The Acid-labile Subunit of the Serum Insulin-like Growth Factor-binding Protein Complexes

STRUCTURAL DETERMINATION BY MOLECULAR MODELING AND ELECTRON MICROSCOPY*

(Received for publication, February 8, 1999, and in revised form, April 27, 1999)

Jackie B. M. Janosi‡, Paul A. Ramsland §§ ¶, Margaret R. Mott †, Sue M. Firth ‡, Robert C. Baxter ‡, and Patric J. D. Delhanty ‡ **

From the ‡Kolling Institute of Medical Research, University of Sydney, Royal North Shore Hospital, St. Leonards, New South Wales 2065, the §University of Technology Sydney, Gore Hill, New South Wales 2065, and the †Key Center for Microscopy, University of Sydney, New South Wales 2006, Australia

The acid-labile subunit (ALS) is a glycosylated 85-kDa member of the leucine-rich repeat (LRR) protein superfamily and circulates in ternary complexes with the insulin-like growth factors (IGFs) and their binding proteins (IGFBPs). These complexes are thought to regulate the serum IGFs by restricting IGF movement out of the circulation. However, little is known about how ALS binds to IGFBP-3 or -5, which link the IGFs to ALS. To investigate potential sites of interaction, the ALS structure has been modeled with the crystal structure of the LRR protein porcine ribonuclease inhibitor as a template. ALS is predicted to be a donut-shaped molecule with an internal diameter of 1.7 nm, an external diameter of 7.2 nm, and a thickness of 3.6 nm. These dimensions are supported by shadowing electron microscopy of ALS. The internal face is lined with a substantial region of electronegative surface potential that could interact with the positively charged region on IGFBP-3 known to be involved in ALS binding. The model also predicts that three potential N-linked oligosaccharide sites within the LRR domain are clustered together, which may be important in light of recent studies showing ALS glycan involvement in complex formation with IGFBP-3.

The majority of serum insulin-like growth factors (IGFs)1 circulate within 130–150-kDa ternary complexes containing either IGF-I or -II, IGF-binding protein (IGFBP)-3, and the acid-labile subunit (ALS), an 85-kDa glycoprotein. It is thought that the size of these complexes prevents IGF access to target cells, while free IGFs and IGFs in binary complexes with the IGFBPs can easily cross the capillary endothelial barrier. Furthermore, the ALS-containing complex significantly increases the serum half-lives of both the IGFs and IGFBP-3 and in this way maintains a circulating store of these molecules (1, 2). Therefore, the association of ALS to the IGF complex is an important event in serum IGF regulation. ALS binding is the limiting step in complex formation, since the affinity of ALS for the IGF-IGFBP-3 complex is up to 2000-fold less than the affinity of IGFBP-3 for the IGFs in physiological salt concentrations, pH and temperature (3). Recently, Twigg and Baxter (4) showed that ALS and the IGFs can also form a ternary complex in vitro with IGFBP-5 (which has high homology to IGFBP-3 in the ALS binding domain) and that this IGFBP-5 complex is found in low concentrations in serum.

Although the structures of the IGFs have been solved (5, 6), the structures of the proteins which interact with and regulate the serum IGFs have not been elucidated. Therefore, it is not yet clear how ALS interacts physically with IGFBP-3 to form the ternary complexes. However, one major structural feature of ALS is that around 75% of its residues are ordered into tandem repeats consisting of 24 amino acids plus two partial repeats, all of which contain the consensus motif for the leucine-rich repeat (LRR) superfamily of proteins. (7). All the members of this superfamily are involved in protein-protein interactions (8). Significantly, in proteins containing multiple domains it is the LRR domain that forms contacts with the partner protein, as occurs with the glycoprotein hormone receptors (9–12). Therefore, it is likely that this domain of ALS is involved in binding to IGFBP-3.

The porcine ribonuclease inhibitor (RI) is the only LRR protein with a solved crystal structure (13). Overall, RI has an unusual, nonglobular horseshoe shape with the internal face exposed to solvent. Each repeat of RI contains a β-strand and an α-helix that alternate with each other when a number of repeats are linked. Consequently, the internal face of RI is lined with a parallel β-sheet lying perpendicular to the plane of the molecule and an external face that is mostly made up of α-helices and loops. The overall shape of RI is determined by the consensus leucines and other aliphatic residues, while the amino acids between these provide the specificity for interactions as demonstrated by the solved structure of RI bound to ribonuclease (14).

