A domain-swapped RNase A dimer with implications for amyloid formation

Yanshun Liu[1], Giovanni Gotte[2], Massimo Libonati[2] and David Eisenberg[1]

[1]*UCLA-DOE Laboratory of Structural Biology and Molecular Medicine, Department of Chemistry and Biochemistry and Biological Chemistry, University of California, Los Angeles, California 90095-1570, USA.* [2]*Department of Neurological Science, Biological Chemistry Section, University of Verona, Verona, Italy.*

Bovine pancreatic ribonuclease (RNase A) forms two types of dimers (a major and a minor component) upon concentration in mild acid. These two dimers exhibit different biophysical and biochemical properties. Earlier we reported that the minor dimer forms by swapping its N-terminal α-helix with that of an identical molecule. Here we find that the major dimer forms by swapping its C-terminal β-strand, thus revealing the first example of three-dimensional (3D) domain swapping taking place in different parts of the same protein. This feature permits RNase A to form tightly bonded higher oligomers. The hinge loop of the major dimer, connecting the swapped β-strand to the protein core, resembles a short segment of the polar zipper proposed by Perutz and suggests a model for aggregate formation by 3D domain swapping with a polar zipper.

The term 3D domain swapping was introduced to describe a dimer of diphtheria toxin[1]. In a 3D domain-swapped protein, two or more protein chains exchange identical 'domains', forming a strongly bound oligomer[1]. To date, some 20 crystal structures of 3D domain-swapped dimers and trimers have been reported but the physiological significance of these tightly bound oligomers is not fully understood. The structure described here of a second 3D domain-swapped form of an RNase A dimer vastly expands the repertoire of types of oligomeric proteins that may form by swapping domains.

In 1962, Crestfield *et al.*[2] reported that RNase A forms dimers and higher order oligomers after lyophilization in 50% acetic acid. From elegant studies of chemical modification of active site residues, they proposed that RNase A forms its dimer by exchanging its N-terminal segment, a mechanism essentially identical to what we now term 3D domain swapping. From the crystal structure of an RNase A dimer[3] we confirmed that the structure is indeed 3D domain-swapped by exchange of the N-terminal helix (residues 1–15).

A follow-up experiment in 1965 showed that RNase A forms two types of dimers, which can be separated by cation exchange chromatography[4]. Study of these two dimers was not pursued until 1996 when Libonati *et al.*[5,6] found that one component predominates (the 'major dimer'). The two dimers show different biophysical properties on gel filtration chromatography, ion exchange chromatography and native gel electrophoresis, suggesting that they have different quaternary structures. Both dimers possess higher enzyme activity on double-stranded (ds) RNA than does the monomer[5,6]. Because our previous RNase A crystals were obtained from a mixture of the two dimers, we seeded solutions of the separated dimers each with the previous crystals. After seeding, crystals grew from the minor dimer solution, but not the major dimer solution. This showed that the dimeric structure determined previously[3] is the minor dimer. A crystal screen then yielded crystals of the major dimer.

Overall structure of the RNase A major dimer

The structure of the major dimer of RNase A was determined by molecular replacement, using the RNase A monomer as a probe, and was refined to 1.7 Å resolution (Table 1). The major dimer is 3D domain-swapped (Fig. 1*c*), but by exchanging its C-terminal β-strand (residues 116–124) with an identical molecule, in contrast to the exchange of the N-terminal helix (residues 1–15) in the minor dimer (Fig. 1*b*). The active site of RNase A contains catalytic residues His 12, Lys 41 and His 119 in the monomer and both dimers. But in both dimers, the active sites are composite, consisting of residue His 12 of the first subunit and residue His 119 of the second subunit. Domain swapping does not disrupt the active site of either dimer, which is consistent with the observation that the RNase A dimers retain enzyme activity[5,6]. The interface between domains that is present in both the monomer and the domain-swapped oligomer is termed the closed interface, the interface found only in the oligomer is termed the open interface, and the loop that links the two domains is termed the hinge loop[1].

