FEBS Letters 507 (2001) 54–58 FEBS 25314

Inhibition of 2′-5′ oligoadenylate synthetase by divalent metal ions

Rune Hartmann, Gernot Walko, Just Justesen*

Department of Molecular and Structural Biology, University of Aarhus, C.F. Møllers alle, Bygn. 130, DK-8000 Aarhus C, Denmark

Received 9 July 2001; revised 12 September 2001; accepted 13 September 2001

First published online 1 October 2001

Edited by Judit Ovádi

Abstract OAS1 is the small form and OAS2 is the medium form of the human interferon-induced 2′-5′ oligoadenylate synthetases. The p42 isoform of OAS1 and the p69 isoform of OAS2 have been expressed in insect cells and purified to give pure, highly active 2′-5′ oligoadenylate synthetase. The catalysis of 2′-5′ oligoadenylate synthesis is strictly dependent on double-stranded RNA and magnesium ions. We have examined the effect of a series of divalent metal ions: copper, iron and zinc ions strongly inhibited the enzymatic activity, cobalt and nickel ions were partly inhibitory whereas calcium and manganese ions were without effect. However, manganese ions can replace magnesium ions as activator. The inhibitory effect of zinc ions was characterised in detail. The inhibitory constants of Zn^{2+} were estimated to be 0.10 mM for OAS1p42 and to 0.02 mM for OAS2p69. Cross-linking experiments showed that zinc ions can control the oligomerisation by enhancing the formation of tetrameric forms of OAS1p42

Key words: Oligoadenylate synthetase; Divalent metal ions; Inhibition

1. Introduction

The 2′-5′ oligoadenylate synthetases (OAS) are a family of interferon-induced antiviral enzymes. They catalyse the oligomerisation of ATP into 2′-5′-linked oligoadenylates (2-5A), which in turn serve as activators of a latent endoribonuclease, named RNase L [1]. Binding of 2-5A to RNase L leads to the dimerisation and activation of this enzyme [2]. Activation of RNase L leads to degradation of a range of RNA molecules within the affected cell, followed by a decrease in cellular protein synthesis [3].

In humans there are three genes encoding functional 2′-5′ oligoadenylate synthetase, OAS1, OAS2 and OAS3, and one gene encoding an OAS-like protein, OASL [4]. The OAS1 gene, which encodes the small OAS, consists of one basal OAS unit, which is repeated twice in the OAS2 gene (encoding medium OAS) [5] and three times in the OAS3 gene (encoding the large OAS) [6]. The OASL p59 protein has one OAS domain and a carboxy-terminal domain homologous to ubiquitin [7,8]. The transcription of all four members of the OAS gene family is strongly induced by interferon-α and to a lesser extent by interferon-γ [9].

The OAS proteins are a unique family of enzymes, as they are the only known proteins to catalyse the formation of 2′- to 5′-linked oligonucleotides. However, OAS is produced as a latent enzyme, which upon interaction with an appropriate RNA activator becomes catalytically active [10]. None of the OAS proteins share significant sequence homology with other known proteins. Nevertheless, a combination of molecular modelling and mutagenesis studies has shown that the architecture of the active site for the nucleotidyl transferase reaction is similar to that known for 3′-5′ nucleotidyl transferases [11]. Three conserved glutamates are assumed to coordinate the catalytic magnesium ion, in a manner similar to that seen in many RNA and DNA polymerases. The mechanism by which RNA activates the enzyme is still largely unknown, but the basal activity of OAS in the absence of an RNA activator is negligible [10].

The involvement of OAS in establishing the antiviral state induced by interferon has been confirmed through several independent experiments. Expression of cDNA encoding OAS1 in mammalian cell lines resulted in an increased resistance towards picornavirus infection. Furthermore the level of OAS expression in different clones correlated with the antiviral effect observed [12]. Later similar results were obtained with OAS2 [13,14]. Expression of OAS1 cDNA together with RNase L cDNA in transgenic plants resulted in increased resistance towards viral infection. In contrast, expression of either OAS1 or RNase L alone had no effect on the antiviral resistance of the plant, demonstrating the need for both genes in the antiviral response [15,16]. Recent data also suggest that activation of the 2-5A system in human cells is involved in at least two pathways leading to apoptosis of the affected cells [17,18]. It appears reasonable that leading virus-infected cells into apoptosis can protect the host against spread of the infection.