Although RI has 28 and 29 residues per repeat, which is more than that for the typical LRR protein, other groups have successfully used RI as a template for homology modeling LRR proteins (15–17). In the case of decorin, the shape of the predicted model was supported by electron microscopy which revealed images of a horseshoe-shaped molecule similar to RI (17, 18). Since serum ALS is difficult to purify above microgram quantities, we also used modeling and electron microscopy to identify regions of ALS that are potentially involved in ternary complex formation. Like many other LRR proteins, ALS has more repeats than RI; ALS has 18 perfect and 2 imperfect LRRs, while RI has only 15. Our model predicts that unlike the
open horseshoe structure of RI, or indeed any LRR protein modeled so far, the 5 extra repeats in ALS have closed the structure so that it more closely resembles a donut. Furthermore, charged regions on ALS were identified which may be involved in IGFBP-3 and -5 binding.

**EXPERIMENTAL PROCEDURES**

**Computational Equipment for Modeling—**All calculations and visualization were performed using an INDY workstation, R5000 CPU, running IRIX version 5.2 (Silicon Graphics Inc., Mountain View, CA). Structural alignments and model visualization were performed using TURBO-FRODO version 5 (Biographics, Marseilles, France). MODELLEUR4 (19) was used to construct all atom models using a sequence alignment and Protein Data Bank template file as inputs (see below). Molecular mechanics simulations were performed using X-PLOR version 3.1 (20) that we have used with or without 0.1% bovine serum albumin (radioimmunochemistry and geometry as assessed by the PROCHECK program (24). The optimized model fulfilled all the essential criteria for acceptable stereochemistry and geometry as assessed by the PROCHECK program (24).

**Rotary Shadowing and Electron Microscopy (EM) of ALS—**

**ALS Model**—The model of human ALS was generated based on the crystal structure of the porcine RI (13) (Protein Data Bank code: 2BNH). ALS contains 20 LRRs; 18 regular LRRs containing 24 residues and 2 irregular COOH-terminal LRRs containing 20 and 22 residues each. Assuming structural homology between the LRRs of RI and ALS a template containing 20 LRRs was constructed. The approach taken was to extend the 14 most structurally conserved LRRs of RI using rigid body alignments of duplicated sections of this molecule to produce an 18 LRR molecule. Numbering the LRRs of RI consecutively from the amino terminus, repeats 2 and 5 were used to extend the amino-terminal region and repeats 14 and 15 were used to extend the carboxy-terminal portion of RI. Two observations bolster our confidence in using this procedure for extending the RI template. First, rigid body alignments of two LRRs immediately after and before the NH₂- and COOH-terminal splice sites gave low root mean squares deviations of 0.80 and 0.59 Å, respectively. Second, the contacts between the LRRs at the splice junction and those of the original template showed that patterns of hydrogen bonding as well as van der Waals interactions were essentially maintained in the extended template (data not shown). Similar arguments were made by Kajava et al. (16) to validate their models of LRR proteins. Initially, the RI repeats 8 and 9 were used to model the two shorter irregular LRRs of ALS (ALS LRRs 19 and 20) in MODELLER. Subsequently, repeats 8 and 9 from RI were spliced onto the 18-RI RI template structure. This modified RI structure, now containing 20 RI LRRs, was used as a starting template for modeling the LRR domain of ALS. A sequence alignment was constructed for ALS and this 20-repeat RI template so that deletions occurred only within the random coils rather than within the helical or β-strand regions of the LRRs. This sequence alignment was used as input into MODELLER4 to construct the full atom model of the LRR domain of human ALS. MODELLER4 uses the methodology of satisfaction of spatial restraints where initial model geometry is optimized using restraints extracted directly from the template molecule (22). The model geometry was optimized using a conjugate-gradient energy minimization and molecular dynamics simulation (X-PLOR version 3.1) (20) that we have previously implemented for modeling antibody fragments (23). The optimized model fulfilled all the essential criteria for acceptable stereochemistry and geometry as assessed by the PROCHECK program (24).

