore the question remains open as to whether or not the enzyme activities are the properties of individual subcomplexes and subunits or, in contrast, are a property of the holocomplex. A striking feature is the metabolic and biochemical stability of prosomes. They have an apparent half-life of 12–15 days in rat liver [24], resist high ionic strength and treatment with strong detergents (1 % sodium lauroylsarcosinate) [1]. Moreover their peptidase activity is unaffected by incubation at 37 °C for up to 7 h [25]. Autodegradation was detected only in the presence of 4 M-urea [25]. We report here that the selective breakdown of the prosome complex in the presence of some bivalent cations leads to the concomitant loss of all of its proteinase activity. Furthermore we show that the prosomal RNA which is resistant to nucleases in the intact prosomes can be digested after dissociation by Zn^{2+} , indicating that it is indeed a genuine constituent.

EXPERIMENTAL

Materials

[^{14}C]Methylcasein (1 MBq/ml, 2.8 μ Ci/mg) was obtained from NEN and the fluorogenic substrates Bz-Val-Gly-Arg-NH-Mec,

Abbreviations used: pRNA, prosomal RNA; Bz, benzoyl; Mec, 4-methylcoumaryl-7-amide; Suc, succinyl; TEA, triethanolamine; the term prosome introduced by our laboratory [1] for the then unknown particle is used here and, for its proteinase activity, the term multicatalytic proteinase or MCP is used following the recommendation of Dahlmann *et al.* [2] and Orlowski & Wilk [3], in preference to the term proteasome suggested by Arrigo *et al.* [4].

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Bz-Ala-Ala-Phe-NH-Mec and Suc-Leu-Leu-Val-Tyr-NH-Mec were purchased from Bachem.

Cell culture

HeLa cells were grown in suspension at 4×10^5 cells/ml with a generation time of about 24 h in Eagle's minimum essential medium supplemented with 10% newborn calf serum.

Cell fractionation and purification of prosomes from HeLa cells and duck erythroblasts

The isolation and purification of postribosomal supernatants from either HeLa cells or duck erythroblasts have been reported [6]. Postribosomal particles were fractionated by centrifugation in isokinetic 5–21% (w/w) sucrose gradients (Beckman Rotor SW 41, 37000 rev./min, 4 °C, 17 h) in high-ionic-strength buffer [10 mM-triethanolamine (TEA)/HCl (pH 7.4), 500 mM-KCl, 5 mM-2-mercaptoethanol]. The peak fractions in the 19S sedimentation zone were pooled and exposed to 0.5% (w/v) sodium lauroylsarcosinate (final concentration) and loaded on a 5–21% (w/w) sucrose-density gradient in detergent buffer [10 mM-TEA/HCl (pH 7.4), 0.1% sodium lauroylsarcosinate, 5 mM-2-mercaptoethanol] and centrifuged (Beckman Rotor SW 41, 37000 rev./min, 4 °C, 16 h). Peak fractions in the 19S zone (i.e. prosomes) were pooled and concentrated in low-ionic-strength buffer [10 mM-TEA/HCl (pH 7.4), 50 mM-KCl, 5 mM-2-mercaptoethanol] on a Centricon 30 microconcentrator (Amicon) and stored at -70 °C. Gel filtration, using an f.p.l.c. Superose 12 column (Pharmacia/LKB), was performed in a buffer containing 20 mM-Tris/HCl (pH 7.4), 50 mM-KCl and 5 mM-2-mercaptoethanol. Protein concentration of the purified particle preparation was determined by the method of Bio-Rad with BSA as standard.

Gel electrophoresis of proteins

SDS/PAGE was carried out by the method of Laemmli [26] in 13% polyacrylamide; two-dimensional electrophoresis (non-equilibrium electrofocusing, SDS/PAGE) was described by O'Farrell *et al.* [27]. M_r markers were phosphorylase *b* (M_r 92000), BSA (M_r 66000), ovalbumin (M_r 45000), carbonic anhydrase (M_r 31000), soya-bean trypsin inhibitor (M_r 21000) and lysozyme (M_r 14000). Non-denaturing gel electrophoresis was performed as described by Konarska *et al.* [28].