Zinc has been proposed to block apoptosis in human cells and several studies have suggested a role for OAS in the induction of apoptosis. Furthermore, the second enzyme in the 2-5A system, RNase L, is also inhibited by Zn^{2+} ions [2].

Mg^{2+} ions are required for the enzymatic activity of the OAS proteins, but can to a certain extent be replaced by Mn^{2+} [4,19]. Other metal ions of the same range are thought to be inhibitory, but the data are rather difficult to assign to the forms of OAS as they are known now.

In this study we are examining the effect of a number of divalent cations on the activity of the OAS1 and the OAS2 proteins, which have been expressed in insect cells. The oligomeric structure of OAS1 (tetramer) and OAS2 (dimer) is important for the enzymatic activity [20–22], and here we demonstrate that this can be controlled by Zn^{2+} and other similar metal ions.

*Corresponding author.
E-mail address: jj@mbio.au.dk (J. Justesen).

PII: S0014-5793(01)02918-0

2. Materials and methods

2.1. Expression and purification of 2′-5′ oligoadenylate synthetase

Human OAS1, p42 isoform: the protein was expressed using the baculovirus system as described elsewhere [10]. 200 ml HiFive cells, infected with the recombinant virus at a multiplicity of infection of 10, were harvested 3 days post infection. Cell pellets were resuspended in 20 ml of lysis buffer (1% (v/v) Nonidet P40, 50 mM HEPES pH 6.8, 300 mM NaCl, 5 mM 2-mercaptoethanol and protease inhibitor cocktail (Complete™, Boehringer Mannheim)) and lysis was performed for 10 min on ice with gentle vortexing. The lysate was centrifuged at $10\,000\times g$ for 20 min at 4°C and the supernatant was applied to 5 ml HiTrap SP column (Amersham Pharmacia Biotech), equilibrated with buffer A (25 mM HEPES pH 6.8 plus 5% glycerol). After loading of the sample, the column was washed with buffer A at a flow of 1 ml/min until the UV_{280} absorption was stable. Bound proteins were eluted with a linear gradient of NaCl from 0 to 0.6 M. 1 ml fractions were collected and samples (5 μl) from every second fraction were tested for OAS activity, using the rapid pyrophosphate assay [23]. Fractions containing enzymatically active OASp42 were pooled and ammonium sulphate was added to a final concentration of 1.5 M and then applied to a HR 10/10 (Pharmacia Amersham Biotech) column packed with phenyl-Sepharose (phenyl-Sepharose 6 FF, Pharmacia Amersham Biotech), equilibrated with buffer B (50 mM HEPES pH 6.8, 50 mM NaCl, 1 mM EDTA) plus 1.5 M ammonium sulphate. The column was washed with buffer B plus ammonium sulphate until the UV_{280} was stable, then again with buffer B without ammonium sulphate until the UV_{280} was stable. Bound proteins were eluted with a linear gradient of an increasing concentration of ethylene glycol (0–50%) in buffer B. Fractions containing enzymatic activity were combined and analysed by SDS–PAGE. The eluted protein was then excessively (48 h) dialysed against buffer E without EDTA at 4°C. Purified protein was stored in aliquots at −80°C. Protein quantification was done using the Bio-Rad protein assay reagent according to the instructions of the manufacturer.

Purification of the OAS2 protein (p69 isoform) was done as described [11,24].

