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On the quaternary assembly of spinach chloroplast thioredoxin *m*

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Abstract

Thioredoxin *m* from spinach chloroplast has been structurally characterized both by X-ray crystallography and by NMR. Thioredoxin *m* is known to be monomeric, a finding which is confirmed by the NMR results. The crystal structure of this protein, however, contains two independent molecules per asymmetric unit. This fact was interpreted as contrasting with the NMR results [Neira et al. (2001) Biochemistry 40: 15246–15256]. Based on computational and biochemical considerations, we show that the presence of two thioredoxin *m* molecules per asymmetric unit bears no biological significance and does not contrast with the NMR results. The non-covalent arrangement of two monomers found in the crystals represents a 'crystallization intermediate' formed under the conditions for crystal growth.

Abbreviations: FPLC – fast protein liquid chromatography; Trx-*m* – thioredoxin *m*

Introduction

Thioredoxins are a large family of redox active proteins found in all living cells (Holmgren 1985). Many of the thioredoxin family members, both prokaryotic and eukaryotic, have been characterized structurally, by means of X-ray crystallography and NMR. For four members, both the crystal structure and the NMR structure are available. These are *Escherichia coli* thioredoxin (Katti et al. 1990; Jeng and Dyson 1994), human thioredoxin (Qin et al. 1994; Weichsel et al. 1996), thioredoxin *h* from *Chlamydomonas reinhardtii* (Mittard et al. 1997; Menchise et al. 2001) and thioredoxin *m* (Trx-*m*) from spinach chloroplasts (Capitani et al. 2000; Neira et al. 2001). In the case of Trx-*m*, two crystal structures of the protein, in the oxidized and in the reduced state (PDB codes 1FB6 and 1FB0, respectively), were published first (Capitani et al. 2000) and were followed one year later by the NMR structure of the oxidized protein (PDB code 1GL8) (Neira et al. 2001). Comparison of 1FB6 and 1GL8 showed that the two structures, albeit obtained using different techniques, are remarkably similar (Neira et al. 2001). The asymmetric unit of the Trx-*m* crystals contains two protein molecules, which form a non-covalent crystal dimer (Capitani et al. 2000). This feature of the Trx-*m* crystals was presented by Neira et al. (2001) as contrasting with NMR data, which pointed to a monomeric state of the protein in solution. The goal of the present article is to clarify that the two techniques do not yield contrasting results on the quaternary state of Trx-*m* in solution. In fact, the presence of two molecules in the asymmetric unit is not necessarily indicative of a dimeric state in solution. The analysis of the crystal packing shows that the arrangement of the two molecules in the crystal structure exhibits features typical of a crystallization dimer, with no biological significance. It is noteworthy that a similar situation, with two molecules per asymmetric unit and a monomeric biological form, had already been described for *C. reinhardtii* thioredoxin *h* (Menchise et al. 2001). In the case of type *m* thioredoxin from