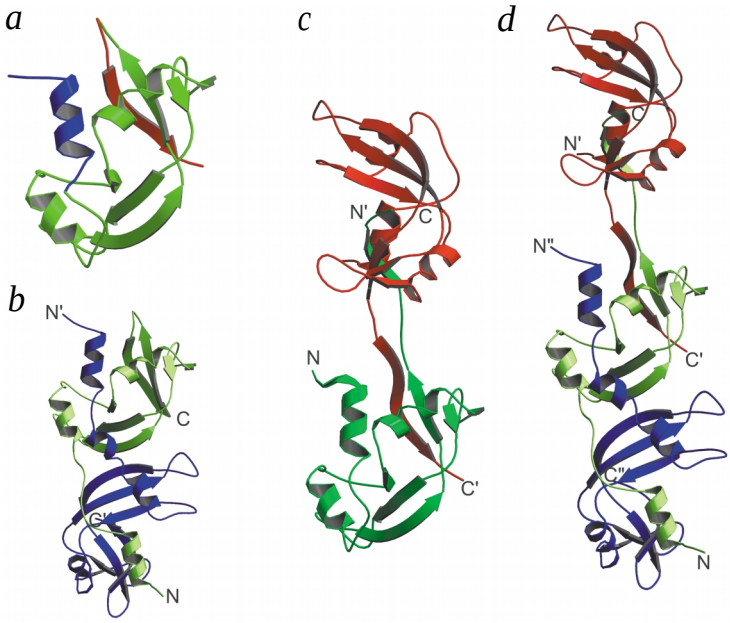

Fig. 1 Ribbon diagrams of the structures of RNase A. ***a***, The monomer[26]; ***b***, the minor dimer[3]; ***c***, the major dimer (this paper); and ***d***, a model of a trimer of (*a*) produced by combining panels (*b*) and (*c*). The N- and C-termini are labeled. In the minor dimer the N-terminal α-helices are swapped, whereas in the major dimer, the C-terminal β-strands are swapped. The closed interfaces are the interface between the blue segment and the green core structure, and the interface between the red segment and the green core structure in (*a*), which are also found in the minor dimer (*b*) and the major dimer (*c*), respectively. The open interface in the minor dimer lies between the green and the blue strands in the middle of the β-sheet in (*b*), and the open interface in the major dimer lies between the red and the green segments in the center of the molecule in (*c*). These open interfaces do not exist in (*a*). The core domain of the green subunit in each molecule has the same orientation. In the model of the trimer, domain swapping takes place at both the N- and C-termini. The figure was created using Raster3D[29].

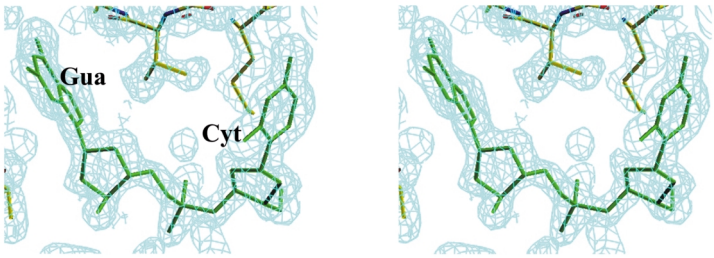

Fig. 2 Stereo view of the $2F_o$ - F_c electron density map of the inhibitor of RNase A, dCpdG, contoured at 1.4 σ to illustrate its detail. The stick model of dCpdG is shown in green. The figure is plotted with the progrem O[27].

The dinucleotide binding site

The dinucleotide (dCpdG) aids crystallization of the major dimer and is clearly seen in the electron density map (Fig. 2). The binding of dCpdG in the active site of the major dimer is called retro-binding (a nonproductive binding), as reported in structures of RNase A monomer in complex with dCpdG[7]. However, the cytosine moiety is not clear in previous structures because of the absence of the interaction of the cytosine moiety with RNase A monomer. In the major dimer, the cytosine moiety forms hydrogen bonds with the protein molecule (Cyt 1 O5′–Arg 85 NE, Cyt 1 N4–Glu 86 O) and with the symmetry-related molecule (Cyt 1 N3–Ser 15 OG, Cyt 1 O2–Ser 15 N). Thus, the structure of the cytosine moiety is well defined. In addition, the guanine moiety also forms hydrogen bonds with symmetry-related molecules (Gua 2 O2P–H_2O–Arg 33 NE, Gua 2 O2P–H_2O–Ser 32 OG, Gua 2 O3′–H_2O–Asp 14 OD2 and Gua 2 O4′–Ser 16 OG). Apparently dCpdG acts as 'glue' for packing of the major dimer crystals.