**Model Structure of ALS—**ALS consists of three separate domains; an amino (NH₂)-terminal domain, a central domain containing the 20 LRRs (which constitutes 75% of ALS) and a carboxyl (COOH)-terminal domain. The terminal domains each contain a cysteine (Cys)-rich portion that flanks the LRR region (Fig. 1). The placement of the Cys residues in the Cys-rich domains resemble those found in the small LRR proteoglycans (SLRPs) of the extracellular matrix. A recent review (26) describes the SLRPs as being unique within the LRR family as they contain 4 similarly spaced Cys residues within a 20-amino acid stretch at the NH₂-terminal end and a further 2 at the COOH terminus. ALS also contains these although there are more than 2 Cys residues at the COOH terminus. The similarity between the SLRPs and ALS is further strengthened, since the ALS LRR consensus fits the LRR consensus of the SLRPs, X-X-I/V/L-X-X-X-X-F/P/I-X-X/I-P/X-X-L-X-X-X-L/I-X-L-X-N-X/I/L. Moreover, as with biglycan and decorin (the two best characterized SLRPs), ALS has a short sequence of highly charged residues which follows the signal peptide. However, unlike the SLRPs, ALS has not been shown to be a protein of the extracellular matrix. Since the Cys-rich regions of other proteins have not been crystallized or solved using NMR approaches, searches of the crystallographic Protein Data Bank (27) failed to locate sequences with similarity to either of the terminal domains of ALS. Because of this, the folds of the terminal domains of ALS could not be predicted and we concentrated our modeling efforts on the LRR domain of ALS.

**LRR Domain of Human ALS—**It has been postulated that there are two branches of the LRR superfamily (16). The majority of LRR proteins have 22–27 amino acids per repeat, such as ALS, and these have been described as the typical LRR protein. There is also a much smaller subset of proteins which have 28–30 residues per repeat and include RI. Therefore, although the ALS repeats have close homology to RI in terms of the placement of leucines and other well conserved residues, there is a difference in the repeat length. However, the structure of two ALS LRRs, which were modeled using two RI LRRs, suggests that they have similar conformation (data not shown). Therefore, the whole ALS LRR domain was modeled using the crystal structure of RI as the template.

Clearly, the most striking feature of the ALS model (Fig. 2A) is that the 20 LRR repeats form a torus or donut with LRR 1 aligning closely to LRR 20. A total of 98 potential atom-pair interactions, including 10 potential hydrogen bonds, stabilize the predicted close association of LRRs 1 and 20. This fold localizes the NH₂ and COOH termini (including the Cys-flanking region) in a precise position relative to the same residues of the LRR domain. The distance between the carbon atoms of the first residue in LRR 1 and the last residue in LRR 20 is 8.1 Å. The external diameter is predicted to be 7.2 ± 0.2 nm, and the diameter of the hole is 1.7 ± 0.2 nm. The thickness of the donut is 3.6 ± 0.2 nm. This model of ALS is the first description of a LRR protein that does not have the open horseshoe shape of RI. The closing of the horseshoe in ALS suggests that 20 LRRs are
probably the maximum number of repeats that can be accom- 
modated by a LRR protein such that the shape remains flat 
along the axis of the curve. In other words, LRR proteins such 
as chaoptin (28), which has 41 repeats, would need to twist, 
spiral, or perhaps straighten out if the general features of the 
alternating α- and β-strand per repeat are conserved.

The helices that form the crest of each LRR loop of RI are also 
predicted for most of the LRRs of ALS (Fig. 2A). However, to 
accommodate the lesser number of residues per repeat in ALS 
compared with RI, the number of residues adopting such con-
formations are reduced. Therefore, the α-helices of ALS contain 
less turns per helix than those of RI, and some have more open 
loop structures than found in RI. In most cases, this conversion 
from helix to random coil was associated with either the ex-
change of a glycine residue for a residue with a bulky side chain 
from helix to random coil was associated with either the ex-
change of a glycine residue for a residue with a bulky side chain 
by the introduction of a proline residue into the template 
repeat. The shorter LRRs 19 and 20 required larger deletions to 
their template repeats to maintain the helical portion of a 
typical LRR. These differences from RI are consistent with 
other models of typical LRR proteins (16). The parallel β-sheets 
lining the inside of the LRR domain are maintained in the ALS 
model. One exception was LRR 20 where the relevant polypep-
tide segment diverged only slightly from optimal geometry for 
a β-strand conformation. Kobe et al. (29) found that the β-sheet 
is the most highly conserved region within the LRR proteins. 
Others who have modeled typical LRR proteins on the RI 
crystal structure have also maintained the parallel β-sheet 
structure of RI (16).

