Molecular conformation of ubiquitinated structures and the implications for regulatory function

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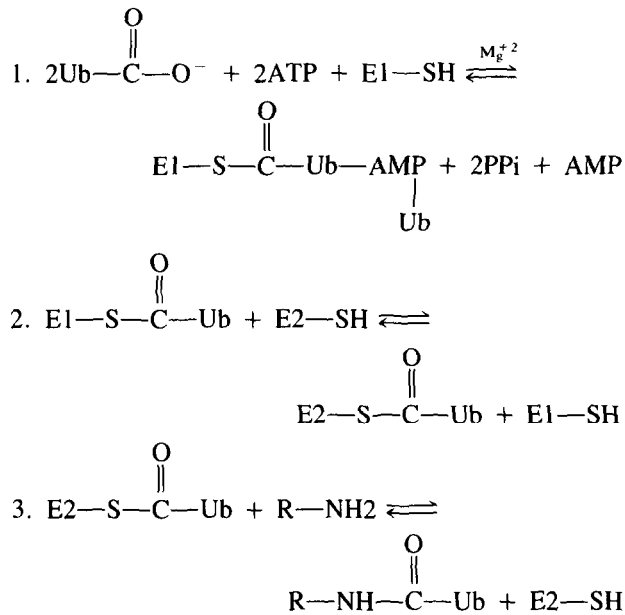
The molecular conformation of ubiquitinated structures and the validity of the N-end rule were examined by simulating the molecular mechanics to ascertain the global energy-minimized structure. We examined the chemical linkage involved in attaching the ubiquitin carboxyl terminus to the N-terminus of three different x*-hexapeptides, where* x *is the amino group of the acceptor peptide—either valine, arginine or glutamic acid—(*x-K *linkage) and to the* ϵ*-amino group of lysine of the acceptor hexapeptide* x-glu_1-his_2-lys_3-gly_4-lys_5-val_6 *(*K-K *linkage) through the formation of an isopeptide bond. Changes in conformation and molecular stability of the multi-ubiquitinated structures were determined by energy-minimization procedures using the SYBYL program developed by Tripos Associates. In the* x-K *linkage, the ubiquitin molecule is stretched in the* β*-pleated sheets and* β*-turns while the* α*-helices expand, as the molecule continues to unfold linearly. In the* K-K *linkage, the ubiquitin molecules have turned into a u-shaped, semicircular alignment, contracting into a compact, folded structure.*

Keywords: ubiquitinated structures

Ubiquitin (Ub) is a highly conserved protein found in all eukaryotes. It has a single-domain, globular structure made up of 5 strands of β-pleated sheets, $3\frac{1}{2}$ turns of an α-helix, a short piece of 3_{10}-helix, and 7 reverse turns. The central core is highly hydrophobic.[1,2] The bulge (unusual G1 β-bulge[3]) is required for proper orientation of the middle strand of the 5-stranded β-sheet. Since β-bulges tend to occur in critical positions at the active or binding sites of a protein,[4] it is possible that this unusual secondary structural feature is important in the interaction of Ub with acceptor proteins.[1]

The best known function of Ub is that it marks protein committed to rapid degradation by an ATP-dependent proteolytic pathway.[5–8] Protein breakdown in this pathway requires the formation of covalent conjugates in which carboxyl terminals of Ub molecules (leu_{73}-arg_{74}-gly_{75}-gly_{76}) extend from the compact structure to form a tail that is enzymatically attached to the acceptor proteins, including a variety of cytoplasmic, nuclear, and integral membrane proteins.[9–31]



Herschko *et al.*[7] reported the isolation of three enzymes required for the ATP-dependent activation of Ub-activating enzyme (Mr, 200,000 KDa)[32] or E1;[33–36] $E2_s$, for Ub-transthiolation to a small carrier protein (Mr, 25,000 KDa)[8] or E2;[7,36] E3, which catalyzes transfer of Ub from the E2 adduct to an ϵ-amino group of the target protein, Ub-protein ligase (Mr, 250,000 KDa) or E3.[7,32,37]