Assay of metal cation sensitivity of prosomes

Prosoemes at a concentration of 0.5–1 $\mu\text{g}/\mu\text{l}$ were incubated in low-ionic-strength buffer [10 mM-TEA/HCl (pH 7.4), 50 mM-KCl, 5 mM-2-mercaptoethanol] at 37 °C in the presence of various cation concentrations; at the times indicated, samples were analysed for either proteolytic activity or protein pattern in gel electrophoresis or filtration.

Assay of proteinase activity

The reaction mixture including the radioactive substrate [^{14}C]methylcasein contained in a volume of 200 μl : 50 mM-Tris/HCl (pH 8), 1 mM-dithiothreitol, 1 mg of poly(L-lysine)/ml (Sigma), 0.05 μCi of [^{14}C]methylcasein/ml and enzyme source as indicated. After incubation at 37 °C for 1 h, the release of radioactive material soluble in 10% (v/v) trichloroacetic acid was determined.

Assay of peptidase activity

The hydrolysis of Bz-Val-Gly-Arg-NH-Mec, Bz-Ala-Ala-Phe-NH-Mec and Suc-Leu-Leu-Val-Tyr-NH-Mec was tested at 37 °C in a volume of 500 μl , containing 125 mM-phosphate/borate buffer, 5 mM-2-mercaptoethanol, 1 mM-MgCl₂ and 10 μM -substrate at pH 9, 7.5 and 7 respectively. The reaction was stopped after 1 h by adding an equal volume of 100 mM-sodium

chloroacetate/70 mM-acetic acid/30 mM-sodium acetate. Liberated aminomethylcoumarin was measured fluorimetrically (excitation 352 nm, emission 442 nm).

Nuclease digestion assay

The samples (10 μg of prosomes) were exposed in a final volume of 50 μl to RNAase V1 digestion at the concentrations indicated, in a buffer containing 0.02 M-TEA (pH 7.4), 0.2 M-NaCl and 10 mM-MgCl₂. The reaction was stopped after 20 min at 37 °C by adding 200 μl of phenol. The RNA was labelled after phenol extraction at the 3' end with [^{32}P]pCp in a reaction catalysed by T4 RNA ligase. The RNA fragments were analysed by 10% (w/v) acrylamide/8 M-urea gel electrophoresis followed by autoradiography.

RESULTS

Effect of bivalent metal cations on the MCP activity of prosomes from different species

During dephosphorylation studies on prosomes of HeLa cells, inhibition of the MCP activity due to the presence of Zn²⁺ ions was observed. The MCP activity was described previously as being cation-sensitive [29–31]. Interestingly, Zn²⁺ has so far been the only bivalent cation to display invariably an inhibitory effect whereas controversial data have accumulated concerning other cations [32]. In order to sort out these controversial findings and to gain further insight into the general inhibitory effect of Zn²⁺, the effects on prosomes of a variety of cations at different concentrations were compared. Particles of either HeLa cells or duck erythroblasts were used and purified by similar procedures.

Prosoemes were incubated for 1 h at 37 °C with different concentrations of various metal ions before assay for proteolytic activity, with Suc-Leu-Leu-Val-Tyr-NH-Mec as substrate. Prosoemes from both sources, i.e. from HeLa cells and duck erythroblasts, behaved identically with regard to stimulation and inactivation (Table 1). Interestingly, Zn²⁺ and Cu²⁺ almost

Table 1. Sensitivity to bivalent cations of the MCP activity of prosomes from HeLa cells and duck erythroblasts

Purified prosomes (final concn. 0.5–1 $\mu\text{g}/\mu\text{l}$) from either HeLa cells or duck erythroblasts were preincubated at 37 °C with the indicated cation concentrations in a final volume of 10 μl . After 1 h, 490 μl of MCP activity assay mix, containing 10 μM -Suc-Leu-Leu-Val-Tyr-NH-Mec, was added. The data given are mean values $\pm$ S.D. of three independent experiments. Results are expressed as percentage activity relative to a control incubation in the absence of bivalent cations (100%).

