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Crystals of the β-subunit of bovine luteinizing hormone and indicators for the involvement of proteolysis in protein crystallization

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The β-subunit of luteinizing hormone (LH), the subunit responsible for the physiological response, has been crystallized beginning with the intact $\alpha\beta$-heterodimeric hormone purified from bovine pituitary glands. The crystals were grown at 310 K in the presence of neutral detergents along with trypsin. The tetragonal bipyramidal crystals diffract to 3 Å resolution and belong to space group $I4_122$, with unit-cell parameters $a = b = 57$, $c = 207$ Å. It is noted that proteins exposed to proteases sometimes yield products that crystallize better than the native molecule and that the β-subunit of LH represents yet another example. Some indicators of when proteolysis may be a factor in crystallization, as well as some consequences, are described.

1. Introduction

Lutropin (luteinizing hormone; LH) is a pituitary glycoprotein hormone that promotes spermatogenesis and ovulation by stimulating the testes and ovaries to synthesize steroids. LH levels in the blood and urine are frequently used for fertility analyses to determine the timing of ovulation to improve chances of conception or during *in vitro* fertilization to determine when eggs are mature and ready for surgical removal from the ovary.

LH is a member of a family of glycoprotein hormones that include two other pituitary hormones, follitropin (follicle-stimulating hormone; FSH) and thyrotropin (thyroid-stimulating hormone; TSH), as well as a placental hormone, chorionic gonadotropin (CG). These hormones are structurally related and consist of two subunits, α and β, which associate noncovalently to form a disulfide-rich heterodimer. Within a species, all glycoprotein hormones share a common α-subunit, while a unique β-subunit confers biological specificity to LH, FSH, TSH and CG. The four β-chains are homologous, with the highest homology being between LH and CG (85%). The glycoprotein hormones function by binding G-protein-linked cell-surface receptors, ultimately stimulating adenylyl cyclase. Indicative of their high homology, LH and CG proteins bind the same receptor, LHCGR, whereas FSH and TSH bind to similar but distinct receptors.

Crystal structures of CG and FSH have revealed that each of the two subunits, α and β, have a similar fold consisting of a core all-β cystine-knot motif from which three elongated hairpin loops extend (Baenzinger, 1994; Lapthorn *et al.*, 1994; Wu *et al.*, 1994; Fox *et al.*, 2001). The cystine-knot motif, which contains three disulfide bridges is known from some protein growth factors. Thus, both subunits of glycoprotein hormones are members of a structural superfamily termed cystine-knot

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cytokines. A total of five disulfide bridges exist in the α-subunit and six in the β-subunit and these are conserved amongst the glycoprotein hormone family. The α/β-heterodimer interaction is noncovalently stabilized by the COOH-terminal polypeptide of the β-subunit (amino acids 91–110), which encircles the α-subunit and links to the core of the β-subunit by forming the disulfide Cys26–Cys110. Residues following Cys110 of the β-subunit comprise a COOH-terminal 'tail' which varies in length for the β-chains of the different pituitary hormones. CG has a notably long carboxy tail of 35 residues, Asp111–Gln145, and in the crystal structure of CG these were disordered, indicating mobility. The α- and β-subunits of bovine luteinizing hormone, the subject of this study, contain 96 and 121 residues, respectively; the β-subunit has an 11-residue carboxy tail.

Bovine LH α-subunit has two N-linked glycosylation sites (Asn56, Asn82) and an O-linked site at Thr43, while the LH β-subunit has a single N-linked glycosylation site at Asn13 (Baenzinger, 1994). Human CG and FSH β-subunits are more highly glycosylated and crystallization required partial deglycosylation of the α/β-dimers by either hydrogen fluoride (HF) treatment, neuraminidase treatment or site-directed mutagenesis (Lapthorn *et al.*, 1994; Wu *et al.*, 1994; Fox *et al.*, 2001). A lower resolution structure of fully glycosylated hCG with two Fv fragments has been determined (Tegoni *et al.*, 1999).

2. Materials and methods

HPLC-purified LH and both the HPLC-purified α- and β-subunits of LH were given to us by Professor John Pierce of the UCLA School of Medicine upon his retirement. The proteins were purified from pooled bovine pituitary glands and had been stored at 193 K for approximately 20–25 y.