Effect of solvent polarity on 3D domain swapping

Examination shows that the closed interfaces of both the major and the minor dimers are composed mainly of apolar side chains engaged in hydrophobic interactions. To examine if the polarity of the solvent affects the yield of dimers, various acids, including hydrochloric acid (HCl), trifluoroacetic acid (TFA) and propionic acid, were substituted for acetic acid during dimer formation by lyophilization. Dimers of RNase A were formed from these four acids, and the yields of the dimers are comparable: 18 ± 2% from HCl, 13 ± 2% from TFA, 21 ± 3% from propionic acid, and 20 ± 1% from acetic acid. In contrast, no dimers are recovered from urea-denatured RNase A. These results indicate that acidic conditions that partially unfold RNase A are required for domain swapping, and the polarity of the solvent has little effect on domain swapping. The more complete unfolding produced by urea prevents 3D domain swapping.

Implications for oligomer formation

The structures of the two dimers of RNase A show that the molecule can swap both its N-terminal α-helix and its C-terminal β-strand. Examination of the RNase A monomer and dimers reveals that one of the four disulfide bonds is adjacent to each of the swapped fragments. These are residues Cys 26–Cys 84 and Cys 58–Cys 110, which apparently limit the size of the 'domain' that can be swapped. Crestfield *et al.*[2] proposed that at acidic pH, the N-terminal fragment is partially unfolded, whereas the core of the structure is stabilized by the disulfide bonds. The structure of the RNase A major dimer indicates that the C-terminal fragment under acidic conditions also breaks its noncovalent bonds with the stable core of the molecule, permitting 3D domain swapping. It is noteworthy that domain swapping does not take place during the renaturation of RNase A from urea solution even though the disulfide bonds are intact. These results suggest that in the case of RNase A, both integrity of the core structure and partial unfolding of the termini, in the sense of breaking their noncovalent bonds with the core, are required for 3D domain swapping. The importance of the termini for domain swapping is reinforced by a survey of the ~20 3D domain-swapped proteins having known structures. This reveals one common feature: all swapped 'domains' regardless of size are at either the N- or C-terminus.

The observation that RNase A forms two distinctly different domain-swapped dimers suggests that the ability of proteins to swap domains may be more general than previously suspected. The swapped fragments of the two RNase A dimers have different primary and secondary structures, different structural environments and different hinge loops. Thus no special molecular property other than a noncovalently constrained terminus

