

Analysis of RNase A refolding intermediates by electrospray/mass spectrometry

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Abstract Electrospray/mass spectrometry (ES/MS) was extensively used to obtain information on disulphide-containing intermediates formed during refolding of bovine pancreatic ribonuclease A. The analysis showed the existence of an equilibrated population of disulphide bonded intermediates, and indicates that intermediates containing two intramolecular S–S are predominant until late stages of the refolding process. Mixed disulphides with exogenous glutathione were also detected, supporting previous evidence of conformational restrictions on the ability of RNase A to form intramolecular disulphides. The results indicate that ES/MS is a suitable technique to detect and characterize refolding intermediates.

Key words: Electrospray/mass spectrometry; Bovine pancreatic RNase A; Refolding intermediate; Alkylation

1. Introduction

Protein folding has been investigated in great detail *in vitro*, providing evidence for the existence of partially structured intermediates along the pathway [1–3]. Although some general characteristics of the refolding pathways are evident, the debate is still open. In fact, there has been no general agreement over any of the proposed models [1–4], mainly because of the incomplete characterization of the refolding intermediates and inadequate understanding of their structures and properties.

For proteins containing disulphides, refolding is coupled to the formation of native disulphide bonds which takes place *in vivo* in the endoplasmic reticulum (ER) [5]. For this class of proteins, refolding intermediates can be trapped in a stable form, by quenching the free SH groups [2,3]. Even though for a reasonable number of such proteins it has been possible to identify and characterize intermediates that accumulate both *in vitro* [6–8] and *in vivo* [9], in most cases a complete understanding of the pathway has not yet been achieved. These observations highlight the need to study protein refolding by a variety of methods to provide more detailed information on protein folding intermediates.

The introduction of electrospray/mass spectrometry (ES/MS) [10] has allowed the determination of molecular weights of proteins within an error of 0.01%. Recently, Miranker et al. [11] used ES/MS to study deuterium/hydrogen exchanges occurring during the refolding of egg-white lysozyme. They provide descriptions of the populations of species on the folding pathway of the protein which it was not possible to obtain with either technique separately.

This paper describes the use of ES/MS to obtain crucial

information on the nature of disulphide-containing intermediates formed during the refolding of a disulphide-bonded protein. The experimental approach is based upon the determination of the molecular weight of the trapped disulphide-bonded intermediates on a time-course analysis of the refolding process by ES/MS. The work has focused on the refolding pathway of bovine pancreatic ribonuclease A (RNase A), which has 124 residues and four disulphide bonds, Cys²⁶-Cys⁸⁴, Cys⁴⁰-Cys⁹⁵, Cys⁵⁸-Cys¹¹⁰ and Cys⁶⁵-Cys⁷². Although RNase A has been one of the most thoroughly studied proteins with regard to refolding [7,8,12,13], several aspects of this pathway have not yet been clarified.

The results obtained by ES/MS provide evidence for the existence of differently balanced populations of disulphide-containing intermediates during the refolding of the reduced and denatured protein. This seems to indicate that the intermediates containing two intramolecular disulphide bonds are predominant until the late stages of the process.

2. Materials and methods

5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB), reduced dithiothreitol (DTT), EDTA, oxidised glutathione (GSSG), reduced glutathione (GSH), guanidinium chloride, iodoacetamide (IAM), iodoacetic acid (IAA), RNase A, Tris, yeast ribonucleic acid (RNA) and urea, were obtained from Sigma Chemical Co. Sephadex G-25 superfine and pre-packed Sephadex G-25M PD10 were acquired from Pharmacia. All other reagents were of analytical grade from Carlo Erba.

RNase A was reduced and denatured at a concentration of 25 mg/ml with a 72-fold molar excess of DTT in 50 mM Tris-HCl, pH 8.6, containing 10 M urea or 6 M guanidinium chloride, for 24 h, at room temperature, under a nitrogen atmosphere. The protein solution was then acidified at pH 4.0 with glacial acetic acid and the protein was separated from the excess DTT and denaturant by gel-filtration chromatography on a Sephadex G-25 column, equilibrated and eluted at 1 ml/min with 0.6% acetic acid. The protein fraction was recovered, tested for SH content, lyophilized and stored at –20°C. The free SH content of reduced and denatured RNase A was estimated by DTNB titration in 0.3 M Tris-HCl, 1 mM EDTA, pH 7.5, containing 6 M guanidinium chloride, and the formation of the 2-nitro-5-thiobenzoate dianion was measured at 412 nm (extinction coefficient 13,600 M⁻¹·cm⁻¹).