Before crystallization, the proteins were analyzed on SDS–PAGE under both reducing (Laemmli, 1970) and non-reducing conditions (Weber & Osborne, 1975). The protein and its subunits were approximately 85% homogeneous, with the remaining 15% of contaminants divided between several bands. Most of the contaminants were of higher molecular weight and judging from their sizes some may well have been covalently linked oligomers of LH. Only a very small amount of hydrolysis was observed as indicated by bands lower than those of the independent subunits. Crystallization experiments were carried out using sitting-drop vapour-diffusion in Cryschem plastic plates (Hampton Research, Laguna Niguel, CA, USA) under conditions described in §3. Sample drops were generally 6–8 µl, with reservoirs of 0.6 ml.

X-ray diffraction data were collected from crystals at room temperature that were conventionally mounted (King, 1954; Rayment, 1985) in quartz capillaries. Successful cryoconditions have not, to this point, been identified for these crystals grown from intact LH, in spite of the malonate mother liquor which usually provides cryoprotection (McPherson, 2001). Two X-ray systems were used for preliminary analysis and for data collection. The first was an Rigaku RU-200 generator fitted with an R-AXIS detector and Osmic mirrors (Rigaku USA, The Woodlands, TX, USA). It was run at 50 kV and 100 mA and data were collected with exposure times of 2–4 min and oscillation angles of 1.5 or 2.0°. The second system was beamline 5.0.1 at the Advanced Light Source at the Lawrence Berkeley Laboratory. Data were collected at room temperature from 15 crystals conventionally mounted in quartz capillaries and were merged during processing. Oscillation angles were 1.5° with exposure times of 1 min using 1 Å wavelength radiation. Approximately five or six images could be obtained from each crystal. The data set, consisting of 3732 independent structure amplitudes, was 100% complete to 3 Å resolution with an $R_{\rm merge}$ of 0.087 and an intensity-to-σ ratio of 3.0 in the outermost shell. Images from the R-AXIS system were processed using the program *DENZO* (Otwinowski & Minor, 1997), while the data from the Advanced Light Source were processed and merged using the program *d*TREK* (Pflugrath, 1999). For the crystals of the β-subunit grown at 277 K from MPD, data were recorded from a single frozen crystal at SSRL beamline 7.1 using a MAR Systems image plate and 1.0 Å wavelength radiation and otherwise the same data-collection parameters. In this case, the MPD mother liquor did provide cryoprotection. Data from the MAR system were processed using the program *DENZO* (Otwinowski & Minor, 1997). The data set of 3355 independent structure amplitudes was 94% complete to 3 Å with an $R_{\rm merge}$ of 0.075 and an intensity-to-σ ratio of 2.6 in the outermost shell. The data which were collected appear to be adequate for structure analysis of the crystals grown from both intact LH and LH β-subunit and this analysis is in progress. However, a detailed statistical analysis of the X-ray data will not be given here because it was only used for the definition of unit-cell parameters, space group and resolution limit of the crystals.

3. Results

The initial observation that ultimately led to the successful crystallization of the β-subunit was based on experiments with the intact hormone, which showed that the LH phase separated and formed oils under a very broad range of crystallization conditions. To address this, the detergent β-thiooctylglucoside was included as an additive in a number of standard screening matrices (Crystal Screens I and II, Index Screen from Hampton Research, Laguna Niguel, CA, USA). Very small needle crystals were observed in Hampton Crystal Screen I trial No. 11, which contained 1 *M* $(NH_4)H_2PO_4$ buffered at pH 5.8 with sodium citrate, but only after 10–12 weeks. Using the condition 1 *M* $(NH_4)H_2PO_4$, a number of other detergents were introduced and small crystals were occasionally obtained, some tablet- or barrel-shaped, but again only after many weeks to months. If no detergent was included, then no crystals were observed, only oils.

At this point, we considered the possibility that slow proteolysis of the LH might be involved. The protein was then set up under the 1 *M* $(NH_4)H_2PO_4$ condition with a broad range of detergents and either trypsin (1 µl 0.5% Gibco-BRL trypsin) or proteinase K (the same concentration, Sigma) was added to every sample. After two weeks at room temperature

no crystals were observed. The trays containing the samples were then transferred to a 310 K incubator. No crystals were observed in those samples to which proteinase K were added, but in many of those to which trypsin had been added crystals such as those in Fig. 1 were seen after 5–7 d. Optimization of the $(NH_4)H_2PO_4$ concentration, pH, detergent and its concentration, protein concentration, trypsin concentration and temperature was then carried out.