2.2. 2′-5′ Oligoadenylate synthetase assay using PEI-thin layer chromatography

Activity assays were performed as follows: 4 μl of buffer C, 4 μl of OAS in buffer B minus EDTA, 10 μl buffer D and 2 μl of a 10× stock of the indicated metal or water. Incubation was performed in thin-walled 100 μl PCR tubes in an incubator at 37°C. Solutions: buffer C (20 mM Tris–HCl pH 7.5, 50 mM $Mg(OAc)_2$, 25 mM ATP pH 7.5, 1 mM dithiothreitol and 10% (v/v) glycerol, 0.025 mCi [α-^{32}P]ATP/μl). Buffer D (10 mM KOAc, 5 mM $Mg(OAc)_2$, 20 mM Tris–HCl pH 7.5, 100 mg/ml poly I poly C (Amersham Pharmacia Biotech)). Separation of ATP and the 2-5A products was performed by thin layer chromatography (TLC) on poly(ethylene)imine–cellulose plates according to [25].

Separated reaction products were visualised either by autoradiography using X-ray films or using a Phosphoimager (Personal Molecular Imager FX, Bio-Rad). Quantification of spots was done using Imagequant software with the Phosphoimager. Activity was calculated as the fraction of ATP converted into 2-5A. Values were corrected for background noise by normalising to a blank lane without enzyme. For determination of IC_{50} values, a minimum of three experiments were carried out for each data point. The data were fitted to the following model: $f(I) = \max A \times (IC_{50}{}^n/(IC_{50}^n + I^n))$ with $f(I)$ as the activity at a given concentration of inhibitor, $\max A$ as the activity without any addition of inhibitor, I as the inhibitor concentration, IC_{50} as the concentration of inhibitor required for half-maximum inhibition and n as the Hill coefficient. The data were fitted to this model using non-linear regression, giving the $\max A$ and IC_{50} values for each data set. In the next step, the experimentally observed activities in each data set were normalised to the corresponding maximum activity. The mean and the mean standard deviation of these normalised values were calculated for each data point, and plotted against the Zn^{2+} concentration.

2.3. Immunoblotting

Proteins were separated by 7.5% SDS–PAGE and blotted onto polyvinylidene difluoride (Immobilon-P, Millipore) membranes. Blots were blocked overnight in phosphate-buffered saline containing 5% dried milk. Blots were probed with a rabbit polyclonal antibody raised against a synthetic peptide corresponding to peptide B from OAS1 used by [22], washed and probed with a secondary antibody (goat anti-rabbit, horseradish peroxidase-conjugated). The blot was finally developed with the enhanced chemiluminescence reagent (ECL, Amersham Pharmacia Biotech) according to the instructions of the manufacturer.