The reduced and denatured proteins (1 mg/ml) were refolded in the presence of 4 mM GSH and 0.4 mM GSSG in 0.1 M Tris-HCl, pH 8.0, containing 1 mM EDTA, at room temperature under a nitrogen atmos-

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Abbreviations: DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); DTT, reduced dithiothreitol; EDTA, ethylenediamine tetraacetic acid, disodium salt; GSSG, oxidised glutathione; GSH, reduced glutathione; IAM, iodoacetamide; IAA, iodoacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; ES/MS, electrospray mass spectrometry.

Dedication: This article is dedicated to the memory of our much missed friend and colleague Gianpaolo Nitti.

phere. Aliquots (100 μ l) of the refolding mixture were withdrawn at different intervals (30 min, 1, 2, 3, 4, 5, 8 and 24 h). Alkylation of the free SH groups present at any time within the intermediate populations was performed according to the procedure described by Gray [14]. The aliquots were added to an equal volume of either a 1 M IAA solution or a 2.2 M IAM solution.

IAA was freshly dissolved in 0.1 M Tris-HCl, containing 1 mM EDTA and buffered with NaOH to pH 8.0. IAM was dissolved in the same buffer at 65°C and cooled down to room temperature before use. During preparation of the reagents, the solutions were protected from light to minimize photolytic production of iodine, which is a very potent oxidizing agent for thiols. The carboxymethylation reaction with IAA was carried out for 5 min, whereas alkylation with IAM was performed for 30 s, in the dark, at room temperature [14]. The protein samples were then removed from the excess blocking reagent by rapid desalting on a pre-packed PD10 column, equilibrated and eluted at 1 ml/min with 0.6% acetic acid. The protein fraction was then recovered and lyophilized.

The concentration of native and reduced/denatured RNase A solutions was determined using an absorption of 0.695 and 0.679, respectively, at 278 nm for a 1 mg/ml solution [15]. RNase A activity was measured as described by Kunitz [16].

ES/MS analyses were carried out using a VG-BIO Q triple quadrupole mass spectrometer equipped with an electrospray ion source. The protein samples were dissolved in a mixture of H_2O/CH_3CN (50/50) containing 1% acetic acid. Protein samples (10 μ l) in concentrations ranging from 20 to 30 pm/ μ l were injected into the ion source via loop injection at a flow rate of 2 μ l/min; the spectra were recorded by scanning the first quadrupole at 10 s/scan. Mass calibration was performed by means of multiply charged ions from a separate injection of horse heart myoglobin (16950.5 Da).

3. Results

RNase A was reduced and denatured in the presence of both 10 M urea and 6 M guanidinium chloride. Before starting each refolding experiment, the reduced and denatured RNase A samples were tested for the content of free SH groups and enzymatic activity, exhibiting 8.1 SH/molecules and no detectable enzymatic activity, thus showing that the reduction and denaturation steps, in the presence of both urea and guanidinium chloride, led to the complete loss of enzymatic activity. The reduced and denatured proteins were then submitted to ES/MS analysis. The spectra showed the presence of a single component exhibiting a molecular weight of 13691.06 ± 0.49 Da, which agrees well with the theoretical value (13690.3 Da). The two fully reduced and denatured RNase samples were allowed to refold under different redox conditions. The reaction was monitored on a time-course basis by sampling aliquots of the incubation mixture at appropriate intervals and measuring the recovery of the enzymatic activity assayed according to Kunitz [16]. The most favourable conditions for promoting the refolding process were found when the reduced and denatured protein samples were incubated with 4 mM GSH and 0.4 mM GSSG. Fig. 1 shows the recovery of enzymatic activity (measured as the % of residual activity relative to a solution of native RNase A at the same concentration and under the same experimental conditions), when the reduced and denatured proteins were incubated in the presence of this redox system. The aliquots withdrawn at time 0, just after the addition of the thiol reagents, showed no detectable ribonuclease activity. As the reaction proceeded, the reduced and denatured proteins regained enzymatic activity which reached a stable value corresponding to 80–90% within 24 h. No marked differences were observed in the recovery of enzymatic activity for the preparation in urea or guanidinium chloride. The two refolding kinetics