After optimization of reagents, concentration and procedures, the best crystals were obtained by mixing the intact luteinizing hormone at 40 mg ml^{-1} with an equal volume of 0.8–1.0 *M* ammonium dihydrogen phosphate and 0.1 *M* sodium citrate containing either 0.5% nonyl-β-D-glucoside or MEGA-9 (nonanoyl-*N*-methylglucamide) at pH 5.8. Crystallization took place by vapor diffusion using Cryschem sitting-drop plates (Hampton Research) with 6 µl drops. After mixing the drops, 1 µl of 0.5% trypsin (Gibco-BRL) was added to the individual samples and the plastic trays were placed in an incubator at 310 K. After approximately one week, crystals began to appear and continued to grow for several days at 310 K, occasionally reaching lengths (between the apexes of the tetragonal bipyramids) of 0.5 mm. Crystals could also be grown by substituting 20–25% saturated sodium malonate for the ammonium dihydrogen phosphate. Malonate has the advantage of being a cryoprotectant (McPherson, 2001). The procedure described here was quite reproducible. The crystals grown in the presence of MEGA-9 detergent were similar to those grown with nonyl-β-D-glucoside, but morphologically distinguishable by their sharper apexes and more acute angles between tetragonal faces. Crystals grown in the presence of each of the detergents are shown in Fig. 1.

Attempts were made to substitute other detergents for those reported here and many also yielded crystals, but none as reproducibly or of as consistently good quality as these two. Doubling the amount of trypsin added to the samples did not induce crystals to form at an earlier time; thus, proteolysis was not the limiting factor for nucleation, although it probably was for growth (see below).

As already noted, if proteinase K was substituted for trypsin at the same concentration no crystals were obtained. If the samples were placed at 295 K rather than 310 K or detergent omitted, no crystals grew. The higher temperature appears to be important for adequate proteolytic activity, which is likely to also be promoted by the presence of the detergent. If the LH is first exposed to an equivalent concentration of trypsin at 310 K in the presence of detergent and then set up to crystallize in the absence of any further enzyme, crystals will grow at 295 K but not at the quality that is obtained at 310 K. The higher temperature not only promotes proteolysis but also seems to favor ordered growth, probably by maintaining the mother liquor at a lower supersaturation. All of the parameters, detergent, salt, pH, temperature and proteolytic enzyme, appear to have an impact on the final product, the crystals.

The crystals belong to space group $I4_122$, with unit-cell parameters $a = b = 57$, $c = 207$ Å. On the home laboratory system used as described above, the diffraction patterns from crystals at 291 K are strong to 3.5 Å resolution but decline rapidly from that point and are barely visible beyond 3 Å. On beamline 5.0.1 at ALS, however, complete data sets of high redundancy to 3.0 Å resolution were obtained at 295 K from 15 different crystals conventionally mounted in quartz capillaries.

An intact LH molecule consisting of an α- and a β-subunit would have a molecular weight of about 24 000 Da. Assumption of one heterodimer as the asymmetric unit would imply a volume-to-weight ratio V_M (Matthews, 1968) of 1.8 $Å^3$ Da^{-1}, which appeared to be unlikely as such values are usually associated with robust well diffracting crystals. This raised suspicions that the crystals were not of the intact molecules, but were of either extensively degraded molecular fragments or one or other of the two subunits.

Crystals collected by centrifugation and the residual mother liquor in the supernatant were each analyzed by SDS–PAGE and this showed the crystals to be composed of the heavier of the two subunits, the β-subunit, while the residual mother liquor contained some LH β, but a preponderance of the lighter α-subunit. With assumption of one β-subunit as the asymmetric unit of the crystals, $V_M = 3.2$ $Å^3$ Da^{-1}, which appears to be more reasonable.