Sites for Potential N-Linked Carbohydrates on ALS—As de-
picted in Fig. 1 there are seven potential N-linked attachment 
sites on ALS, four reside within the LRR domain and the 
remaining three are contained within the Cys-rich regions that 
flank the LRR domain. Fig. 2B depicts the carbohydrate an-
choring asparagines within the ALS LRR domain in green. One 
site (Asn441) is isolated and appears to jut straight into the hole 
of the donut. The other three sites within the LRR domain are 
clustered near the LRR 1 and LRR 20 junction. The three 
N-linked sugar sites, which are in the Cys-rich regions flanking 
the LRR domain, are also likely to be brought close to the 
carbohydrate. Therefore, up to six sites are potentially located in very 
close proximity to each other. Recently, we demonstrated that 
enzymatic removal of all of the N-linked sugars in ALS abol-
ished ternary complex formation although the loss of any single 
N-linked site could not prevent the complex from forming (30). 
This suggested that more than one glycan was involved. The 
clustering of the sugars in the model may, in part, explain this,
since it is likely that the loss of any single carbohydrate would 
be compensated by a number of other carbohydrates in the region.

We also showed that the enzymatic removal of negatively 
charged sialic acids on the N-linked sugars of ALS reduced the 
affinity of ALS for the IGF-IGFBP-3 binary complex by at least 
50% (30). From earlier studies, it had been demonstrated that 
polyanions, polycations, and high ionic strength all decrease 
the affinity of ALS for the IGF-IGFBP-3 complex, and therefore 
charge-charge interactions were expected to play a role (31). 
In support of this, Firth et al. (32) have since demonstrated that 
the affinity of IGFBP-3 for ALS is decreased 10-fold when basic 
residues in the carboxyl-terminal region of IGFBP-3 are re-
moved by mutagenesis. Therefore, negative charges on ALS are 
likely to be involved in the interaction and may explain why the 
loss of the negatively charged sialic acids affected ternary com-
plex formation. Since all of the sialic acids on ALS were deter-
mained to be contained on the N-linked sugars, the clustering of 
the N-linked sugars in one region, as seen in the model (Fig. 2B), 
suggests that a concentration of negative charge may form. 
It may be this charge that plays a part in the ionic interactions 
of the complex.

Electrostatic Surface of the Leucine-rich Repeat Domain—
Since the removal of the negatively charged sialic acids did not 
completely disrupt complex formation, we reasoned that the 
negatively charged regions on the protein backbone of ALS 
were also involved. Therefore we used the ALS model to iden-
tify potential binding sites. Fig. 2C depicts the electrostatic potentials at the molecular surface of the LRR domain of hu-
man ALS. Although the charged residues are relatively evenly 
distributed on the outer regions of the domain, the center hole 
of the donut is notably lined with a large region of electroneg-
ative surface depicted in red. The amino acids that contribute 
to this electronegatively charged face are shown in Table I. Since 
we have described a positively charged region on IG-
FBP-3 as being crucial for ternary complex formation (32), this 
negatively charged surface on ALS is a prime target for IG-
FBP-3 binding.

Rotary Shadowing EM—Fig. 3 shows images of purified hu-
man serum ALS in different orientations. The circular profiles 
in Fig. 3, A and B, and indicated by the arrows, are interpreted 
to be ALS molecules lying flat on the EM grid. Fig. 3C depicts 
what we infer to be two molecules of ALS viewed side-on 
demonstrating the nonspherical shape of ALS. Together, these 
images support the donut shaped model of ALS. Table II shows 
the dimensions of ALS predicted by the model compared with
AEM grid are illustrated, demonstrating the typically circular image of ALS. This electron micrograph also reveals the darkened, probably raised center, seen in many of the EM images of ALS. In C, the images are interpreted to represent two molecules of ALS lying side-on rather than flat against the EM grid. These molecules are not circular, demonstrating that ALS is not spherical but is more like a donut as is predicted by the model. In each panel the scale represents 1 nm.