Metal	Final ion concn. (mM)	Activity (%)	
		HeLa-cell prosomes	Duck erythroblast prosomes
None	–	100	100
Ca ²⁺	10.0	156 $\pm$ 14.7	130 $\pm$ 10.5
Ca ²⁺	1.0	125 $\pm$ 12.2	131 $\pm$ 10.4
Mg ²⁺	10.0	126 $\pm$ 13.8	108 $\pm$ 6.2
Mg ²⁺	1.0	130 $\pm$ 12	99 $\pm$ 8.8
Fe ²⁺	10.0	2 $\pm$ 2.5	6 $\pm$ 3.4
Fe ²⁺	1.0	113 $\pm$ 10.2	40 $\pm$ 8.1
Fe ²⁺	0.1	113 $\pm$ 10.7	69 $\pm$ 4.6
Cu ²⁺	1.0	7 $\pm$ 6.6	3 $\pm$ 1
Cu ²⁺	0.1	20 $\pm$ 4.3	6 $\pm$ 5
Cu ²⁺	0.01	110 $\pm$ 5.5	81 $\pm$ 7.1
Zn ²⁺	1.0	2 $\pm$ 1	1 $\pm$ 1.1
Zn ²⁺	0.1	3 $\pm$ 2.6	2 $\pm$ 2.5
Zn ²⁺	0.01	19 $\pm$ 5.1	28 $\pm$ 5.7
Zn ²⁺ /EDTA	1.0/2.0	86 $\pm$ 5.1	96 $\pm$ 3.2

Fig. 1. Electrophoretic analysis in non-denaturing conditions of metal-cation-treated prosomes

(a) HeLa cell prosomes (10 μg) treated as indicated in the legend of Table 1 were loaded on a 5% polyacrylamide gel in non-denaturing buffer and stained with Coomassie Blue after electrophoresis. Lane 1, 10 mM-Fe²⁺; lane 2, 10 mM-Co²⁺; lane 3, 1 mM-Cu²⁺; lane 4, 10 mM-Mg²⁺; lane 5, 10 mM-Ca²⁺; lane 6, 1 mM-Zn²⁺; lane 7, control. (b) HeLa cell prosomes were incubated with 1 mM-Zn²⁺ (lane 1) or 1 mM-Zn²⁺ in the presence of one of the four substrates: 0.05 μCi of [¹⁴C]methylcasein/ml (lane 3), 10 μM-Suc-Leu-Leu-Val-Tyr-NH-Mec (lane 4), 10 μM-Bz-Val-Gly-Arg-NH-Mec (lane 5) or 10 μM-Bz-Ala-Ala-Phe-NH-Mec (lane 6) for 1 h before electrophoresis in a non-denaturing gel. Lane 2, control. The gel was stained with Coomassie Blue.

Fig. 2. Characterization by SDS/PAGE of prosome proteins after metal cation treatment

Prosome purified from HeLa cells (10 μg) were incubated with metal cations for 1 h at 37 °C. Lane 1, control; lane 2, 1 mM-Zn²⁺; lane 3, 1 mM-Cu²⁺; lane 4, 10 mM-Fe²⁺; lane 5, 10 mM-Mg²⁺; lane 6, 10 mM-Ca²⁺. Before SDS/PAGE analysis, an equimolar amount of EDTA was added to chelate cations. Proteins were stained with Coomassie Blue. *M_r* markers were: phosphorylase *b* (*M_r* 92000), BSA (*M_r* 66000), ovalbumin (*M_r* 45000), carbonic anhydrase (*M_r* 31000), soya-bean trypsin inhibitor (*M_r* 21000) and lysozyme (*M_r* 14000).