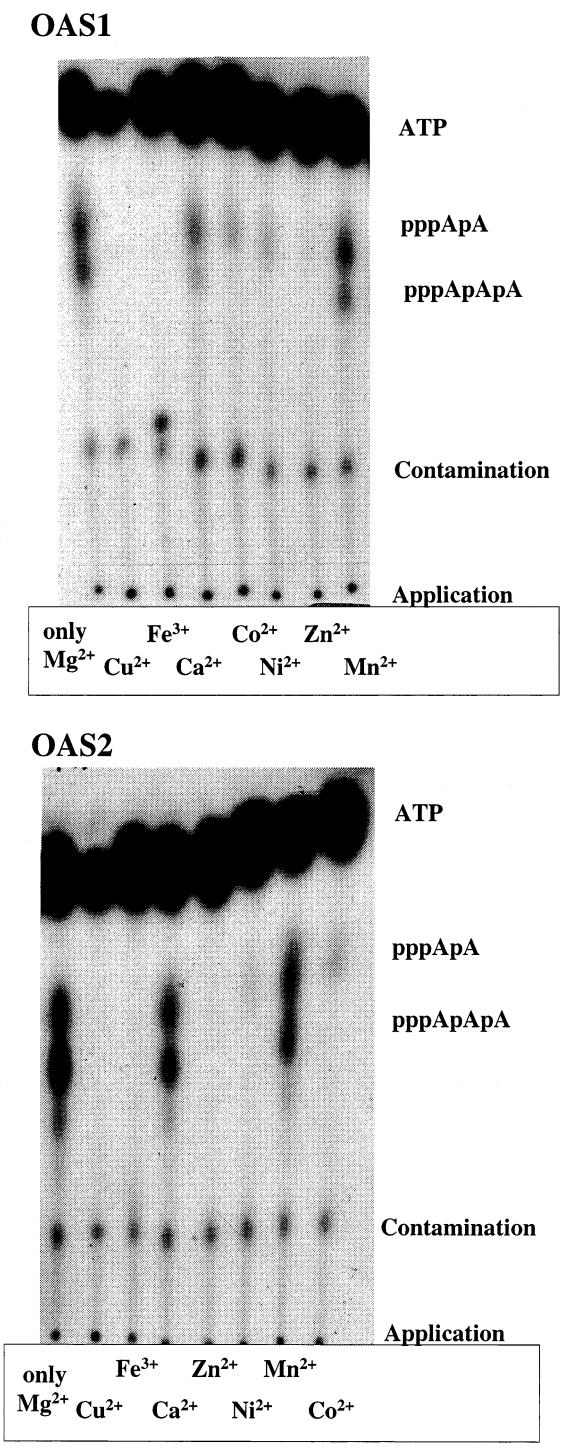

Fig. 1. Inhibition of OAS1 (p42 isoform) and OAS2 (p69 isoform) by various divalent metal ions. Standard 2′-5′ oligoadenylate synthetase assays were performed in the presence of 1 mM of the indicated divalent metal ions or water (only Mg^{2+}). All metals were added as chloride salts. ATP was separated from the produced 2-5As using thin layer chromatography.

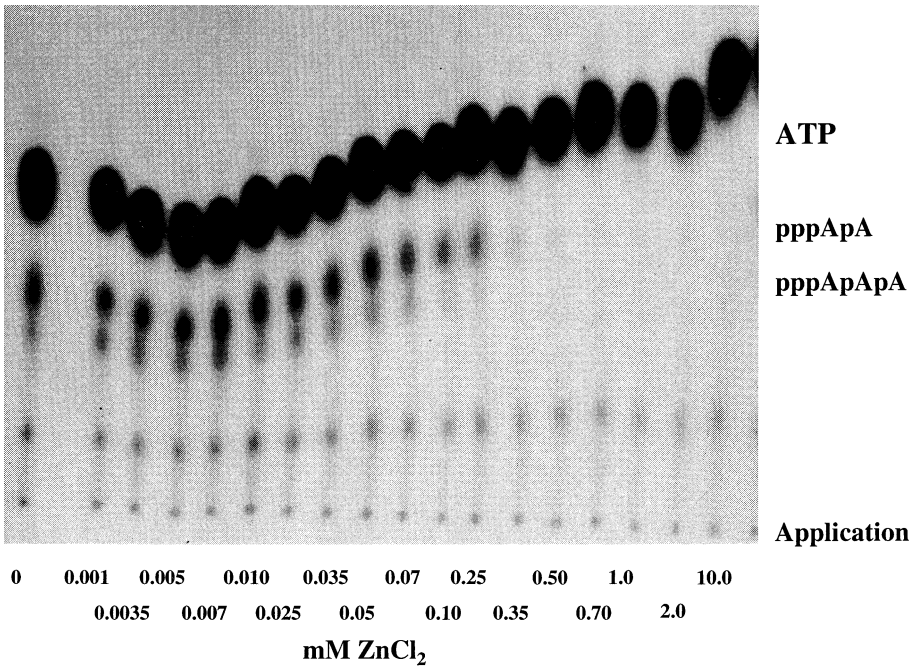

Fig. 2. Dose–response experiment for OAS1 with increasing concentrations of zinc ions. $ZnCl_2$ was added to standard 2′-5′ oligoadenylate synthetase assays. The amount of 2-5A produced was quantified and normalised to the ATP spot.

2.4. Protein cross-linking with dimethyl suberimidate (DMS)

Chemical cross-linking was done as described by [26] using DMS as the cross-linking reagent. The reaction mixture contained 5 μl OAS (20 μg/ml)+3 μl DMS (40 μg/ml), and metal ions at the indicated concentration, in a total volume of 10 μl. Incubation was performed for 1 h at room temperature. Reactions were stopped by addition of 10 μl of sample-loading buffer containing 4% SDS and boiling at 95°C for 10 min followed by SDS–PAGE and immunoblotting.