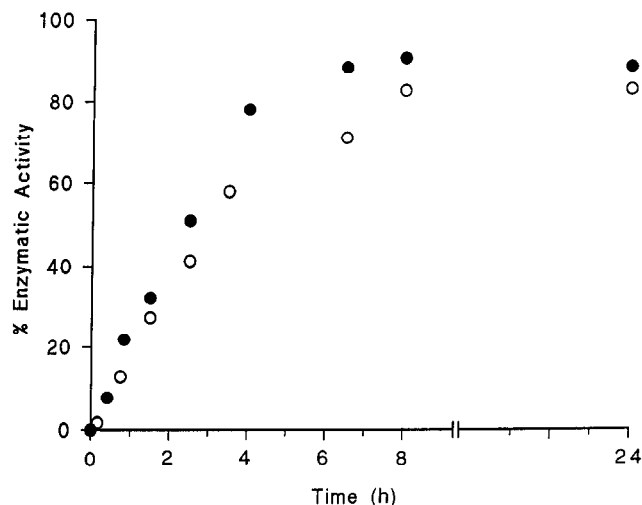


Fig. 1. Recovery of enzymatic activity of reduced and denatured RNase A incubated with 4 mM GSH and 0.4 mM GSSG. Solid (●) and open (○) circles refer to samples denatured in 10 M urea and 6 M guanidinium chloride, respectively.

approximate well to a single first-order reaction, with the kinetics of the refolding of the substrate denatured in urea being slightly faster ($k = 1.01 \times 10^{-4} \text{ s}^{-1}$) than the kinetics measured for the refolding of the sample denatured in guanidinium chloride ($k = 8.27 \times 10^{-5} \text{ s}^{-1}$). The two preparations were then used in the structural characterization of the disulphide bonded intermediates formed during refolding.

In order to test the efficiency of different alkylation procedures in trapping the free SH groups during refolding, two samples were incubated under the same experimental conditions of refolding (such as protein concentration, redox buffer, pH and temperature), and alkylated with a final concentration of 0.5 M IAA or 1.1 M IAM, as described in section 2. The alkylated samples were then submitted to ES/MS analysis in order to provide an identification of the species formed during the quenching reaction. The same analysis was also performed on the native protein as a reference. The ES/MS spectrum of the native RNase A (Fig. 2a) showed a single component the molecular mass of which was measured as 13682.83 ± 1.78 Da, which completely agrees with that predicted (13682.2 Da). Fig. 2b and 2c show the electrospray spectra of samples alkylated with 0.5 M IAA and 1.1 M IAM, respectively. The ES/MS spectrum of the sample carboxymethylated with 0.5 M IAA (Fig. 2b) showed the presence of a major component, named A, exhibiting a molecular weight of 14155.14 ± 0.64 Da, corresponding to the protein carrying 8 carboxymethyl groups (theoretical value 14154.5 Da), thus demonstrating that no re-formation of disulphide bonds occurred as no species containing disulphide bonds were detected. In fact the spectrum showed the presence of only another minor component, named B, exhibiting a molecular weight of 14213.99 ± 1.39 Da, corresponding to the protein carrying 9 carboxymethyl groups (theoretical value 14213.6 Da). Under the drastic carboxymethylation conditions used, in fact, histidine residues might also be modified by IAA [17]. Fig. 2c shows the ES/MS spectrum of the sample alkylated with 1.1 M IAM: a single component, named A, was detected, the calculated molecular weight of which, 14145.23 ± 0.58 Da, agrees well with the theoretical molecular