Two distinct types of Ub protein conjugates have been identified. One type contains the Ub carboxyl terminus joined via an isopeptide bond to the N-terminus of the α-amino group of the acceptor protein.[38–40] The second type of conjugate is formed by ubiquitination of the ϵ-amino groups of protein lysine residues.[5,7,40]

Proteins with a basic or bulky-hydrophobic residue in the amino-terminal position are recognized by the ligase, marked by Ub, and degraded. This is not true, however, for proteins with an acidic residue in the same position. Ciechanover[41] suggested that tRNA is required for the conjugation of Ub to acidic amino-terminal substrates.

Color Plates for this article are on pages 18–20.

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Bachmair *et al.*[39] proposed that the protein's metabolic stability depends on the nature of its acceptor's amino-terminal residues, conferring short halflives on the corresponding *x*-acceptor protein. (The code or rule that relates the metabolic stability of the protein to the nature of its amino-terminal residues has been called the "N-end rule."[40]) The halflife of arginine upon ubiquitination to β-galactosidase (β-gal) is reported to be 2 minutes; that of glutamic acid, 30 minutes; and that of valine, less than 20 hours.[40]

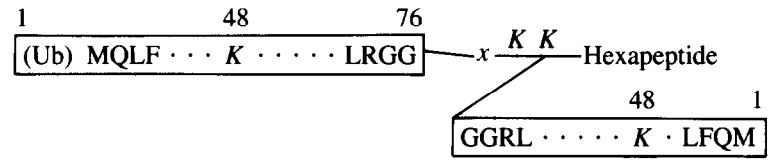
To test the applicability of the N-end rule of molecular stability to energy minimization procedures, we have examined the chemical linkage involved in the attachment of the Ub carboxyl terminus to two model systems through the formation of an isopeptide bond.

CHEMICAL LINKAGE MODELS

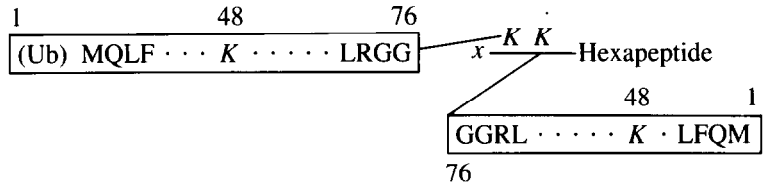
(1) The *X-K* linkage model (C-terminal glycine-Nα-amide bond), where attachment is through the N-terminus of one of three hexapeptides:

X-glu$_1$-his$_2$-lys$_3$-gly$_4$-lys$_5$-val$_6$
X-val$_1$-his$_2$-lys$_3$-gly$_4$-lys$_5$-val$_6$
X-arg$_1$-his$_2$-lys$_3$-gly$_4$-lys$_5$-val$_6$

X_1 is the α-amino group of the acceptor peptide containing two lysine groups, lys$_3$ and lys$_5$. The C-terminal of another Ub is conjugated with lys$_5$ of the hexapeptide.



(2) The *K-K* linkage model (C-terminal glycine-Nϵ-amide bond), where the C-terminus of Ub is attached directly to the ϵ-amino group of lys$_3$ of X-glu$_1$-his$_2$-lys$_3$-gly$_4$-lys$_5$-val$_6$. The C-terminal of another Ub is conjugated with lys$_5$ of the hexapeptide. In both model systems, upon multi-ubiquitination, the Ub C-terminus is covalently attached to lys$_{48}$ (*K48*) of Ub.



ENERGY MINIMIZATION PROCEDURE

The changes in conformation and molecular stability of the multi-ubiquitinated structures were examined by simulating the molecular mechanics using the SYBYL 5.32 program developed by Tripos Associates (St. Louis) with the Silicon Graphics 4D/210 GTX graphics module. The Ub structure used in this work was Brookhaven Protein Data Bank entry 2UbQ.Ent.[1]

The CRYSIN function allows the entry of crystallographic data into the SYBYL environment and permits the normal fractional-to-Cartesian and Cartesian-to-fractional coordinate conversions, symmetry operations, connectivity scans, and expansions of molecules to form surfaces or arrays in space.