3. Results

3.1. Inhibition of OAS1 and OAS2 by various divalent metal ions

In order to establish the effect of divalent metal ions on the OAS we have tested the influence of different chloride salts of these metals on the activity of the OAS1 and OAS2 proteins (Fig. 1). The various metals were added to standard OAS activity reactions at a final concentration of 1 mM, and the formation of 2-5A oligomers was monitored after separation by thin layer chromatography. Transitions state metals such as copper, iron and zinc showed an almost complete inhibition, whereas cobalt and nickel gave a strong but somewhat weaker inhibition. Calcium gave a weak inhibition and manganese had no significant influence.

We therefore chose to investigate the inhibition exerted by zinc in further detail. We performed dose–response experiments with both the OAS1 (Fig. 2) and OAS2 enzymes, and determined the IC_{50} values (Figs. 3 and 4). Both enzymes were strongly inhibited by Zn^{2+}, with an IC_{50} value of 0.10 mM for OAS1 (Fig. 3) and an IC_{50} of 0.02 mM for OAS2 (Fig. 4). Estimation of the Hill coefficient revealed a clear difference between the two enzymes (Figs. 3 and 4). We estimated the Hill coefficient for the Zn^{2+} inhibition of OAS2 to be approximately 1, indicating a non-co-operative binding of Zn^{2+} to OAS2. In contrast, the inhibition of OAS1 by Zn^{2+} had a Hill coefficient of 1.8, indicating co-operative binding of Zn^{2+} with at least two binding sites on the molecule.

3.2. Zn^{2+} leads to oligomerisation of OAS1

OAS1 has previously been reported to exist in an oligomeric state [27]. We therefore chose to examine the influence of Zn^{2+} on the oligomeric state of OAS1. The oligomerisation was measured by chemical cross-linking of the OAS1 protein under conditions identical to the ones used for activity assays. The reaction mix was preincubated for 10 min before addition of the cross-linker DMS. Oligomers were then separated by SDS–PAGE and detected by Western blots (Fig. 5). The amount of oligomeric protein clearly increases with increasing Zn^{2+} concentrations and the concentration dependence of the cross-link with regard to Zn^{2+} correlates with the concentration required for inhibition. In the cross-linking experiment

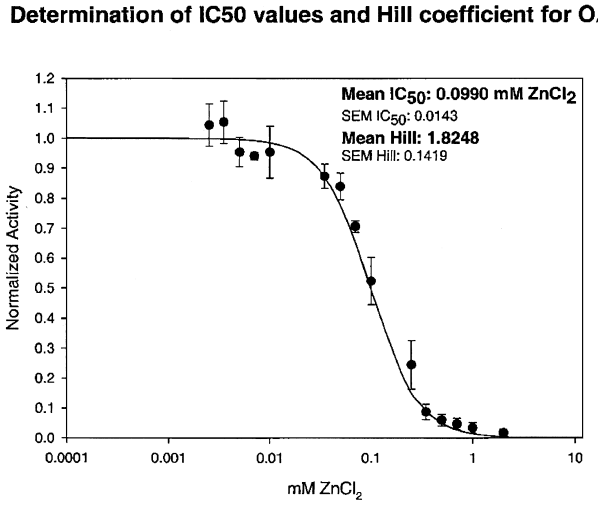

Fig. 3. Dose–response curve for OAS1. The mean activity of at least three independent measurements was plotted versus the $ZnCl_2$ concentration. The IC_{50} and Hill coefficient were estimated using nonlinear regression analysis.