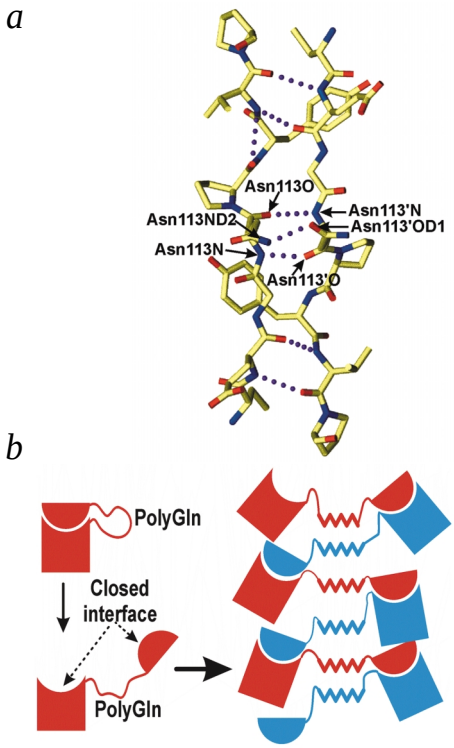

Fig. 3 Structure of the hinge loops of the RNase A major dimer with implications for amyloid fiber formation. ***a***, Structure of the open interface of the RNase A major dimer, showing the atomic structure for residues 110–117 of both molecules of the dimer. These segments correspond to the central portion of Fig. 1*c*. Carbon, nitrogen and oxygen atoms are in yellow, blue and red, respectively. Hydrogen bonds are shown as purple dots. Three hydrogen bonds are formed between Asn 113 residues in the two hinge loops. The atoms involved in these three hydrogen bonds are labeled. The figure is plotted with SETOR[30]. ***b***, Speculative 3D domain-swapping, polar-zipper model for formation of aggregates, with polyamide expansion loops. With the expansion of polyglutamine in the hinge loop between two domains, a fluctuation that breaks the noncovalent bonds between the two domains exposes the polyglutamine loop as well as the two halves of the closed interface. The polyamide segment is able to form a β-sheet, with three hydrogen bonds per residue pair, as observed for residue 113 in (*a*). This stabilized sheet grows as additional polyamide β-strands are added, defining the fiber axis perpendicular to the β-strands, shown at the right. The sheet may be further stabilized by 3D domain swapping formed by additional closed interfaces at the two sides of the sheet. A less stable sheet could be formed by other (non-polyamide) sequences, and could account for the observation that proteins that undergo transition to the amyloid state exhibit enhanced β-structure.

Table 1 Data collection and refinement of the RNase A major dimer

Data collection			
Crystals	RNase A major dimer	RNase A major dimer	
Temperature	Room temperature	Cryo	
Cryo protectant	none	25% glycerol	
Wavelength (Å)	1.5418	1.072	
Resolution range (Å)	40–2.4	40–1.7	
Total reflections	60,587	813,673	
Unique reflections	13,566	76,047	
Completeness (%)	92.1	95.0	
R_{merge} (%)[1]	8.0	5.2	
Space group	C2	$P2_1$	
Unit cell dimensions	a = 83.58 Å,	a = 83.57 Å,	
	b = 97.83 Å,	b = 96.34 Å,	
	c = 48.86 Å,	c = 48.24 Å,	
	β = 107.1º	β = 107.48º	
Refinement			
Resolution range (Å)	10–1.7	No. of reflections	75,824
R-factor(%)[2]	18.4	No. of protein atoms	3,803
R_{free} (%)[3]	21.3	No. of water molecules	556
R.m.s. deviations			
Bond lengths (Å)	0.019	No. of phosphate ions	4
Bond angles (º)	2.072	No. of dCpdG	4
Average B factor(Å²)	25.14	No. of glycerol molecules	24

[1]$R_{merge} = \Sigma_{hkl} \Sigma_i |I(hkl)_i - <I(hkl)>| / \Sigma_{hkl} \Sigma_i <I(hkl)_i>$.
[2]R-factor = $\Sigma_{hkl} |F(hkl)_o - <F(hkl)_c>| / \Sigma_{hkl} F(hkl)_o$.
[3]R for 10% of reflections not used in refinement.

appears essential for 3D domain swapping. This leads to the suggestion that every protein may form domain-swapped oligomers under conditions of high concentration in which the core structure of the protein is preserved and the noncovalent bonds between a terminus and the core are weakened.

The suggestion that the mechanism of domain swapping depends on the weakening of the interaction of the swapped domain with the core is supported by measurements by others of the binding of the N-terminal fragment of RNase S with the core. K_d of RNase S (RNase A cleaved by subtilisin between residues Ala 20 and Ser 21) changes from 3.1×10^{-11} M at pH 8.3 to 1.1×10^{-6} M at pH 2.7 (ref. 8). This indicates that the interaction between the S-peptide (the N-terminal fragment of RNase A after the cleavage by subtilisin) and the S-protein (the C-terminal fragment of RNase A after the cleavage by subtilisin) is significantly weakened under acidic conditions. In addition, S-peptide is required for the complete refolding of S-protein[9,10], suggesting an early and tight association of S-peptide with S-protein. Noting that S-peptide consists of the swapped N-terminal helix and part of the hinge loop of the minor dimer, we speculate that this association prevents the N-terminal segment of RNase A from domain swapping during renaturation at neutral pH. However, under acidic conditions, the interaction between the N-terminal segment and the core structure is weakened and the core structure remains intact, permitting domain swapping at the N-terminal helix. A similar mechanism could also apply to the 3D domain swapping at the C-terminal strand in the major dimer.