The energy is a function of the atomic coordinates and the program attempts to generate the coordinates that correspond to a minimum of energy. This is accompanied by a minimization procedure—an iterative method in which the atomic coordinates are modified from one iteration to the next to decrease energy.

Given a surface potential function, it is desirable to find the minimum energy configuration of a system. In some cases, minimization is performed to relieve strain in conformations obtained experimentally or by the averaging of several structures. In other cases, finding a local or global energy minimum may be of prime interest, e.g., for determining the configuration of a peptide. For macromolecular systems, the number of local minima and the cost of computations prevent an exhaustive search of the energy surface, so it is frequently necessary to determine the global energy minimum in the neighborhood of X-ray diffraction structures. The first and second derivatives of the energy function are used to minimize the energy of the protein or to find its response to a perturbation.

The energy E of the molecule in the force field arises from deviations from "ideal" structural features, and can be approximated by a sum of energy contributions.

$$E = \Sigma E_{str} + \Sigma E_{bend} + \Sigma E_{oop} + \Sigma E_{tors} + \Sigma E_{vdw} + \Sigma E_{ele} + \Sigma E_{dis+c} + \Sigma E_{ang-c} + \Sigma E_{tor-c} + \Sigma E_{range-c}$$

where the sums extend over all bonds, bond angles, torsion

angles, and nonbonded interactions between atoms not bound to each other or to a common atom (i.e., 1,4-interaction and higher).

The bond-stretching, angle-bending torsional, 6–12 Lennard-Jones potential parameters[42] and out-of-plane bending terms for the Tripos 5.32 force field were described by Clark *et al.*[43] The Gasteiger and Marsili method[42,44] was used to calculate the σ and π charges. Here, the total σ charge of an atom after the kth iteration is equal to the sum charge of increments Q from all bonds, including this atom, and the value of the charge of previous iterations.

The standard SYBYL energy minimizer, MAXIMIN2, was used under the following conditions: If the force of an atom exceeded 5 millidynes, an atom-by-atom Simplex minimization was performed until all forces fell below this threshold. After this threshold, conjugate-gradient minimization proceeded until the convergence criterion of a root-mean-square gradient over all atoms of less than 0.1 kcal/mole Å was reached. The hexapeptide structures used in this work were built from the literature as a β-sheet. For these structures the crystallographic neighbors were generated using the crystallographic functions of SYBYL so that the reference peptide was completely surrounded by a lattice including all molecules with atoms within 6 Å of any atom in the reference molecule.[44] The average errors for the Phi and Psi angles using the MAXIMIN2 force field are -1.5 and 1.3°, respectively. The removal of steric hindrance using energy minimization procedures resulted in a structure remarkably similar to the β-pleated sheet. The final delta energy was approximately -118.6 kcal/mole and the acceptor hexapeptide was free from van der Waals overlappings.

RESULTS AND DISCUSSION

The *X-K* linkage model, in which the C-terminal end of Ub is attached to the α-amino group of the acceptor peptide, ubiquitinated on the lys5 (K5) position of the acceptor molecule, is shown as a ribbon model, before and after energy minimization (Color Plate 1). Before energy minimization, the hexapeptide is on a nonlinear plane with the two Ub molecules aligned at a 90° angle,[1,2] while the alignment of the Ub molecules is distinctly linear after energy minimization, resulting in conformational alteration in the β-pleated sheets and distortion of the β-turns. The alignment of the α-helices of the second molecule is nearly parallel to the linear plane. As is apparent from Color Plate 2, it is impossible to distinguish in the ribbon model, any conformational differences between *X*-arg$_1$-hexapeptides, after energy minimization. Both show a distinctly linear alignment. In Color Plate 3A, the molecules in the lefthand photograph have been scanned to relieve steric interaction and it is assumed that there is no van der Waals contact between bonds. The structure of this ribbon model already shows deformation, with only the third molecule of the group showing a resemblance to the original molecule. After energy minimization, the Ub is further stretched in the β-pleated sheets and β-turns while the α-helices expand, as the molecules continue to unfold linearly. At left in Color Plate 3B, the molecules are seen before scanning and before energy minimization. After energy minimization, the α-helices and the β-sheets are deformed, but when only two Ub molecules are attached at the *K5* position the stretching is not as pronounced, as is seen in Color Plate 3A.