Additional crystals were recovered from collected droplets of mother liquor by centrifugation and the material in the crystals subjected to mass-spectrometric analysis. This was carried out on native crystalline protein and separately on crystalline protein that had been reduced and alkylated. LH β-subunit purified by HPLC (also from Professor John Pierce) was run as a standard. These analyses revealed that the crys-

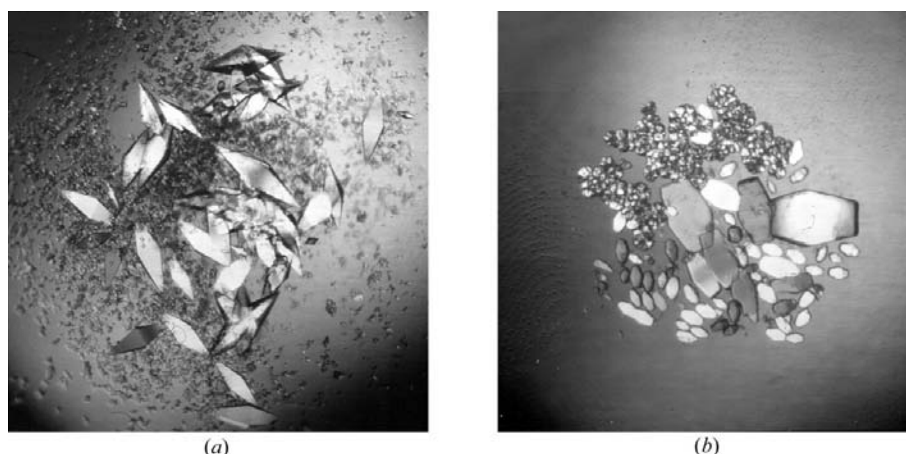

Figure 1
Crystals of the β-subunit of luteinizing hormone grown in the presence of neutral detergent and trypsin. (*a*) Crystals grown using the detergent MEGA-9 and (*b*) those grown using nonyl-β-D-glucoside. While the unit cell is identical for both crystals and both are tetragonal bipyramids, those from MEG-9 exhibit sharp apexes while those from nonyl-β-D-glucoside are truncated bipyramids. The crystals diffract equally well.

tals were composed almost entirely of only LH β-subunit and that the β-subunits were intact, including the oligosaccharide moieties. A mixture of smaller fragments was also present in the samples, but no one fragment predominated or was even present in significant amounts. On the other hand, the crystals were also completely free of the higher molecular-weight contaminants present in the starting LH preparations.

To further confirm the composition of the crystals, purified β-subunit, lacking any α-subunit component was set up for crystallization using exactly the same conditions including detergent but excluding trypsin, as described above. After one week at 310 K, crystals identical in appearance to those seen in Fig. 1 were obtained. X-ray diffraction analysis confirmed their identity.

Small crystals of the free β-subunit that was not exposed to trypsin were also obtained under somewhat more conventional conditions and in the absence of detergent. Crystals were grown at 277 K when drops of 40 mg ml^{-1} protein in water were mixed with equal volumes of 55% MPD containing 0.05 *M* sodium citrate at pH 5.5 and equilibrated by vapor diffusion in Cryschem sitting-drop plates (Hampton Research, Laguna Niguel, CA, USA) with the buffered 55% MPD solution.

While crystals were yielded reproducibly, these were invariably extremely small needles. In spite of extensive efforts to further optimize conditions, only rarely did a crystal grow of sufficient size for X-ray analysis. Nevertheless, the mother liquor of 55% MPD was an excellent cryoprotectant in itself and in a few rare cases small crystals could be taken onto cryoloops (Hampton Research) and frozen directly in a cryostream. The crystals diffracted to approximately 3.5 Å resolution and also belonged to the tetragonal space group $I4_122$, with unit-cell parameters $a = b = 64$, $c = 149$ Å. The volume-to-weight ratio V_M = 2.45 Å^3 Da^{-1} indicates the asymmetric unit to consist of one β-subunit of LH.

4. Discussion

Although low pH dissociates LH into α- and β-subunits, 1 *M* $(NH_4)H_2PO_4$ pH 5.8 will not do so. Furthermore, both the presence of detergent and exposure to trypsin for several days at 310 K is required before crystallization of the LH β-subunit occurs; neither the detergent nor the trypsin alone are sufficient. Finally, based on SDS–PAGE and mass spectrometry, it is clear that the LH β-subunit is not itself cleaved by the protease nor degraded. This is also confirmed by the isomorphous crystallization of the purified LH β-subunit under identical conditions but in the absence of any protease.