**TABLE I**

List of charged residues found on the internal face of ALS

| LRR     | \(-ve\) | \(+ve\) |
|---------|---------|---------|
| 1       | Asp\(^{15}\) | Arg\(^{105}\) |
| 3       | Glu\(^{103}\) | Glu\(^{267}\) |
| 5       | Asp\(^{147}\) | Glu\(^{286}\) |
| 6       | Glu\(^{171}\) | Asp\(^{319}\) |
| 7       | Glu\(^{185}\) | Asp\(^{267}\) |
| 8       | Glu\(^{197}\) | Arg\(^{387}\) |
| 10      | Asp\(^{225}\) | Arg\(^{503}\) |
| 11      | Arg\(^{269}\) | Arg\(^{311}\) |
| 12      | Glu\(^{286}\) | Arg\(^{367}\) |
| 14      | Glu\(^{314}\) | Glu\(^{367}\) |
| 15      | Asp\(^{319}\) | Asp\(^{387}\) |
| 16      | Arg\(^{311}\) | Arg\(^{503}\) |
| 20      | 0.1     | 0.2     |
| 0.2     | Not visible in EM |

Fig. 3. Rotary shadowing electron micrographs of ALS. In A and B, images interpreted to be ALS molecules lying flat against the EM grid are illustrated. A depicts a cluster of three molecules of ALS highlighted by the three arrows. In B, a single molecule of ALS is shown demonstrating the typically circular image of ALS. This electron micrograph also reveals the darkened, probably raised center, seen in many of the EM images of ALS. In C, the images are interpreted to represent two molecules of ALS lying side-on rather than flat against the EM grid. These molecules are not circular, demonstrating that ALS is not spherical but is more like a donut as is predicted by the model. In each panel the scale represents 1 nm.

**TABLE II**

Comparison of dimensions predicted by the model compared with those calculated from the electron micrographs

For each dimension predicted by the ALS model, 10 measurements were taken as described under “Experimental Procedures,” and these were averaged. For the EM dimensions, 21 circular images were used to calculate the external diameter, while 18 images of ALS lying side-on were used to average the thickness of ALS. Results are given as mean ± S.E.

| Structure       | Model | EM |
|-----------------|-------|----|
| External diameter | 7.2 ± 0.2 | 7.2 ± 0.1 |
| Thickness       | 3.6 ± 0.2 | 3.9 ± 0.1 |
| Internal diameter | 1.7 ± 0.2 | Not visible in EM |

those measured from 39 profiles in four separate electron micrographs. The external diameters from both are the same and the thickness of the EM images is only marginally larger than the predicted thickness. However, the centers of the donuts in the EM images are not empty. Instead, many of the images contain a dark, metal-shadowed dot in the center, which may suggest that the hole is filled or obscured. There are at least two possible reasons for this and the first is suggested by the model depicted in Fig. 2B. One potential site of N-glycan attachment occurs at Asn\(^{341}\), which faces straight into the hole of the donut. Therefore, if the site were used, the sugar would fill at least part of the hole. Another explanation may be that the NH\(_2\)- and COOH-terminal domains may be extending over the hole of the donut.

In conclusion, our model of human ALS predicts a donut-shaped molecule with an external diameter of 7.2 nm, an internal diameter of 1.7 nm, and a thickness of 3.6 nm. This is schematically shown in Fig. 4. The model is based on the solved crystal structure of the ribonuclease inhibitor, and despite differences between the two proteins (including individual repeat size and the total number of repeats), the external dimensions of the ALS model are supported by physical data from rotary-shadowing EM. These data suggest that our model is a valid representation of serum ALS although crystallographic confirmation is required to establish a definitive structure. The predicted region of negative charges on the internal face of ALS may be involved in binding the electro-positive COOH-terminal domain of IGFBP-3, which has been shown previously to be essential for this interaction. Moreover, our model predicts that 20 is likely to be the largest number of LRR repeats in a molecule if the shape of the protein is to remain circular and flat.