The effects of multi-ubiquitination through the internal lys$_{48}$ (K48) of two adjacent Ub molecules conjugated with an *X*-hexapeptide (*X-K*) linkage to which four additional Ub molecules are attached through the *K3* linkage, before and after energy minimization, may be seen in Color Plate 4. After energy minimization, the α-helices and β-sheets of the four Ub molecules are expanded. While the original pair of Ub molecules shows expansion only in the β-pleated sheets and β-turns, the α-helices appear to be unaffected. On the whole, the formation resembles a linear string of beads, much as is seen in Color Plate 3.

To destabilize the ubiquitin-*X*-hexapeptide conjugates through the *X-K* linkage, it appears that the most efficient means is through the attachment of additional Ub to the lysine group of the acceptor molecule, in this case the 3- or 5-position of a hexapeptide. The more ubiquitinated the structure, the more elongated and unfolded it will become. Chau *et al.*[45] postulated that multi-ubiquitination through internal lys 48 of an adjacent Ub in β-galactosidase (through lys 15 or lys 17 within the amino-terminus of β-gal) is essential for the degradation of that protein. By our energy minimization procedure, for the β-galactosidase to be conformationally unstable, the *X*-β-gal must be conjugated with the C-terminus of Ub, followed by internal multi-ubiquitination through lys 15 or lys 17. The *K-K* linkage model, in which attachment of two Ub molecules is through the ϵ-amino group of the two lysines of the hexapeptide, is shown as a ribbon model before and after energy minimization (Color Plate 5). The two Ub molecules are aligned at a 135° turn from one another before energy minimization, with evidence of expanded α-helices (Color Plate 5A). In the energy-minimized form, the two molecules have assumed a u-shaped, semicircular alignment with contraction of the helical segments, which are nearly parallel (Color Plate 5B). Such an alignment appears to be typical of the *K-K* linkage, while the *X-K* linkage results in a linear alignment after energy minimization.

As more Ub is attached through either side of the *K-K* linkage, the molecule becomes increasingly compact, even before energy minimization. The molecule tends to spread into a more relaxed form upon energy minimization, but still retains its compact form (Color Plate 6A). We next examined the structural changes in the multi-ubiquitinated form upon energy minimization when no acceptor molecule is present. We still observe a compact, fairly circular structure (Color Plate 6B).

Our survey of global energy-minimized structures indicates that the *K-K* linkage results in a compact structure that is somewhat circular in form, which would probably be highly resistant to proteolysis. The energy-minimized form from the *X-K* linkage is a linear structure that is more likely to be subject to enzyme degradation.

These studies on multi-ubiquitination have demonstrated that energy minimization is an effective technique for simulating protein unfolding, provided that the protein in question is conjugated by an *X-K* linkage to an anchoring polypeptide. The linear stretching observed upon energy minimization is an indicator of the change in a particular

protein's conformation upon unfolding. When the protein is conjugated by a *K-K* linkage, it is possible to examine the compaction characteristic of the folding process.

In the nucleus, Ub is conjugated to the ϵ-amino group of lys 119 in histone H2A and lys 120 in H2B.[46,47] This conjugate is not degraded and is thought to play a role in regulating both chromatin structure and gene expression,[48,49] in accordance with our *K-K* model of ubiquitination.