Our conclusion is that the detergent is essential for solubilization of the LH in the mother liquor and to maintain the protein soluble while trypsin exhibits its activity. The detergent is also likely to be important in determining crystal contacts and promoting the ordered assembly of crystals. It appears that the function of the trypsin may be twofold: to cleave and perhaps ultimately degrade the α-subunit and promote its dissociation from the β-subunit, its primary function, and possibly also to degrade the contaminating proteins present in the initial preparation. These contaminants may consist of other proteins, but also aggregates of LH and its subunits and denatured copies of those same components. It does not appear, although we cannot be completely certain, that there are residual fragments of the LH α-subunit bound to the β-subunits in the crystal. The mass-spectrometric results would suggest not, but such small fragments are sometimes difficult to identify.

Some additional points are also worth noting in passing. Proteinase K, a much more vigorous and non-specific protease, capable even of degrading kerritin (hence the K), could not substitute for trypsin in producing crystals, in this case probably because it damaged the LH β-subunit. Proteinase K is more similar, in general, to the proteases secreted by bacterial and fungal contaminants of crystallization mother liquors. Proteinase K is the protease secreted by a common fungal contaminant and is also a subtilisin-like protease typical of secreted bacterial proteases. Secondly, the overall morphology, size and quality of the crystals we obtained were definitely sensitive to the detergent employed in the mother liquor. Thus, detergents must be thoroughly investigated and screened with the same care as other crystallization variables.

The protein volume-to-weight ratio V_M is 3.2 Å^3 Da^{-1} for crystals grown from 1 *M* $(NH_4)H_2PO_4$ at 295 K in the presence of detergent, but 2.45 Å^3 Da^{-1} for those grown from 55% MPD at 277 K in the absence of detergent. The difference is almost certainly owing to the presence of detergent in the former crystals and the propensity of detergents to promote the incorporation of additional solvent along with it. Indeed, we routinely find that when detergent is an essential component of the mother liquor the V_M is significantly higher than for protein crystals grown in the absence of detergents. This is also supported by analysis of crystallization conditions and V_M values for the entries found in the Biological Macromolecule Crystallization Database (Gilliland *et al.*, 2001).

It has been recognized for some time that proteolytic cleavage of proteins may in some instances be beneficial to their crystallization (McPherson, 1982, 1999). Indeed, many domains or other large proteolytic fragments of proteins have been crystallized when the intact macromolecule fails to do so or when its crystals otherwise prove intractable. The Klenow fragment of DNA polymerase (Ollis *et al.*, 1985), the catalytic domain of HIV integrase (Dyda *et al.*, 1994) and the myosin subfragment (Rayment *et al.*, 1993) are only a few noteworthy examples; the Protein Data Bank (Berman *et al.*, 2000) contains many more. The directed application of proteolysis by specific enzymes to produce crystallizable fragments, such as using papain to degrade IgG to Fab and Fc domains, has also been exploited for many years. More recently, directed proteolysis has been used to identify discrete domains or folding units in proteins whose limits are subsequently refined using recombinant DNA constructs to yield crystallizable protein units (reviewed by Dale *et al.*, 2003).

At the same time as more or less rational and systematic proteolysis was being applied to specific proteins, X-ray crystallographers were also finding that serendipitous

proteolysis, generally a consequence of bacterial or fungal contamination, also occasionally produced favorable results, sometimes yielding crystals of modestly degraded or cleaved proteins when the intact molecule resisted crystallization. Canavalin, one of the first seed storage proteins ever to be crystallized (Sumner & Howell, 1936; McPherson, 1999), is an early example, as is ribonuclease S (Wyckoff *et al.*, 1970); more recent experiences include, among many others, Ef-Tu (Jurnak *et al.*, 1980) and numerous soluble domains of membrane or membrane-associated proteins.