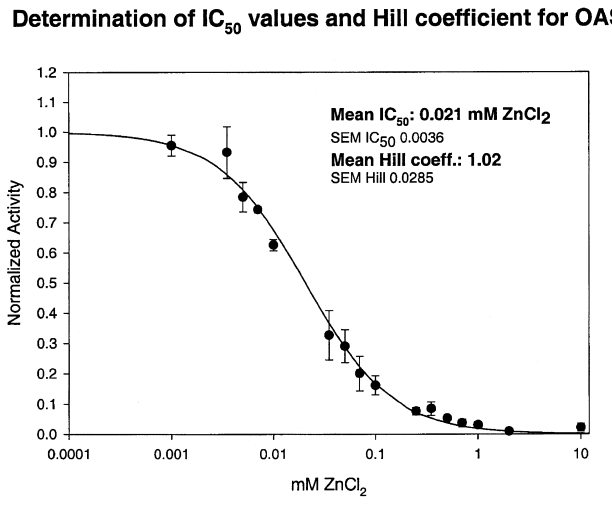

Fig. 4. Dose–response curve for OAS2. Experiments were performed as in Fig. 3.

we used a low concentration of the cross-linker DMS, in order to avoid extensive cross-linking which leads to clotting in the gel slots. This means that only a fraction of the oligomeric protein will cross-link under the conditions used here. If standard concentrations of DMS were used together with a Zn^{2+} concentration of 1 mM, an almost complete cross-linking would be observed. However, under these conditions it was not possible to separate the protein properly in the gel and most of the protein would remain in the stacking gel (data not shown). Without the addition of metal in addition to Mg^{2+}, a weak cross-linking is observed.

In order to rule out the possibility that Zn^{2+} activates the cross-linking process rather than inducing oligomerisation of OAS1, we performed cross-linking of creatine kinase in the presence or absence of zinc. Rabbit creatine kinase forms a tetrameric complex, which will cross-link partially under the conditions used here. We examined if Zn^{2+} would influence the efficiency of the cross-linking reaction. There was no effect of zinc ions on the DMS cross-linking reaction with creatine kinase (data not shown). In a similar experiment with OAS2 the eventual oligomerised protein was below the detection limit (data not shown).

4. Discussion

4.1. Inhibition of 2′-5′ oligoadenylate synthetase by divalent metal ions

We have identified a number of metals that strongly inhibit the activity of the OAS1 as well as the OAS2 protein, and we have examined the effect of zinc ions in further detail. Addition of copper, iron or zinc ions at a concentration of 1 mM inhibited the enzyme almost completely leaving less than 5% of the original activity. It should be stated that 10 mM Mg^{2+} is present for full 2-5A synthetase activity and that 1 mM Mg^{2+} will only give 10% of full activity. Cobalt and nickel ions also showed an inhibition at the same concentration, although roughly 15% of the original activity remained. The primary ligands of these metals in proteins are the nitrogen atom of the imidazole ring in histidine, and the thiol group of cysteine. Co-ordination of Zn^{2+} by the carboxyl groups of the side chains of aspartic and glutamic acids can occur even though this is less common. We have also confirmed that Mg^{2+} and Mn^{2+} are activators and that Ca^{2+} has no effect.

Even though both OAS1 and OAS2 are strongly inhibited by zinc and by other metal ions as well, only OAS1 displays some co-operativity in the metal ion-mediated inhibition. No structural information is yet available on the OAS proteins, and hence we do not know the zinc-binding site. The Zn^{2+}-induced oligomerisation seen with OAS1 suggests that binding of Zn^{2+} to the protein leads to a conformational change within the OAS1 protein which promotes the oligomerisation.

Zinc has previously been proposed to inhibit apoptosis under certain conditions and zinc is also capable of inhibiting the important proapoptotic protein caspase 3 [28]. The intercellular concentration of Zn^{2+} has been estimated to be around 0.10 mM [29], but the concentration of free Zn^{2+} might be as low as 0.01 mM. However, iron also exists in significant amounts within the cell and might play a role under conditions with high levels of free iron ions. We can only speculate that the inhibition of OAS1 and 2 described here represents a mechanism for cellular regulation of OAS activity in addition to the regulation by RNA.