RNase A also forms trimers and higher order oligomers[5] whose structures are presently unknown. Based on the structures of the two RNase A dimers, we have constructed a model of an RNase A trimer with both types of domain swapping taking place in the same molecule (Fig. 1*d*). By this mechanism a protein can form linear oligomers without exposing closed interfaces (that is, no dangling domains), in contrast to our earlier model[11].

The minimal polar zipper

3D domain swapping has been proposed as a mechanism for amyloid fiber formation[3,12,13] and this proposal is supported by the observations of this paper. It was previously noted[14] that 3D domain swapping and amyloid fiber formation share common features: (i) both have high specificity in that they are each formed from a single type of protein; (ii) both amyloid-forming proteins and domain-swapping proteins have two stable forms, separated by a high energy barrier; and (iii) both amyloids and domain-swapped oligomers can form linear aggregates. In addition, both domain-swapped oligomers and amyloids were once believed to form from only a few types of proteins, but Dobson and coworkers[15] have proposed that every protein may form amyloid fibers at high concentration under partially destabilizing conditions. Here we propose that every protein may be domain-swapped at high concentration under partially destabilizing conditions.

There is another feature of the domain-swapped RNase A major dimer that is reminiscent of the polyglutamine aggregates such as those formed by the protein huntingtin[16,17]. This feature is the pattern of hydrogen bonding observed (Fig. 3*a*) in the 'open interface' formed between the two hinge loops of the major dimer. This hinge loop essentially forms a two-stranded antiparallel β-sheet, except that it is interrupted by Pro 114, which lacks the amide NH necessary for participation in the sheet. In addition, each strand of the hinge loop (residues 112–115) contains Asn 113, whose main chain groups form two hydrogen bonds exactly as in antiparallel β-sheets. Also the two Asn 113 side chains form a third hydrogen bond. The hydrogen bonding between the two Asn residues is highly similar to the minimal unit of the antiparallel β-sheet 'polar zipper' model of poly-glutamine structures proposed by Perutz *et al.*[16] as stabilizing the aggregates of huntingtin. If additional residues of Asn (or Gln) were inserted in the hinge loop of the RNase A major dimer, it seems likely that the antiparallel β-sheet would be extended and stabilized by additional triple hydrogen bonds. This is so because there is no interaction at the open interface of the major dimer that would interfere with such hydrogen bonds, which would merely extend the bonding seen between the Asn 113 residues of the major dimer. In the case of huntingtin, polyglutamine expansion has been reported to induce amyloid-like fiber formation in the expanded protein[18]. In the yeast prion Ure3, polymerization is mediated by a highly asparagine-rich domain

Table 2 Comparisons of the major and minor dimers of RNase A

Dimer	Major dimer	Minor dimer[3]
Swapped fragment	C-terminal	N-terminal
	β-strand	α-helix
	residues 116–124	residues 1–15
Hinge loop	Residues 112–115	Residues 16–22
Total molecular surface per dimer (Å²)	13,411	12,236
Area of closed interface per dimer (Å²)	1,716	1,592
Area of open interface per dimer (Å²)	200	558
Largest molecular dimension (Å)	85	77
Molar ratio of yield[5]	3	1

of Ure2p protein[19], indicating that polyasparagine, like polyglutamine, could also form a polar zipper structure.

By combining the observations of 3D domain swapping and 'polar zipper' hydrogen bonding in the RNase A major dimer, we propose a 3D domain swapping zipper model for fiber formation of huntingtin (Fig. 3*b*) and of other proteins. Native protein structures fluctuate with occasional breaking of noncovalent bonds between domains. This fluctuation is enhanced by mild acid in the case of RNase A. In huntingtin, we postulate that after polyglutamine expansion, a 'domain' adjacent to the expansion breaks its noncovalent bonds with the core of the protein. The separation of this domain from the core exposes three surfaces: the two halves of the closed interface and the hinge loop, which is polyglutamine in this case. In our model, the polyglutamine segments from several molecules form a β-sheet, which is stabilized both by the triple hydrogen bonds of the polar zipper and by 3D domain swapping as in the schematic drawing of Fig. 3*b*. This domain-swapping zipper model may also represent other amyloidogenic proteins. Many protein segments form β-sheets. If the hinge loop between the protein core and a terminal segment of secondary structure is a β-forming segment, hydrogen bonding between hinge loops can form a β-sheet and the structure can be stabilized by 3D domain swapping at both sides of the sheet. Because hinge loops are flexible, the domain-swapped portion at the sides of the sheet would not generally be well ordered and would not contribute strong reflections to the X-ray fiber diffraction pattern. This model for amyloid formation by a polar zipper with 3D domain swapping is consistent with the observation of increased β-sheet structure upon formation of amyloid[13,20].