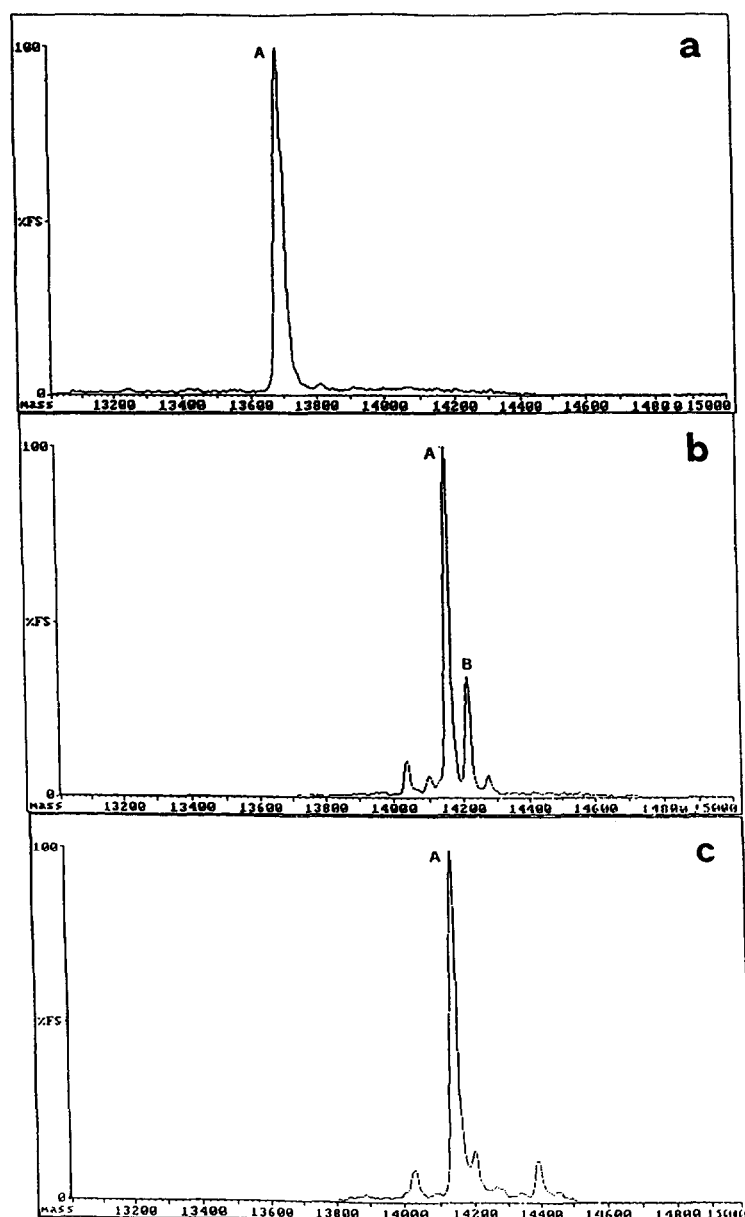


Fig. 2. Electrospray mass spectrometric analysis of native RNase A (a) and aliquots of the refolding mixture withdrawn at time 0, just after the addition of thiol reagents and alkylated with a final concentration of 0.5 M IAA (b) and 1.1 M IAM (c).

weight of the protein carrying 8 carboxyamidomethyl groups (14146.7 Da). No other components carrying extra carboxyamidomethyl groups, in addition to the eight linked to the cysteine residues, were detected in the spectrum.

Once the quenching conditions were defined, the reduced and denatured ribonuclease was incubated at 1 mg/ml in the presence of 4 mM GSH and 0.4 mM GSSG in 0.1 M Tris-HCl, pH 8.0, containing 1 mM EDTA, at room temperature under a nitrogen atmosphere. Aliquots of the refolding process were withdrawn at different intervals, assayed for ribonuclease activity, trapped as described, and then analysed by ES/MS to identify the disulphide bonded intermediates formed. The alkylation reaction used to quench the free SH groups provides an excellent tool to increase the molecular weight of the intermediates by a fixed amount (each additional carboxymethylation

and carboxyamidomethylation in fact increases molecular mass by 58 and 57 Da, respectively), and therefore provides a possibility of separating the populations containing different numbers of disulphide bonds by their masses and to determine their relative concentration during refolding.