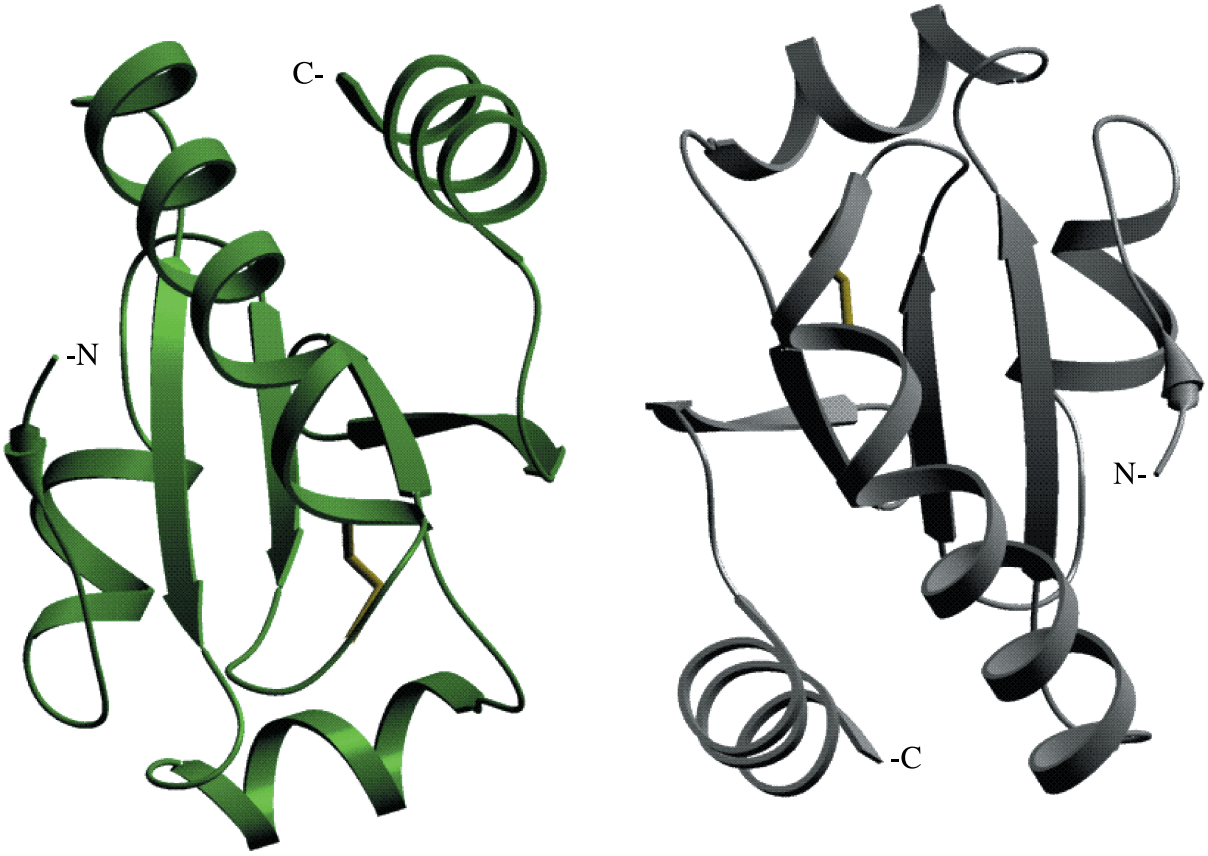

Figure 1. Cartoon representation of the two Trx-*m* molecules found in the crystal asymmetric unit. The direction of the view is along the non-crystallographic two-fold axis. Figure prepared with SETOR (Evans 1993).

C. reinhardtii, solved by NMR (Lancelin et al. 2000), analysis of the ^{15}N–^{1}H relaxation data had provided evidence for a monomeric state in solution.

Analysis of the crystal packing of thioredoxin *m*

The crystals of Trx-*m* belong to the space group $P3_121$, with comparatively small unit-cell lengths ($a = b = 74.7$ Å, $c = 74.0$ Å) and two molecules per asymmetric unit, with a V_M value of 2.6. Trx-*m* had long been known to be a monomeric protein (Schürmann et al. 1981). The presence of two molecules in the asymmetric unit was reported but not discussed in Capitani et al. (2000) since it was not considered to be a biologically relevant feature. Was this choice justified?

The interface area for the two Trx-*m* molecules in the asymmetric unit, as calculated with the Protein–Protein Interaction Server (http://www.biochem.ucl.ac.uk/bsm/PP/server/index.html) (Jones and Thornton 1996), is 1159 Å^2. How does this value compare to that of a typical crystal contact interface? Janin and Rodier (1995) studied 1320 pairwise interfaces formed by crystallographic symmetry in 152 crystal forms where the asymmetric unit contains a monomeric protein. The studied pairwise interfaces are therefore a result of the crystallization process and do not possess biological significance. Their average area was calculated to be 570 Å^2, and the bulk of the values was in the range of 200–1200 Å^2 (Janin and Rodier 1995). Thus, the value for Trx-*m* is in the normal range (albeit on the high side) for a crystal-contact interface. To confirm this indication, the packing of 1FB6 was analyzed using the PQS server (http://pqs.ebi.ac.uk/) (Henrick and Thornton 1998). PQS uses an empirical, weighted score of several factors to determine whether the protein contacts are specific (a true macromolecular oligomer) or non-specific (crystal packing). The contributing factors are the size of the solvent-accessible surface area buried in the oligomeric