completely abolished the enzyme-activity at a concentration of 0.1 mM, and more than two-thirds inactivation was found at 0.01 mM-Zn²⁺ but not Cu²⁺, whereas Fe²⁺ displayed this effect only at a concentration of 10 mM. Of the other cations tested, Ca²⁺ and Mg²⁺ showed either no influence on the activity or produced stimulation. Zn²⁺, Cu²⁺ and Fe²⁺ inhibited all three proteinase activities assayed to the same extent, i.e. the proteinase activity, the chymotrypsin-like and the trypsin-like activities, probed respectively with [¹⁴C]methylcasein, Bz-Ala-Ala-Phe-NH-Mec and Bz-Val-Gly-Arg-NH-Mec (results not shown). The analysis of different salts and the protection against inactivation when EDTA was added simultaneously with the cations (shown for Zn²⁺ in Table 1) demonstrated that the effect observed was cation-specific and independent of the anions used. None of these inhibitory effects could be reversed by addition of EDTA before the MCP activity assay. Most importantly, no inactivation was observed when the substrates were added simultaneously with the metal cations, i.e. when preincubation was omitted (see below).

Dissociation of prosomes treated with metal ions

To test whether the loss of enzyme activity might be due to changes in the prosome structure, we examined the integrity of the particles by non-denaturing gel analysis. Surprisingly,

Fig. 3. Two-dimensional electrophoretic protein pattern of prosomes incubated with or without 1 mM-Zn²⁺

Prosome from HeLa cells (30 μg) were preincubated with or without 1 mM-Zn²⁺ under standard conditions. After 1 h, a sample was assayed for its MCP activity to check for inactivation by Zn²⁺. The remainder of the sample was analysed by two-dimensional gel electrophoresis, using non-equilibrium electrofocusing in the first and SDS/PAGE in the second dimension. (a) Prosomes without treatment with Zn²⁺. (b) Prosomes after inactivation by Zn²⁺. The gel was stained with Coomassie Blue.

Fig. 4. MCP inactivation kinetics and non-denaturing gel analysis after time-dependent dissociation of the complex

(a,b) 10 μ g of prosomes from duck erythroblasts were incubated with the indicated Zn²⁺ concentrations under standard conditions. At the times indicated, samples were assayed for MCP activity using Suc-Leu-Leu-Val-Tyr-NH-Mec, and the corresponding level of dissociation was examined by electrophoresis in non-denaturing gel. (a) Non-denaturing gel analysis of time- and dose-dependent dissociation. (b) MCP inactivation kinetics at 1 mM-Zn²⁺.

Coomassie Blue staining revealed a total disappearance of the band corresponding to the prosome particle incubated in the presence of Zn²⁺, Cu²⁺ or Fe²⁺ (Fig. 1a). This result indicates the dissociation of the complex and the absence of discrete subcomplexes of M_r higher than 100 000. The protein subunits migrating with M_r 20 000–36 000 run out of the gel, as judged from M_r standards. In contrast, Mg²⁺, Ca²⁺ and Co²⁺ had no effect on the integrity of the complex. The slight smear seen in the presence of Co²⁺ is probably due to the effect of the high ionic concentration (10 mM) on the migration (Fig. 1a).

Protection from dissociation of the MCP activity by proteinase substrates

The fact that Zn²⁺ or Cu²⁺ inactivated the proteinase only after preincubation in the absence of substrate hinted at the possibility that the complex was stabilized and protected by the substrate. To address this issue, prosomes were incubated in a mixture containing Zn²⁺ and one of the four substrates, i.e. [¹⁴C]-methylcasein, Suc-Leu-Leu-Val-Tyr-NH-Mec, Bz-Ala-Ala-Phe-NH-Mec or Bz-Val-Gly-Arg-NH-Mec before non-denaturing gel analysis. As expected, little if any dissociation was detected (Fig. 1b).

Analysis of the dissociated protein subunits

Having thus identified the dissociation of the particle as the primary cause of the inhibition of the MCP activity by some bivalent cations, we concentrated our interest on the elucidation of the mechanism of inactivation by Zn²⁺. The enzyme activity was most susceptible to this cation. Kinetic studies showed no change in either K_m or V_{max} caused by Zn²⁺, when the proteolytic activity was measured in the presence of substrates (results not shown). These results and the finding of Tanaka & Ichihara [25] of autodigestion of 'proteasomes' in the presence of 4 M-urea raised the possibility that the proteins are broken down in the particle.