4.2. Zn^{2+}-mediated oligomerisation of OAS1

Mutational studies have shown that an invariant cysteine found in all OAS proteins, except that of *Geodia cydonium*, is crucial for the enzymatic activity as well as important for the ability to form multimers [11,20]. When a three amino acid motif including this cysteine was mutated to alanine, OAS1 (the p48 isoform) lost the ability to form tetramers and OAS2 (the p69 isoform) lost its ability to form dimers. Both the OAS1 and the OAS2 proteins were inactivated by this mutation. These observations lead to the conclusion that the formation of oligomers is required for the activity of OAS1 and OAS2. The authors isolated monomeric and tetrameric OAS1 (p48 isoform) protein by gel filtration, and demonstrated that the active protein was found in the tetrameric fraction [20].

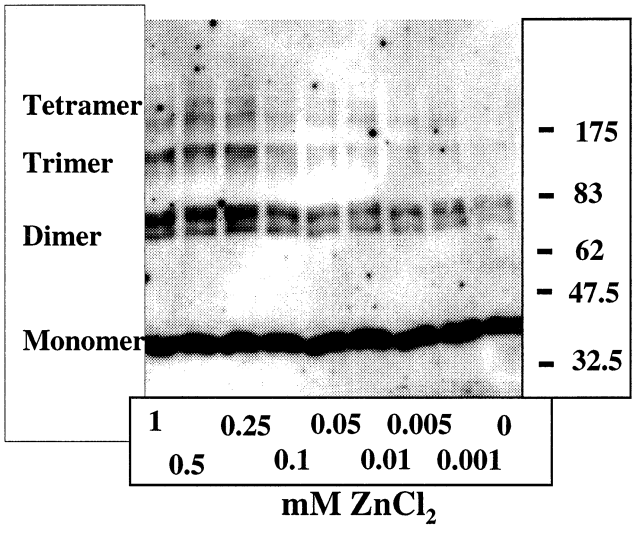

Fig. 5. Zinc promotes the oligomerisation of OAS1. DMS cross-linking of p42 under normal assay condition, using increasing amounts of zinc ions. After cross-linking the proteins were separated on SDS–PAGE and detected by immunoblotting. A clear increase in multimeric OAS1 (p42 isoform) is seen as increasing concentrations of zinc ions are used.

However, the isolation of monomeric and fully active human OAS1 (p42 isoform) protein by glycerol gradient has been reported [21]. The protein used in this study was isolated from insect cells infected with a recombinant baculovirus expressing the gene of interest. Human OAS1 protein, p42 isoform, produced in insect cells is mainly monomeric, with 5–20% of multimeric protein. However, in our hands the monomeric p42 protein can be isolated with no loss of activity by gel filtration (data not shown). Furthermore, we have reproduced the results obtained with the OAS1 protein (both the Zn^{2+} inhibition and the Zn^{2+}-induced cross-linking) using a porcine orthologue of the human OAS1. This porcine protein is produced in *Escherichia coli* and is exclusively monomeric as judged by gel filtration.

There are several possible explanations for the discrepancies discussed above, inherent differences between the p42 and p48 isoforms and the different assay methods used. Arundhati Ghosh and her colleagues [20] used enzyme immobilised on agarose beads whereas we used soluble enzyme. Nevertheless, we have demonstrated that Zn^{2+} leads to the formation of inactive multimers of the OAS1 enzyme. As noted above, the invariant cysteine found in the carboxy-terminus of the OAS proteins is involved in the multimerisation of both the OAS1 and OAS2 proteins as well as being important for the activity of these proteins. Since Zn^{2+} leads to both inhibition of both OAS1 and OAS2, and the multimerisation of OAS1, it might interact with this cysteine and disorders its normal function.

Acknowledgements: The authors thank Inger Bjørndal for excellent technical assistance. We thank Niels Ole Kjeldgaard and Pia Møller Martensen for helpful discussions and for help with baculovirus expression. The work was supported by the EU-Socrates programme (G.W.), the Danish Natural Science Research Council and The Danish Cancer Society.