It should also be noted that studies on Alzheimer's disease have shown that the ubiquitinated paired helical filaments, which are the principal components of the neurofibrillary tangles associated with the disease, are also not degraded, but instead accumulate as large perikaryol masses.[50,51] The nature of these paired helical filaments has been closely examined because of a possible correlation between their concentration in the brain and degree of dementia experienced by the Alzheimer's patient.[51]

Mori *et al.*[52] suggest that the failure of paired helical filaments (PHF) to be degraded upon ubiquitination could be due to a defective ATP-dependent protease or to an unusual resistance of PHF to the protease.[53]

Our energy minimization studies suggest that it is the nature of the chemical linkage between Ub and an element of the acceptor molecule which may be responsible for the failure of the conjugate to be degraded. We hypothesize that the isopeptide linkage between Ub and the microtubule-associated phosphoprotein τ, identified as the major antigenic determinant of PHF, follows the *K-K* model, so that the Ub conjugate no longer degrades readily.

ABBREVIATIONS

The equations of the Tripos force field consist of harmonic bond-stretching and angle-bending terms. The out-of-plane bending terms depend harmonically on the distance of the central atom from the plane defined by its three attached neighbors, and the torsional function consists of a single cosine term. The bond-stretching and torsional parameters used depend on the bond type to correctly represent both double and single bonds between Sp^2-hybridized atoms. Since the electrostatic term is extremely large and negative when the interatomic distance is very small, a linear extrapolation is used at a cutoff of 0.5 Å to prevent the energy from causing a numeric overflow. No explicit hydrogen bond terms are included. Hydrogen attached to atom types designated as hydrogen-bond donors are given a radius of 0 in the 6–12 nonbonded term for their interaction with atom types designated as hydrogen-bond acceptors. The terms of the force field equation are defined as follows:

E_{dist-c} Energy associated with distance constraints
E_{ang-c} Energy associated with angle constraints
E_{tor-c} Energy associated with torsion angle constraints
$E_{range-c}$ Energy associated with range constraints
E_{str} Energy of a bond stretched or compressed from its natural bond length
E_{bend} Energy of bending bond angles from their natural values
E_{oop} Energy of bending planar atoms out of the plane
E_{tor} Torsional energy due to twisting about bonds
E_{vdw} Energy due to van der Waals nonbonded interactions
E_{ele} Energy due to electrostatic interactions

MAXIMIN2 in the SYBYL 5.32 uses a combination of first-derivative and nonderivative methods. The Simplex method,[54] a nonderivative based procedure, is used on an atom-by-atom basis until the maximum force on any atom is below some specified value. In highly distorted structures the potential energy surface and its derivatives are often discontinuous. Simplex can handle these areas while derivative-based procedure cannot. MAXIMIN2 provides a quasi-Newton procedure called BFGS.[54] This procedure approximates the inverse of the Hessian matrix by accumulating information from the first derivatives at each iteration.

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REFERENCES

1 Vijay-Kumar, S., Bugg, C.E. and Cook, W.J. *J. Mol. Biol.* (1987) **194**, 531–544
2 Vijay-Kumar, S., Bugg, C.E., Wilkinson, K.D. and Cook, W.J. *Proc. Natl. Acad. Sci. U.S.A.* (1985) **82**, 3582–3585
3 Richardson, J.S. *Adv. Protein Chem.* (1981) **34**, 167–339
4 Richardson, J.S., Getzoff, E.D. and Richardson, D.C. *Proc. Natl. Acad. Sci. U.S.A.* (1978) **75**, 2574–2578
5 Hershko, A., Ciechanover, A., Heller, H., Haas, A.L. and Rose, I.A. *Proc. Natl. Acad. Sci. U.S.A.* (1980) **77**, 1783–1786
6 Hershko, A., Eytan, E., Ciechanover, A. and Haas, A.L. *J. Biol. Chem.* (1982) **257**, 13 964–13 970
7 Hershko, A., Heller, H., Elias, S. and Ciechanover, A. *J. Biol. Chem.* (1983) **258**, 8206–8214
8 Pickart, C.M. and Rose, I.A. *J. Biol. Chem.* (1985) **260**, 7903–7910
9 Bond, U. and Schlesinger, M.J. *Mol. Cell. Biol.* (1985) **5**, 949–953
10 Dworkin-Rastl, E., Shrutkowski, A. and Dworkin, M.B. *Cell* (1984) **39**, 321–325
11 Finley, D., Ciechanover, A. and Varshavsky, A. *Cell* (1984) **37**, 43–55
12 Finley, D. and Varshavsky, A. *Trends Biochem. Sci.* (1985) **10**, 343–346
13 Ciechanover, A., Finley, D. and Varshavsky, A. *Cell* (1984) **37**, 57–66
14 Busch, H. and Goldknopf, I.L. *Mol. Cell. Biochem.* (1981) **40**, 173–187
15 Levinger, L. and Varshavsky, A. *Cell* (1982) **28**, 375–385