**REFERENCES**

1. Guler, H. P., Zapf, J., Schmid, C., and Froesch, E. R. (1989) Acta Endocrinol. 121, 753–758
2. Lewitt, M. S., Saunders, H., and Baxter, R. C. (1995) Endocrinology 133, 1797–1802
3. Holman, S. R., and Baxter, R. C. (1996) Growth Regul. 6, 42–47
4. Twigg, S. M., and Baxter, R. C. (1998) J. Biol. Chem. 273, 6704–6709
5. Sato, A., Nishimura, S., Ohtsuki, T., Kyo, Y., Koyama, S., Kobayashi, M., Yasuda, T., and Kobayashi, Y. (1995) Int. J. Pept. Protein Res. 41, 435–440
6. Hua, Q. X., Narhi, L., Jin, W., Arakawa, T., Rosenfeld, H., Hawkins, N., Miller, J. A., and Weiss, M. A. (1996) J. Mol. Biol. 259, 297–313
7. Leong, R. B., Baxter, R. C., Camerato, T., Dai, J., and Wood, W. I. (1992) Mol. Endocrinol. 6, 870–876
8. Kobe, B., and Deisenhofer, J. (1995) Curr. Opin. Struct. Biol. 5, 409–416
9. Nagayama, Y., Wadsworth, H. L., Russo, D., Chazenbalk, G. D., and Rapoport, B. (1991) J. Clin. Invest. 88, 336–340
10. Nagayama, Y., Russo, D., Wadsworth, H. L., Chazenbalk, G. D., and Rapoport, B. (1991) J. Biol. Chem. 266, 14926–14930
11. Xie, Y. B., Wang, H., and Segaloff, D. L. (1996) J. Biol. Chem. 265, 21411–21414
12. Braun, T., Schofield, P. R., and Sprengel, R. (1991) EMBO J. 10, 1885–1890
13. Kobe, B., and Deisenhofer, J. (1993) Nature 366, 751–756
14. Kobe, B., and Deisenhofer, J. (1995) Nature 374, 183–186
15. Jiang, X., Dreano, M., Buckler, D. R., Cheng, S., Yihier, A., Wu, H., Hendrickson, W. A., and El Tayar, N. (1995) Structure ( Lond.) 3, 1341–1353
16. Kajava, A. V., Vassart, G., and Wodak, S. J. (1995) Structure ( Lond.) 3, 867–877
17. Weber, I. T., Harrison, R. W., and Iooss, R. V. (1996) J. Biol. Chem. 271, 31767–31770
18. Scott, J. E. (1996) Biochemistry 35, 8795–8799
19. Sali, A., and Blundell, T. L. (1993) J. Mol. Biol. 234, 779–815
20. Brünger, A. T. (1992) X-FLOR-A System for X-ray Crystallography and NMR, Version 3.1, Yale University Press, New Haven, CT
21. Nicholls, A. (1993) GRASP: Graphical Representation and Analysis of Surface Properties, Columbia University, New York
22. Sali, A. (1996) Mol. Med. Today 1, 270–277
23. Ramsland, P. A., Guddat, L. W., Edmundson, A. B., and Raison, R. L. (1997) 
   *J. Comp. Aided Mol. Des.* 11, 453–461
24. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) 
   *J. Appl. Crystallogr.* 26, 283–291
25. Baxter, R. C., Martin, J. L., and Beniac, V. A. (1989) *J. Biol. Chem.* 264, 
   11843–11848
26. Hocking, A. M., Shinomura, T., and McQuillan, D. J. (1998) *Matrix Biol.* 17, 
   1–19
27. Abola, E. E., Bernstein, F. C., Bryant, S. H., Koetzle, T. F., and Weng, J. (1987) 
   in *Crystallographic Databases-Information Content, Software Systems, 
   Scientific Applications* (Bergerhoff, A. G., and Sievers, R., eds) pp. 107–132, 
   Data Commission of the International Union of Crystallography, Bonn
28. Krantz, D. E., and Zipursky, S. L. (1990) *EMBO J.* 9, 1969–1977
29. Kobe, B., and Deisenhofer, J. (1994) *Trends Biochem. Sci.* 19, 415–421
30. Janosi, J. B. M., Firth, S. M., Bond, J. J., Baxter, R. C., and Delhanty, P. J. D. 
   (1999) *J. Biol. Chem.* 274, 5292–5298
31. Baxter, R. C. (1990) *Biochem. J.* 271, 773–777
32. Firth, S. M., Ganeshprasad, U., and Baxter, R. C. (1998) *J. Biol. Chem.* 273, 
   2631–2638
33. Koradi, R., Billeter, M., and Wuthrich, K. (1996) *J. Mol. Graphics* 14, 51–55