To address this issue, prosomes were incubated for 1 h in the presence of metal ion concentrations that would produce a maximal effect on the MCP activity, before analysis of the proteins by one-dimensional SDS/PAGE. As shown in Fig. 2, there was no difference in the protein pattern when prosomes inactivated by Zn²⁺ or Cu²⁺ were compared with the control. Even when the incubation was prolonged to 5 h in 1 mM-Zn²⁺ and at 37 °C, there was no degradation (result not shown). This result indicates that the proteins that ran out of the gels in non-denaturing conditions (Fig. 1) were quantitatively and, apparently, qualitatively preserved during the dissociation of the complex by Zn²⁺.

Digestion and disappearance of all proteins was observed, however, in the case of prosomes treated with 10 mM-Fe²⁺ (Fig. 1). The results were the same for both HeLa cell and duck erythroblast prosomes. This phenomenon is probably a fairly common feature of the particle found *in vitro*, as Tanaka & Ichihara reported the same effect in the presence of 4 M-urea [25]. Considering the apparent autodigestion of the particles in the presence of high concentrations of Fe²⁺, we tested whether or not the inhibition by Zn²⁺ was due to a partial autodegradation of one or several prosomal proteins, an event that could have escaped detection by one-dimensional SDS/PAGE (Fig. 2). Therefore prosomes inactivated by Zn²⁺ were analysed by two-dimensional gel electrophoresis. No significant difference in the protein pattern was detected when compared with the control (Fig. 3). Treatment of prosomes with Zn²⁺ thus leads merely to dissociation of the complex without disappearance, autodigestion or damage of any of the subunits.

Time- and dose-dependent dissociation and identification of the dissociation products

The interesting question arose as to whether the disintegration of the particle into its subunits was complete or whether a residual core complex could be detected. As no discrete break-

Fig. 5. Gel filtration of non-treated and Zn-dissociated prosomes

HeLa cell prosomes (50 μ g) were preincubated with or without 1 mM- Zn^{2+} under standard conditions. After 30 min, the samples were adjusted to 5 mM-EDTA and a sample was assayed for its MCP activity to check for inactivation by Zn^{2+} . The remainder of the sample was loaded on to a Superose 12 column. ---, prosomes without treatment with Zn^{2+} ; —, prosomes after inactivation by Zn^{2+} .

down products of the Zn^{2+} -dissociated prosomes could be observed by non-denaturing gel analysis, even using the more sensitive silver stain, we studied the kinetics of dissociation. Prosomes were examined after different preincubation times with Zn^{2+} in the absence of substrate for both their MCP activity and their level of dissociation in a non-denaturing gel. Prosomes treated with 1 mM- Zn^{2+} at 37 °C were inactivated very rapidly. As shown in Fig. 4(b), 90 % of the proteinase activity was lost within 5 min. This result was confirmed by the rapid disappearance of the prosome band in a non-denaturing gel; even after this short time, no intermediates of dissociation could be detected by this method (Fig. 4a). Dose-dependent dissociation studies in which prosomes were incubated in increasing concentrations of Zn^{2+} ions for 20 min confirmed the absence of discrete intermediates (Fig. 4a). Furthermore, to check carefully the absence of distinct intermediates by other methods, similar kinetics of Zn^{2+} dissociation were analysed by gel filtration. The majority of the prosomal proteins shifted after Zn^{2+} treatment from M_r 700000 to M_r 55000–15000, as judged from M_r standards; again, no discrete intermediates were observed (Fig. 5). These results, together with the analysis of the prosomal proteins after Zn^{2+} treatment by two-dimensional gel electrophoresis, indicate that the treatment of the particles with either Cu^{2+} or Zn^{2+} dissociates the complex into components inferior to trimers, as judged from the M_r standards, without damaging the individual protein subunits.