interaction (Δasa), the number of buried residues at the interface and the difference in solvation energy of folding (Eisenberg and McLachlan 1986) between the complete assembly and that for each chain, and the number of interchain salt and disulfide bridges (Henrick and Thornton 1998). The PQS prediction for 1FB6 was that the presence of two molecules in the asymmetric unit resulted from a non-specific interaction (OLIGOMER of two independent molecules). An interesting feature of the Trx-*m* dimer interface is that it possesses a point-group symmetry, as the two molecules are related by a non-crystallographic two-fold axis (Capitani et al. 2000) (Figure 1). The residues participating in the molecular contacts do not correspond to those found in the asymmetric unit of the *C. reinhardtii* thioredoxin *h* crystals (Menchise et al. 2001). In a systematic study of protein–protein interactions at crystal contacts, Wang and Janin (1993) showed that some unexpectedly large interfaces occur in crystals of monomeric proteins, and are nearly always associated with point-group symmetry. The same phenomenon was observed for crystals of monomeric proteins having more than one molecule per asymmetric unit (Wang and Janin 1993; Menchise et al. 2001). Janin and Rodier (1995) pointed out that such comparatively large interfaces, with point-group symmetry, may result from dimeric (or multimeric) intermediates in the crystallization process. Since the interface between the two molecules in the asymmetric unit of Trx-*m* crystals is on the high side of the range for a non-specific interaction and it does possess two-fold symmetry, we decided to investigate whether a significant percentage of Trx-*m* dimer could be detected in solution at a concentration and ionic strength similar to those of crystallization.

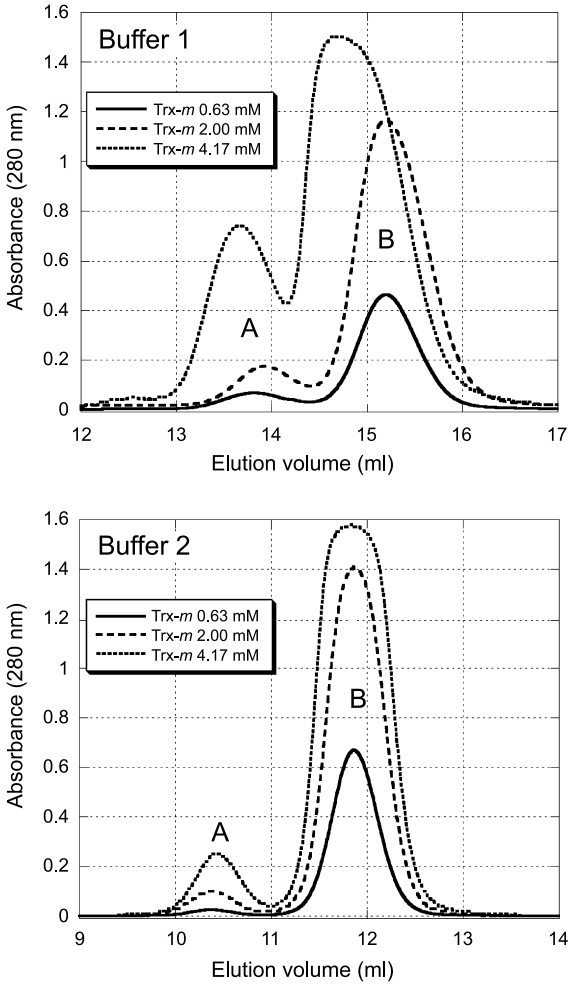

Figure 2. Analytical gel filtration of recombinant thioredoxin *m* by FPLC on a Superdex 75 HR 10/30 column. Sample volume: 25 µl. Flow rate: 1.0 ml/min. Buffer 1: 100 mM Tris-HCl buffer pH 7.3, 200 mM $(NH_4)_2SO_4$ and 0.02% (w/v) NaN_3. Buffer 2: 50 mM Tris-HCl pH 8.0 and 0.02% (w/v) NaN_3. The peak areas were integrated using the FPLC manager software. A – dimer peak; B – monomer peak.