This 3D domain swapping zipper model for amyloid is consistent with the characteristic cross-β structure of amyloid: β-strands perpendicular to the amyloid fiber axis[21]. During molecular aggregation, the aggregates tend to grow in the direction perpendicular to the surface that provides the greatest interaction[22]. In the 3D domain swapping zipper model, the polar zipper provides the strongest interaction between the adjacent molecules. Thus, the molecules tend to aggregate along the direction perpendicular to the β-strands formed by the polar zipper, creating a fiber with its long axis perpendicular to the β-strands. This is consistent with X-ray fiber diffraction data[21]. Our model can also interpret electron microscopic results from the yeast prion Sup35. Fibers from the full-length Sup35 appear as a rigid, rod-like structure decorated by amorphous material along its sides, yet Sup35 without C-terminal domain forms fibers with only the rod-like structure[23]. The rod-like structure could be the β-sheet formed from polar zippers and the amorphous material could be the globular domains along both sides of the sheet in the 3D domain swapping zipper model. Our model suggests that domain swapping may help to stabilize the polar zipper during aggregation, which does not conflict with the observation that pure polyglutamine segments are sufficient to cause aggregation[24]. Considering that amyloid proteins may not consist of only the polar zipper segment *in vivo*, domain swapping may in fact contribute to the development of amyloid diseases.

In this 3D domain swapping zipper model, native proteins undergo the transition to amyloid in three steps: (i) thermal fluctuations break noncovalent interactions between two domains of a protein present at high concentration, exposing a hinge loop that favors formation of β-structure; (ii) the hinge loops from identical molecules stack into a β-sheet; and (iii) the sheet grows perpendicular to the direction of the β-strands. Portions of the molecules forming domain-swapped units on either side of the sheet pair are not necessarily ordered with respect to the sheet. The result is a protein fiber built from β-sheets with the β-strands running perpendicular to the fiber axis, stabilized at its edges by domain swapping.

Methods

The RNase A major dimer was formed and purified as described[5,6]. Diffraction quality crystals were obtained using the hanging-drop vapor diffusion method by mixing a 1:1 ratio of protein solution with reservoir solution. The protein solution contains 10 mg ml^{-1} of RNase A major dimer and 2 mM dCpdG in 0.1 M phosphate buffer (pH 6.5). The reservoir solution contains 0.1 M phosphate buffer (pH 6.5), 16% PEG 4000, 2% dioxane. X-ray diffraction data were collected on a home source at room temperature to 2.4 Å. The space group is C2, with 2 monomers per asymmetric unit. Then diffraction data to 1.7 Å were collected at -170 ºC at synchrotron beam line X8C at Brookhaven National Laboratory. Upon fast freezing, the space group of crystals was changed to $P2_1$, with 4 monomers per asymmetric unit. The structure was solved using the molecular replacement program AMoRe[25] from room temperature data, using a monomer[26] as the search model. The model from the room temperature data was then used as search model to solve the structure from cryo data. Alternating cycles of model building with the program O[27] and refinement with the program CNS[28] were used to determine the final structure. Continuous electron density was observed for all residues in the protein and dinucleotides.

Coordinates. The atomic Coordinates of the major dimer of RNase A have been submitted to the Protein Data Bank (accession code 1F0V).