16 Olson, M.O. Jr., Goldknopf, I.L., Guetzow, K.A., James, G.T., Hawkins, T.C., Mays-Rothberg, C.J. and Busch, H. *J. Biol. Chem.* (1976) **251**, 5901–5903
17 Hunt, L.T. and Dayhoff, M.D. *Biochem. Biophys. Res. Commun.* (1977) **74**, 650–655
18 Rechsteiner, M. *Ann. Rev. Cell Biol.* (1987) **3**, 1–30
19 Wilkinson, K.D. *Anticancer Drug Design* (1987) **2**, 211
20 St. John, T., Gallastin, W.M., Siegelman, M., Smith, H.T., Fried, V.A. and Weissman, I.L. *Science* (1986) **231**, 845–849
21 Bonner, W.M., Hatch, C.L. and Wu, R.S. Ubiquitinated histones and chromatin. In: *Ubiquitin* (Martin and Rechsteiner, Eds.) Plenum, New York, 1988, 157–168
22 Siegleman, M. and Weissman, I.L. Lymphocyte homing receptors, ubiquitin and cell surface proteins. In: *Ubiquitin* (Martin and Rechsteiner, Eds.) Plenum, New York, 1988, 239–263
23 Yarden, Y., Escobedo, J.A., Kuang, W.-J., Yang-Feng, T.L., Daniel, T.O., Tremble, P.M., Chen, E.Y., Ando, M.E., Harkins, R.N., Francke, U.L., Fried, V.A., Ullrich, A. and Williams, L.T. *Nature* (1986) **323**, 226–232
24 Finley, D., Bartel, B. and Varshavsky, A. *Nature* (1989) **338**, 394–401
25 Hershko, A. and Ciechanover, A. *Prog. Nucleic Acids Res. Mol.* (1986) **33**, 19–56
26 Wilkinson, K.D., Cox, M.J., Mayer, A.N. and Frey, T. *Biochemistry* (1986) **25**, 6644–6649
27 Siegelman, M., Bond, M.W., Gallatin, W.M., St. John, T., Smith, H.T., Fried, V.A. and Weissman, I.L. *Science* (1980) **231**, 823–829
28 Hershko, A., Heller, H., Eytan, E., Kakly, G. and Rose, I.A. *Proc. Natl. Acad. Sci. U.S.A.* (1984) **81**, 7021–7025
29 Wilkinson, K.D., Lee, K.M., Des Ande, S., Deurksen-Hughes, P., Boss, J.M. and Pohl, B. *Science* (1989) **246**, 670–673
30 Özkaynak, E., Finley, D. and Varshavsky, A. *Nature* (London) (1984) **312**, 663–666
31 Wilborg, O., Pedersen, M.S., Wind, A., Berglund, L.E., Marker, K.A. and Vuust, J. *EMBO. J.* (1985) **4**, 755
32 Ciechanover, A., Elias, S., Heller, H. and Hershko, A. *J. Biol. Chem.* (1982) **257**, 2537–2542
33 Ciechanover, A., Heller, H., Katz-Etzion, R. and Hershko, A. *Proc. Natl. Acad. Sci. U.S.A.* (1981) **78**, 761–765
34 Haas, A.L. and Rose, I.A. *J. Biol. Chem.* (1982) **257**, 10 329–10 337
35 Haas, A.L., Warms, J.B.V., Hershko, A. and Rose, I.A. *Biochemistry* (1983) **22**, 4388–4394
36 Haas, A.L., Warms, J.B.V., Hershko, A. and Rose, I.A *J. Biol. Chem.* (1982) **257**, 2543–2548