Fig. 3 shows the spectra of the samples withdrawn at 2 h (a), 4 h (b), 8 h (c) and 24 h (d) of the refolding process and alkylated with 1.1 M IAM final concentration. The ES/MS spectrum of the aliquot withdrawn after 2 h of the refolding process revealed the enormous complexity of the refolding mixture, showing the simultaneous presence of several molecular species listed in Table 1. The measurement of the accurate molecular mass allowed the identification of all the components; species A, B, C and D in Fig. 3a represent a population of intermediates containing, respectively, 4 disulphide bonds

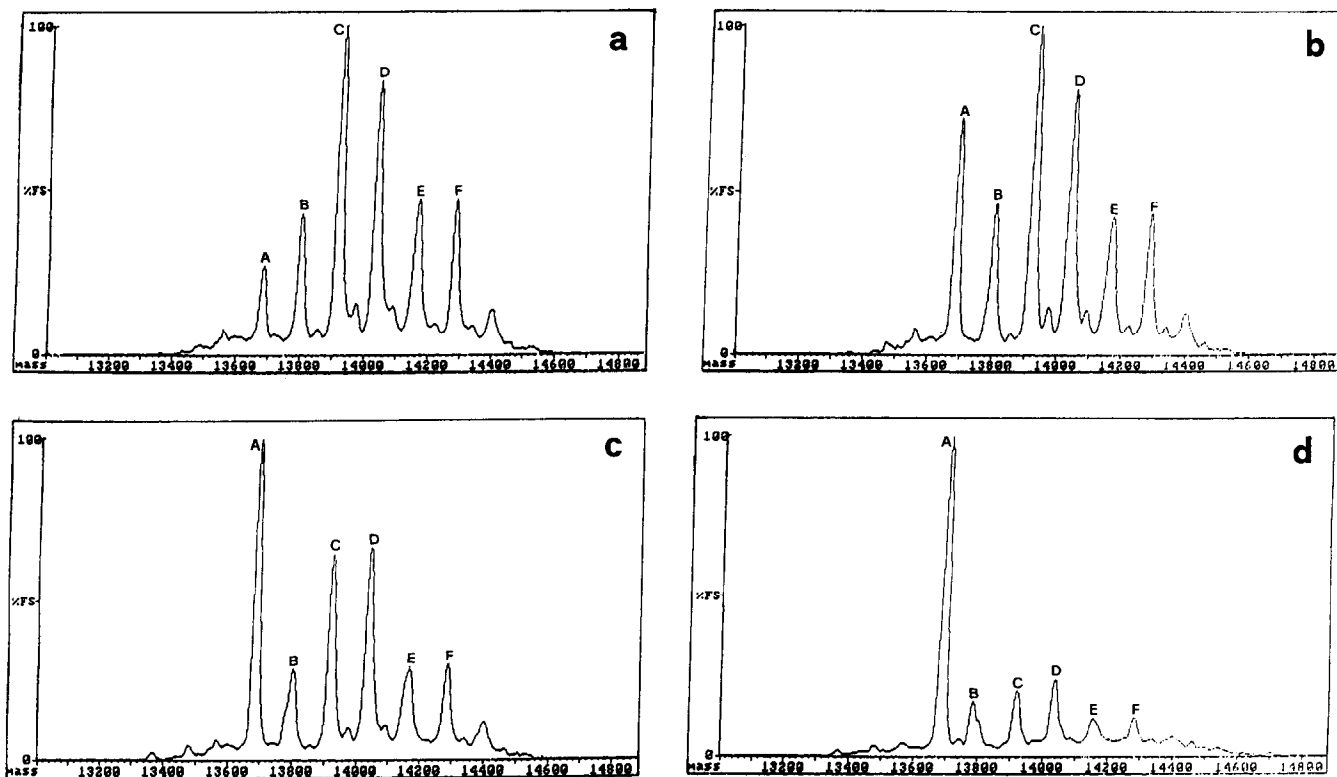


Fig. 3. Electrospray mass spectrometric analysis of aliquots of the refolding mixture in the presence of 4 mM GSH and 0.4 mM GSSG, withdrawn at time 2 h (a), 4 h (b), 8 h (c) and 24 h (d), and alkylated with 1.1 M IAM.

(no carboxyamidomethyl groups), 3 disulphides (two carboxyamidomethyl groups), 2 disulphides (four carboxyamidomethyl groups) and one disulphide (6 carboxyamidomethyl groups). It should be underlined that the component carrying 8 carboxyamidomethyl groups corresponding to the fully reduced RNase A completely disappeared after 2 h of refolding. Moreover, the ES/MS spectrum also revealed the formation of species carrying mixed disulphides with the exogenous glutathione: component E contains a mixed disulphide and two intramolecular disulphide bonds (3 carbox-

yamidomethyl groups) and component F contains a mixed disulphide together with an intramolecular one (five carboxyamidomethyl groups). As shown in Fig. 3, the detected molecular species were present at different concentrations during the process. The analysis of the samples withdrawn at 4 h (Fig. 3b) and 8 h (Fig. 3c) basically showed the presence of the same components detected at 2 h but revealed a progressive alteration in their relative abundances, in that the intensity of the species A increased and that of all the other components decreased.