Acknowledgments

We thank D. Cascio, O. Dym, D. Anderson and M. Sawaya for technical advice, I. Xenario, M. J. Bennett and G. Kleiger for useful discussion, and A. McPherson for generously providing coordinates of RNase A and $d(pA)_4$ complex. This work was supported by NSF, NIH, DOE, and Italian MURST-Prin 1999.

Correspondence should be addressed to D.E. *email: david@mbi.ucla.edu*

Received 27 October, 2000; accepted 20 December, 2000.

1. Bennett, M. J., Choe, S. & Eisenberg, D. S. *Proc. Natl. Acad. Sci. USA* **91**, 3127–3131 (1994).
2. Crestfield, A. M., Stein, W. H. & Moore, S. *Arch. Biochem. Biophys.* Suppl. **1**, 217–222 (1962).
3. Liu, Y., Hart, P. J., Schlunegger, M. P. & Eisenberg, D. S. *Proc. Natl. Acad. Sci. USA* **95**, 3437–3442 (1998).
4. Fruchter, R. C. & Crestfield, A. M. *J. Biol. Chem.* **240**, 3868–3874 (1965).
5. Gotte, G., Bertoldi, M. & Libonati, M. *Eur. J. Biochem.* **265**, 680–687 (1999).
6. Libonati, M., Bertoldi, M. & Sorrentino, S. *Biochem. J.* **318**, 287–290 (1996).
7. Lisgarten, J. N., Maes, D., Wyns, L., Aguilar, C. F. & Palmer, R. A. *Acta Crystallogr. D* **51**, 767–771 (1995).
8. Schreier, A. A. & Baldwin, R. L. *J. Mol. Biol.* **105**, 409–426 (1976).
9. Haber, E. & Anfinsen, C. B. *J. Biol. Chem.* **236**, 422–426 (1961).
10. Kato, I. & Anfinsen, C. B. *J. Biol. Chem.* **244**, 1004–1007 (1969).
11. Bennett, M. J., Schlunegger, M. P. & Eisenberg, D. S. *Protein Sci.* **4**, 2455–2468 (1995).
12. Klafki H. W. *et al. Biol. Chem. Hoppe-Seyler* **374**, 1117–1122 (1993).
13. Cohen, F. E. & Prusiner, S. B. *Annu. Rev. Biochem.* **67**, 793–819 (1998).
14. Schlunegger, M. P., Bennett, M. J. & Eisenberg, D. S. In *Advances in protein chemistry (*eds Richards, F. M. Eisenberg, D. S. & Kim P. S.) **50**, 61–122 (Academic Press, New York; 1997).
15. Chiti F. *et al. EMBO J.* **19**, 1441–1449 (2000).
16. Perutz, M. F., Johnson, T., Suzuki, M. & Finch, J. T. *Proc. Natl. Acad. Sci. USA* **91**, 5355–5358 (1994).
17. Perutz, M. F. *Trends Biochem. Sci.* **24**, 58–63 (1999).
18. Scherzinger E. *et al. Cell* **90**, 549–558 (1997).
19. Maddelein, M. L. & Wickner, R. B. *Mol. Cell. Biol.* **19**, 4516–4524 (1999).
20. Fink, A. L. *Folding & Design* **3**, R9–R23 (1998).
21. Sipe, J. D. *Crit. Rev. Clin. Lab. Sci.* **31**, 325–354 (1994).
22. Bunn, C. W. *Chemical crystallography.* 278–280 (The Clarendon Press, Oxford, UK; 1952).
23. Glover, J. R. *et al. Cell* **89**, 811–819 (1997).
24. Satyal, S. H. *et al. Proc. Natl. Acad. Sci. USA* **97**, 5750–5755 (2000).
25. Navaza, J. *Acta Crystallogr. A* **50**, 157–163 (1994).
26. Birdsall, D. L. & McPherson, A. *J. Biol. Chem.* **267**, 22230–22236 (1992).
27. Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. *Acta Crystallogr. A* **47**, 110–119 (1991).
28. Brunger A. T. *et al. Acta Crystallogr. D* **54**, 905–921 (1998).
29. Merritt, E. A. & Bacon, D. J. *Methods Enzymol.* **277**, 505–524 (1997).
30. Evans, S. E. *J. Mol. Graphics* **11**, 134–138 (1993).