Analysis of the protection of prosomal RNA against nuclease digestion by intact and Zn^{2+} -dissociated prosomal particles

To test stringently the controversial question about the presence of RNA in the particle, we took advantage of the dissociation of the particle on Zn^{2+} treatment. Dineva and co-workers [15] gave evidence for high nuclease resistance of 80–90 % of the RNA in the intact prosomes. Taking this result as a basis, we addressed the question as to whether the pRNA could be digested by nuclease after dissociation of the complex into its protein subunits by Zn^{2+} . Zn^{2+} -dissociated or non-dissociated prosome particles were incubated with increasing amounts of RNAase V1 before RNA analysis. As seen in Fig. 6, most of the RNA was digested after dissociation of the particles, whereas two nuclease-resistant RNA bands in the range of 80 and 85 nucleotides,

Fig. 6. Urea/polyacrylamide-gel electrophoresis of pRNA after nuclease protection assay in presence or absence of Zn^{2+}

HeLa cell prosomes (10 μ g) were incubated at 37 °C in the absence or presence of 1 mM- Zn^{2+} in a final volume of 20 μ l. After 1 h, 1 mM-EDTA was added before nuclease digestion. After the reaction, the samples were extracted with phenol, the RNA subsequently 3' end labelled, separated on an 8 M-urea/10 % polyacrylamide gel, and visualized by autoradiography (12 h). Lanes 1, 3 and 5, prosomes incubated in the absence of Zn^{2+} before RNAase digestion with 12.5, 26 and 37.5 units of RNAase V1/ml respectively. Lanes 2, 4, and 6, prosomes incubated in the presence of 1 mM- Zn^{2+} before RNAase digestion with 12.5, 25 and 37.5 units of RNAase V1/ml respectively. Lanes 7 and 8, prosomes incubated in the absence or presence of 1 mM- Zn^{2+} respectively without RNAase digestion.

characteristic of pRNA, were found after RNAase digestion of the non-treated particles.

The relative enrichment of the 85-nucleotide-long band after digestion with high doses of V1 nuclease, as compared with the controls, needs further elucidation. A conceivable possibility is that RNAase V1 digestion reveals another prosomal RNA by cleavage of a protected 3' end otherwise inaccessible to RNA ligase. The speculation that it is not the integrity of the complex

per se that protects the RNA, but that its MCP activity might cleave the V1 enzyme seems to be ruled out since (i) increasing amounts of RNAase V1 did not change the digestion pattern and (ii) the internal control of copurified RNA was digested in the presence of the intact prosome-MCP particle. This result demonstrates clearly that it is the intact particle, and not the individual prosomal proteins, that protect the RNA from digestion. It therefore strongly supports the idea that at least some of the small RNAs found in the prosome-MCP particle preparations are an intrinsic part of the prosome particle.

DISCUSSION

This paper relates to two main issues concerning the prosome-MCP particles. The first one concerns the question of whether or not individual prosomal subunits are associated with the individual proteinase activities of the MCP. The other relates to the RNA content of the particle, a problem that remains an issue of controversy to this date. We demonstrate here that, on dissociation of the particle in conditions that do not denature the secondary structure of the individual protein chains, the proteinase activities are lost. Therefore the prosome-MCP complex does not contain individual proteinases that give rise to the multicatalytic activity but displays this feature only when present as a holoparticle. As far as the RNA content is concerned, we show here that small RNA is a genuine component of at least a part of the prosome particles.

The basis of the new observations reported here is the inducible dissociation of the prosome particle by addition of Zn^{2+} or Cu^{2+} without damaging the protein subunits. This observation allowed us to demonstrate that the integrity of the prosome-MCP complex is a prerequisite for its enzyme activity, since none of the isolated protein constituents has any proteinase activity. The possibility that active centres on subunits exist and are only inhibited by the presence of Zn^{2+} is almost ruled out, since even 5 mM-EDTA, a concentration that can chelate Zn^{2+} out of Zn^{2+} finger proteins [33], does not restore the MCP activity (results not shown).