Finally the ES/MS spectrum recorded at 24 h shows the presence of a major component, A, containing four S-S bonds and corresponding to the native protein. At this stage of the refolding process the sample is showing a recovery of more than 80% of enzymatic activity. The low intensity components also detected at 24 h were due to species containing carboxyamidomethyl groups and therefore free SH groups, thus demonstrating that, at this stage, some molecular species exist which have not completed the refolding process, yet.

The same kind of analysis described above was carried out by trapping the refolding intermediates with 0.5 M final concentration of IAA. Fig. 4 shows the relative intensities of the refolding intermediates detected by ES/MS analysis in both the experiments (alkylation with IAM, Fig. 4A; alkylation with IAA, Fig. 4B) plotted vs. the reaction time. The time-course plotted in Fig. 4 shows very clearly that the intermediates containing two intramolecular disulphide bonds are predominant from the beginning of the reaction up to 5 h, when the intensity of the species containing four intramolecular disulphide bonds increases. The two alkylation procedures produced a very sim-

Table 1
ES/MS identification of the disulphide-bonded intermediates formed during the refolding of RNase A in the presence of 4.0 mM GSH and 0.4 mM GSSG

| Component | Measured M.W. (Da) | Theoretical M.W. (Da) | Description |
|-----------|--------------------|-----------------------|--------------------------------------|
| A | 13,682.44 ± 0.26 | 13,682.2 | 4 S-S 0 CAM* groups |
| B | 13,798.39 ± 1.19 | 13,798.3 | 3 S-S 2 CAM groups |
| C | 13,915.01 ± 0.69 | 13,914.4 | 2 S-S 4 CAM groups |
| D | 14,031.50 ± 0.76 | 14,030.6 | 1 S-S 6 CAM groups |
| E | 14,160.98 ± 0.96 | 14,161.4 | 2 S-S 1 mixed S-S 3 CAM groups |
| F | 14,279.64 ± 1.02 | 14,277.5 | 1 S-S 2 mixed S-S 5 CAM groups |

*CAM = carboxyamidomethyl.

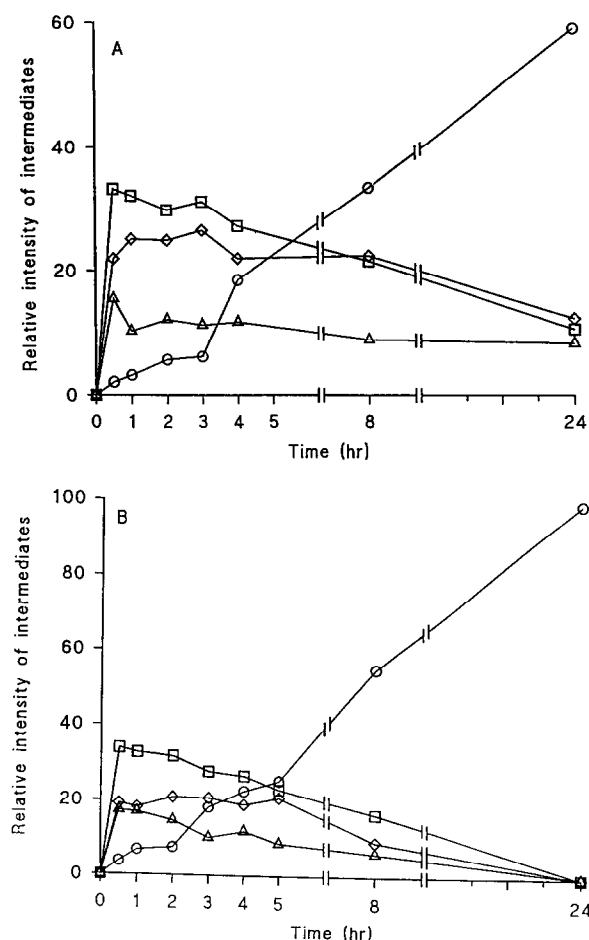


Fig. 4. Time-course of the relative intensity of the species detected by ES/MS analysis along the refolding pathway of reduced and denatured RNase A in the presence of 4 mM GSH and 0.4 mM GSSG. (A) Aliquots alkylated with IAM; (B) aliquots alkylated with IAA. (○) 4 S-S bonds; (◊) 3 S-S bonds; (□) 2 S-S bonds; (△) 1 S-S bond.

ilar distribution of disulphide-containing intermediates (within the experimental error), providing evidence for the accuracy of the quenching reaction in reflecting the original composition of the refolding mixture, since it does not depend on the kind of alkylating reagent used.