References

[1] Silverman, R.H. (1997) in: Ribonucleases: Structures and Functions (D'Alessio, G. and Riordan, J.F., Eds.), pp. 515–551, Academic Press, New York.
[2] Dong, B. and Silverman, R.H. (1997) J. Biol. Chem. 272, 22236–22242.
[3] Silverman, R.H. and Williams, B.R. (1999) Nature 397, 208–209, 211.
[4] Justesen, J., Hartmann, R. and Kjeldgaard, N.O. (2000) Cell Mol. Life Sci. 57, 1593–1612.
[5] Marie, I. and Hovanessian, A.G. (1992) J. Biol. Chem. 267, 9933–9939.
[6] Rebouillat, D., Hovnanian, A., Marie, I. and Hovanessian, A.G. (1999) J. Biol. Chem. 274, 1557–1565.
[7] Hartmann, R., Olsen, H.S., Widder, S., Jorgensen, R. and Justesen, J. (1998) Nucleic Acids Res. 26, 4121–4128.
[8] Rebouillat, D., Marie, I. and Hovanessian, A.G. (1998) Eur. J. Biochem. 257, 319–330.
[9] Kalvakolanu, D.V. and Borden, E.C. (1996) Cancer Invest. 14, 25–53.
[10] Hartmann, R., Nørby, P.L., Martensen, P.M., Jørgensen, P., James, M.C., Jacobsen, C., Moestrup, S.K., Clemens, M.J. and Justesen, J. (1998) J. Biol. Chem. 273, 3236–3246.
[11] Sarkar, S.N., Ghosh, A., Wang, H.W., Sung, S.S. and Sen, G.C. (1999) J. Biol. Chem. 274, 25535–25542.
[12] Chebath, J., Benech, P., Revel, M. and Vigneron, M. (1987) Nature 330, 587–588.
[13] Marie, I., Rebouillat, D. and Hovanessian, A.G. (1999) Eur. J. Biochem. 262, 155–165.
[14] Ghosh, A., Sarkar, S.N. and Sen, G.C. (2000) Virology 266, 319–328.
[15] Ogawa, T. (1996) Nature Biotechnol. 14, 1566–1569.
[16] Mitra, A., Higgins, D.W., Langenberg, W.G., Nie, H., SenGupta, D.N. and Silverman, R.H. (1996) Proc. Natl. Acad. Sci. USA 93, 6780–6785.
[17] Castelli, J.C., Hassel, B.A., Maran, A., Paranjape, J., Hewitt, J.A., Li, X.L., Hsu, Y.T., Silverman, R.H. and Youle, R.J. (1998) Cell Death Differ. 5, 313–320.
[18] Diaz Guerra, M., Rivas, C. and Esteban, M. (1997) Virology 236, 354–363.
[19] Johnston, M.I. and Torrence, P.F. (1984) in: Interferon 3. Mechanisms of Production and Action (Friedman, R.M., Ed.), pp. 189–298, Elsevier, Amsterdam.
[20] Ghosh, A., Sarkar, S.N., Guo, W., Bandyopadhyay, S. and Sen, G.C. (1997) J. Biol. Chem. 275, 33220–33226.
[21] Wathelet, M., Moutschen, S., Cravador, A., DeWit, L., Defilippi, P., Huez, G. and Content, J. (1986) FEBS Lett. 196, 113–120.
[22] Chebath, J., Benech, P., Hovanessian, A., Galabru, J. and Revel, M. (1987) J. Biol. Chem. 262, 3852–3857.
[23] Justesen, J. and Kjeldgaard, N.O. (1992) Anal. Biochem. 207, 90–93.
[24] Sarkar, S.N. and Sen, G.C. (1998) Methods 15, 233–242.
[25] Justesen, J., Ferbus, D. and Thang, M.N. (1980) Nucleic Acids Res. 8, 3073–3085.
[26] Davis, J.P. and Stark, G.R. (1970) Proc. Natl. Acad. Sci. USA 66, 651–656.
[27] Rebouillat, D. and Hovanessian, A.G. (1999) J. Interferon Cytokine Res. 19, 295–308.
[28] Perry, D.K., Smyth, M.J., Stennicke, H.R., Salvesen, G.S., Duriez, P., Poirier, G.G. and Hannun, Y.A. (1997) J. Biol. Chem. 272, 18530–18533.
[29] Pattison, S.E. and Cousins, R.J. (1986) Fed. Proc. 45, 2805–2809.