Biochemical evidence for monomeric state

Trx-*m*, prepared as described in Schürmann (1995), was analyzed for oligomeric state by analytical gel filtration on a Superdex 75 HR 10/30 FPLC (Amersham Pharmacia Biotech, UK) column (Figure 2). Three different concentrations of the protein (8.4, 27.4 and 55.6 mg/ml) were used, with 100 mM Tris-HCl buffer pH 7.3, 200 mM $(NH_4)_2SO_4$ and 0.02% (w/v) NaN_3 (buffer 1). As a comparison, Trx-*m* had been crystallized by mixing 1 µl of protein (50 mg/ml in 50 mM Tris-HCl buffer pH 7.3 and 0.02% (w/v) NaN_3) with 1 µl of precipitating solution containing 200 mM $(NH_4)_2SO_4$, 0.1 M sodium acetate pH 4.6 and 30% (w/v) PEG monomethylether 2000 (Capitani et al. 2000). Trx-*m* crystals formed at a concentration higher than 25 mg/ml, due to the equilibration process in the vapor diffusion method. Buffer 1 provides conditions of comparatively high ionic strength, similar to those of the Trx-*m* crystallization experiment. Trx-*m* solutions at the same concentrations as above (8.4, 27.4 and 55.6 mg/ml) but in 50 mM Tris–HCl pH 8.0 and 0.02% (w/v) NaN_3 (buffer 2) were also prepared and analyzed.

The results of the gel filtrations, as obtained by peak integration, are summarized in Table 1 and

Table 1. Percentage of Trx-*m* dimer in different conditions.

Trx-*m* conc (mM, mg/ml)	% dimer, buffer 1	% dimer, buffer 2
0.63 mM, 8.4 mg/ml	12	2
2.00 mM, 27.4 mg/ml	10	5
4.17 mM, 55.6 mg/ml	24	9

clearly indicate that there is a small percentage of dimer, rising with protein concentration, both in buffer 1 and in buffer 2. Thus, there is evidence of a monomer–dimer equilibrium, in which the dimer is a minor species. The amount of dimer is larger (even though still small in absolute terms) in buffer 1 than in buffer 2 (Figure 2), probably due to hydrophobic interactions promoted by the higher ionic strength of buffer 1. Neira et al. (2001) carried out preparative gel filtration on their Trx-*m* samples in the range of concentrations 50–200 μM (no buffer details were given). They used a Superdex 75 (26/60) column and did not detect dimerization in the 50–200 μM concentration range, which was much lower than that described in this work (0.63–4.17 mM). It is conceivable that, under the conditions explored by Neira et al. (2001), the amount of Trx-*m* dimer is so low that it is undetectable.

Conclusions

In conclusion, the non-covalent dimeric arrangement in the asymmetric unit of the Trx-*m* crystals possesses the features defined by (Janin and Rodier 1995) as indicative for a 'crystallization intermediate'. In fact, the two Trx-*m* molecules have an interface area in the normal range, but on the high side, for a crystal contact interface. Thus, bioinformatic tools, like the PQS server, recognize this arrangement to be a non-specific dimer, which also possesses point-group symmetry (a two-fold axis). In solution, at high concentration and ionic strength, a monomer–dimer equilibrium is detectable, with small but significant percentages of dimer. It is conceivable that the crystals of Trx-*m* have assembled from the small amount of dimer present in solution. The dimeric arrangement observed in the asymmetric unit appears, therefore, not to be biologically significant but rather to be a possible 'crystallization' intermediate under the conditions for crystal growth. In Capitani et al. (2000), the discussion about the structural and functional properties of Trx-*m* assumed that the protein was monomeric in solution. The presence of two molecules per asymmetric unit was described, but not discussed, since it was considered to bear no biological relevance. That choice appears to be justified in the light of the considerations and results described above.

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