Finally, the preparations in urea and guanidinium chloride behaved similarly with regard to the distribution of disulphide bonded intermediates, suggesting that if RNase A has different properties under different denaturing conditions [18–20], they are not reflected in marked differences in the refolding pathway. However, a fully consistent analysis should involve a complete structural characterization to determine whether or not the different properties of the denatured states subsequently affect the refolding process.

4. Discussion

Reduced and denatured RNase A was refolded under different redox conditions and the recovery of enzymatic activity was monitored on a time-course analysis. As previously demonstrated [13], in the presence of oxidised DTT no recovery of enzymatic activity was detected, while in the presence of oxidised glutathione a recovery of about 70% was observed. Better

results were obtained when the reaction was carried out in the presence of mixtures of oxidised and reduced glutathione at concentrations in the range of 0.1–0.5 mM GSSG and 1–10 mM GSH. The best recovery of enzymatic activity, corresponding to 80–90%, was observed when the reduced and denatured RNase A was incubated with 4 mM GSH and 0.4 mM GSSG. These results confirm that only a mixture of reduced/oxidised glutathione [21–23] can effectively promote the entire process of the disulphide folding pathway. There is now good evidence [24] showing that glutathione is the major thiol-disulphide buffer in the secretory organelles of eukaryotic cells, and that the ER lumen is in a more oxidative state than the cytosol, although how it is maintained is still unknown [25].

The reduced and denatured RNase A was then refolded in the presence of 4 mM GSH and 0.4 mM GSSG. Aliquots of the refolding mixture were withdrawn at different intervals and trapped with IAA or IAM. The greatest risk of S–S reshuffling, and hence alteration of the cysteine pairing process, occurs during the alkylation step: therefore, kinetically forcing conditions were used [14] to enhance intermolecular alkylation, thus suppressing intramolecular exchange. ES/MS analysis revealed that quenching with 0.5 M IAA or 1.1 M IAM gave rise to a species in which all the eight cysteine residues were blocked by the carboxymethyl and carboxyamidomethyl groups, respectively, confirming that the alkylation conditions set up provided an accurate quench [3,14].

The trapped samples were then analysed by ES/MS. The alkyl group introduced with the quenching reaction increased the molecular weight of the intermediates by a fixed mass, thus allowing the separation by mass of the populations containing different numbers of disulphide bonds. ES/MS analysis of the refolding process provided crucial data for the definition of the disulphide-bonded species along the pathway. It revealed that the pairing of the cysteine residues proceeded via three groups of balanced intermediates containing disulphide bonds, namely one-disulphide, two-disulphides and three-disulphides. These intermediates could be highly heterogeneous and may consist of nearly all possible disulphide species. However, it is clearly evident that species containing two disulphide bonds predominate until the late stages of the process when the component containing four disulphide bonds increases. ES/MS analysis is not enough to determine whether the component containing four disulphide bonds consists of any scrambled species together with the native one. The FAB/MS analysis (unpublished data) of the samples withdrawn at 24 h showed the existence of all four native disulphide bonds and very few weak signals corresponding to non-native disulphides, confirming that some molecular species exist which have not completed the refolding process. However, at this stage it is not possible to determine whether scrambled intermediates are present along the pathway.

The predominance of the species containing two disulphide bonds along the pathway appears to be significant. On a purely statistical basis, in fact, a larger number of intermediates containing three S–S bonds was expected, whereas their concentration remains lower than that of the components containing two disulphides along the entire process. Therefore, the accumulation of the two disulphides-containing intermediates along the refolding pathway could be ascribed either to their intrinsic thermodynamic stability compared to other species or to a kinetic control of the whole process.