As little is known currently about the assembly, maintenance and catabolism of the particle, one can only speculate about the dissociating effects of these cations. Conceivable mechanisms for the dissociation are: (i) substitution by Zn^{2+} or Cu^{2+} of one or several different cations positively involved in the integrity of the complex; (ii) involvement of the bifunctional cations at the level of the protein-protein interaction site(s), and oversaturation of the latter by an excess of Zn^{2+} or Cu^{2+} ; (iii) introduction of a conformational change by interaction of the cations with some prosomal proteins outside their protein-protein binding site(s). We cannot rule out the first hypothesis, but two lines of evidence argue against it: (i) prosomes are resistant to treatment with 10 mM-EDTA [6]; (ii) a 5-fold excess of different bivalent cations (Mg^{2+} , Ca^{2+} or Co^{2+}) tested as competitors to Zn^{2+} did not prevent the loss of the MCP activity (results not shown).

An interesting observation is the fact that the presence of MCP substrates protect the particle from dissociation. We cannot exclude the possibility that this protection is based on the shielding of Zn^{2+} interactive site(s) by the substrates. However, as a protective effect by the substrate was also described by Tanaka *et al.* when they examined the influence of SDS in the presence or absence of substrate [34], it is possible that prosomes rather undergo structural modification by binding the substrate, which leads to general stabilization of the enzymically active complex. The latter hypothesis rather points to the importance of the particle conformation and changes therein. Slight structural modifications were, for example, thought to be responsible for the conversion from a so-called 'latent' form into the activated

form [35], a suggestion later confirmed by Saitoh *et al.* [36]. Furthermore, at least three stable and functionally distinct states of the complex, which are based on changes in the conformation rather than the subunit composition, have been recently described by Mykles & Haire [37]. It might be thus that Zn^{2+} or Cu^{2+} induce an unstable conformation in the absence of substrate, which leads to dissociation of the particles. Furthermore, the presence of a high concentration of Fe^{2+} like 4 M-urea [25], might induce yet another conformation, which initiates autodigestion.

One of the most controversial points with regard to the prosome-MCP particle was the presence of small RNAs in the complex. Recent results from the groups of Rivett [11] and Kloetzel [21] and our own laboratory [38], however, strongly support the observation concerning the presence of small RNA (pRNA) in the prosome-MCP particle, originally made by Schmid and colleagues for prosomes of duck erythroblasts and HeLa cells [1,6]. Isolation of the particle by different purification procedures systematically led to copurification of small RNA species with the prosome-MCP particle. Furthermore, Dineva and co-workers [15] showed that most of the prosomal RNA is digested by nucleases only after phenol extraction, i.e. after elimination of the prosomal proteins. Our observation that the specific dissociation of the particle by Zn^{2+} is a prerequisite for nuclease digestion of the prosomal RNA confirms this result. In addition, it demonstrates that it is the intact particle, and not the individual prosomal proteins, that protects the RNA from digestion. Therefore this result strongly supports the idea that the small RNAs found in prosome-MCP particle preparations are an intrinsic part of prosomes, and thus gives proof that at least a fraction of the prosome-MCP particles is genuine ribonucleoprotein.

The mild dissociating condition produced by Zn^{2+} may be of great advantage for future reconstitution experiments. It permits disruption of the complex in the absence of high concentrations of urea or detergents, which could denature the proteins of the complex and lead to subsequent inactivation of structurally or enzymically important sites.

In conclusion, the data reported here confirm the presence of RNA as a genuine component of the prosome particle. This does not imply, however, that all particles necessarily contain RNA. It is likely that only a subpopulation of them has RNA at any given time. On the other hand, it has become evident that none of the prosomal proteins has proteinase activity themselves but that it is exclusively a property of the holoparticle. More extensive investigations will be necessary to clarify the functional significance of the presence of RNA, as well as to understand the generation of the various MCP-proteinase activities from certain of the protein subunits. Finally, to understand the mechanism of the Zn^{2+} effect and its prevention by substrates, extensive structural studies will be necessary to determine the targets of Zn^{2+} ions, as well as of the substrates, within the peptide chains.

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