37 Hershko, H., Heller, H., Eytan, E. and Reiss, Y. *J. Biol. Chem.* (1986) **261**, 11 992–11 999
38 Ciechanover, A., Heller, H., Elias, S., Haas, A.L. and Hershko, A. *Proc. Natl. Acad. Sci. U.S.A.* (1980) **77**, 1365–1368
39 Bachmair, A., Finley, D. and Varshavsky, A. *Science* (1986) **234**, 179–186
40 Gonda, D.K., Bachmair, A., Wunning, J., Tobias, J.W., Lane, W.S. and Varshavsky, A. *J. Biol. Chem.* (1989) **264**, 16 700–16 712
41 Ciechanover, A. In: *Current Communications in Molecular Biology* The ubiquitin system. (M. Schlesinger and A. Hershko, Eds.) Cold Spring Harbor Monographs, Cold Spring Harbor, NY, 1988, 84–90
42 Gasteiger, J. and Marsili, M. *Tetrahedron* (1980) **36**, 3219–3228; Gasteiger, J., Saller, H. and Low, P. *Anal. Chim. Acta* (1986) **191**, 111–123; Gasteiger, J., Röse, P. and Saller, H. *J. Mol. Graphics* (1988) **6**, 87–92
43 Clark, M., Cramer III, R.D. and Van Opdenbosch, N. *J. Comp. Chem.* (1989) **10**, 982–1012
44 *Sybyl Molecular Modeling Software V5.32 Theory Manual.* Tripos Associates, St. Louis, MO, USA, 1989
45 Chau, V., Tobias, J.W., Bachmair, A., Marriott, D., Ecker, D.J., Gonda, D.K. and Varshavsky, A. *Science* (1989) **243**, 1579–1583
46 Goldknopf, I.L. and Busch, H. *Proc. Natl. Acad. Sci. U.S.A.* (1977) **74**, 864–868
47 Thorne, A.W., Sautiere, P., Briand, G. and Crange-Robbinson, C. *EMBO. J.* (1987) **6**, 1005–1010
48 Matsui, S., Seon, B.K. and Sandberg, A.A. *Proc. Natl. Acad. Sci. U.S.A.* (1979) **76**, 6386–6390
49 Levinger, L. and Varshavsky, A. *Cell* (1982) **28**, 375–386
50 Kidd, M. *Nature* (London) (1963) **197**, 192; Terry, R.D., Gonatas, N.R. and Weiss, M. *Am. J. Pathol.* (1964) **44**, 269–281; Wisniewski, H., Narang, H.K. and Terry, R.D. *J. Neurol. Sci.* (1976) **27**, 173–181
51 Thomlinson, B.E., Blessed, G. and Roth, M. *J. Neurol. Sci.* (1970) **11**, 205–242; Wilcock, G.K. and Esiri, M.M. *J. Neurol. Sci.* (1982) **56**, 343–356
52 Mori, H., Kondo, J. and Ihara, Y. *Science* (1987) **235**, 1641–1644
53 Selkoe, D.J., Ihara, Y., Abraham, C., Roscool, C.G. and McCluskey, A.H. *Banbury Rep.* (1983) **15**, 125–144
54 Press, W.H., Flannery, B.P., Teukolsky, S.A. and Vetterling, W.T. *Numerical Recipes, The Art of Scientific Computing.* Cambridge Univ. Press, London, 1986, 307–312; Simplex, 312–327