ES/MS analysis also revealed the formation of species containing intramolecular S–S bonds and mixed disulphides with the exogenous glutathione. The tendency of disulphide intermediates to accumulate as mixed disulphides with glutathione along the refolding pathway of reduced/denatured RNase A has already been observed [26–28] and was claimed to be clear evidence for conformational restrictions on the ability of the protein to form intramolecular disulphides.

Finally, the fast developments taking place in the field of ES/MS indicate that this technique will play an increasingly important role in studying protein refolding and will significantly contribute to a better understanding of a process that is still a topic of debate.

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References

- [1] Creighton, T.E. (1988) *Proc. Natl. Acad. Sci. USA* 85, 5802–5806.
- [2] Creighton, T.E. (1990) *Biochem. J.* 270, 1–16.
- [3] Weissman, J.S. and Kim, P.S. (1991) *Science* 253, 1386–1393.
- [4] Scheraga, H.A., Konishi, Y., Rothwarf, D.M. and Mui, P.W. (1987) *Proc. Natl. Acad. Sci. USA* 84, 5802–5806.
- [5] Creighton, T.E., Bagley, C.J., Copper, L., Darby, N.J., Freedman, R.B., Kemmink, J. and Sheikh, A. (1993) *J. Mol. Biol.* 232, 1176–1196.
- [6] Creighton, T.E. and Goldenberg, D.P. (1984) *J. Mol. Biol.* 179, 497–526.
- [7] Rothwarf, D.M. and Scheraga, H.A. (1993) *Biochemistry* 32, 2671–2679.
- [8] Rothwarf, D.M. and Scheraga, H.A. (1993) *Biochemistry* 32, 2680–2689.
- [9] Braakman, L., Helenius, J. and Helenius, A. (1992) *EMBO J.* 11, 1717–1722.
- [10] Smith, R.D., Loo, J.A., Edmonds, C.G., Barinaga, C.J. and Udseth, H.R. (1990) *Anal. Chem.* 62, 882–899.
- [11] Miranker, A., Robinson, C.V., Radford, S.E., Aplin, R.T. and Dobson, C.M. (1993) *Science* 262, 896–900.
- [12] Anfinsen, C.B., Haber, E., Sela, M. and White Jr., F.H. (1961) *Proc. Natl. Acad. Sci. USA* 47, 1309–1314.
- [13] Wearne, S.J. and Creighton, T.E. (1988) *Proteins: Struct. Funct. Genet.* 4, 251–261.
- [14] Gray, W.A. (1993) *Protein Sci.* 2, 1732–1748.
- [15] Schaffer, S.W., Ahmed, A.K. and Wetlauffer, D.B. (1975) *J. Biol. Chem.* 250, 8483–8486.
- [16] Kunitz, M.J. (1946) *J. Biol. Chem.* 164, 563–572.
- [17] Gurd, F.R.N. (1972) *Methods Enzymol.* 25, 424–441.
- [18] Sosnick, T.R. and Trewhella, J. (1992) *Biochemistry* 31, 8329–8335.
- [19] Makhatadze, G.I. and Privalov, P.L. (1992) *J. Mol. Biol.* 226, 491–505.
- [20] Pace, C.N., Laurents, D.V. and Thomson, J.A. (1990) *Biochemistry* 29, 2564–2572.
- [21] Creighton, T.E. (1986) *Methods Enzymol.* 131, 83–106.
- [22] Jaenicke, R. and Rudolph, R. (1989) in: *Protein Structure* (Creighton, T.E., Ed.) pp. 191–122, IRL Press, Oxford.
- [23] Lyles, M.M. and Gilbert, H.F. (1991) *Biochemistry* 30, 613–619.
- [24] Hwang, C., Sinskey, A.J. and Lodish, H.F. (1992) *Science* 257, 1496–1502.
- [25] Freedman, R.B. (1990) in: *Glutathione: Metabolism and Physiological Functions* (Vina, J., Ed.) pp. 125–134, CRC Press, Boca Raton, FL.
- [26] Creighton, T.E. (1977) *J. Mol. Biol.* 113, 329–341.
- [27] Creighton, T.E. (1979) *J. Mol. Biol.* 129, 411–431.
- [28] Konishi, Y., Ooi, T. and Scheraga, H.A. (1981) *Biochemistry* 20, 3945–3955.