It might be expected that uncontrolled proteolysis would be inherently deleterious as far as crystal growth is concerned and indeed we frequently incorporate azide or other inhibitors of microbial growth specifically to prevent its occurrence. One might well expect undesired proteolysis to produce a range of nicked, cleaved and partially degraded products that, as a heterogeneous mixture, would be antithetical to the formation of crystals. Indeed, examples exist, such as trypsin inhibited with benzamidine or subtilisin with BPN, of autocatalytic proteases that are best crystallized or only crystallized after rigorous inhibition and where autoproteolysis products inhibit success.

On the other hand, a surprisingly large number of macromolecules appear to have crystallized or to have formed better crystals as a consequence of limited proteolysis and, in many cases, of severe proteolysis. As has been remarked before, limited proteolysis appears to eliminate mobile terminal polypeptide segments or perhaps trim disordered or dynamic loop elements and this might in fact aid crystallization (McPherson, 1982, 1999). A question which should be carefully considered is whether far more crystallographic analyses and structure determinations have not benefited from this process than is commonly recognized.

In this laboratory we have, over the years, crystallized ten different macromolecules which benefited from intentional or serendipitous proteolysis. In reviewing the instances from our own laboratory where polypeptide cleavage has played a role, a number of indicators emerge in retrospect as signifying that proteolysis may be a factor in the nucleation and growth of the crystals. While these are not absolutely indicative, they should serve as clues that proteolysis may be playing a role.

(i) A long lag time precedes the appearance of any crystals. Here, we are speaking of times that far exceed those necessary for the sample to come to equilibrium with its reservoir by vapor diffusion or dialysis. When many weeks or even months pass without the formation of protein crystals and then they suddenly appear, this is a near-certain sign that the molecule underwent slow change and the most likely source is microbial proteases. In most instances, macromolecular crystals nucleate during the course of equilibration and grow relatively rapidly once crystallization begins. An unusually long lag time indicates a molecular process, not one associated with lattice development.

(ii) Irreproducibility among batches of protein from the same preparation. This is particularly the case when an aged batch produces crystals but the introduction of a fresh batch leads to failure. Then, after sufficient time, it too may begin to yield crystals. Similarly, a batch of protein may initially or after some time yields crystals and, as time goes on, become increasingly prolific. Often this evolutionary improvement is misinterpreted as increased proficiency of the investigator.

(iii) In some cases, transient exposure (a day or more) to higher temperature, particularly 310 K or its environs, results in the appearance of crystals. A possibility is that the temperature rise does not lead to any increase or other modification of supersaturation, but that the microbial production of proteases and/or proteolysis is promoted.

(iv) In some instances, crystals are observed in a crystallization sample but the amount of protein represented by the crystals only accounts for a small percentage of protein present in the sample. The crystals may represent a minor component, a proteolytic fragment or some cleaved form of the molecule.

(v) There is an unexpected change in the unit-cell parameters, symmetry, morphology or the entire unit cell of crystals. Is the new crystal of a proteolytically modified form of the protein?

(vi) Crystals grow but diffract to only limited resolutions. The possibility should be considered that the crystals may be a mixture of proteolytic forms.

(vii) If indications of microbial activity are observed (streaking of mother liquor on agar plates is one way of detecting this) in samples where crystals appear, then proteolysis should be considered. Azide and other inhibitors of microbial growth occasionally, perhaps frequently fail and cannot be completely relied upon.

(viii) Crystals suddenly begin appearing that diffract to significantly higher resolution. Although this might be considered as looking a gift horse in the mouth, proteolytic modification may be responsible.

(ix) A crystal structure does not refine well and/or certain polypeptide elements appear disordered or weak. Discontinuities appear. Nicks or cleavages may have been introduced and some segments of the polypeptide may be missing from most (reduced occupancy) or all molecules in the crystal.

(x) If crystals grow very slowly, then the time-dependence of growth may reflect the rate of proteolysis and not the kinetics for incorporation of molecules into growth steps. There are clearly other good reasons why crystals may develop slowly after nucleation, but generally growth is a relatively rapid kinetically favored process. Proteolysis, on the other hand may be quite slow, particularly at 295 K or less.

A reasonable question is whether there have been other instances of proteolytic intervention, not only in our own laboratory, but elsewhere. How many structures in the Protein Data Bank, ostensibly intact, are in fact partially cleaved, nicked or even missing segments, loops or terminal strands? How many structures have failed to refine or have refined poorly because the crystals or crystal used in data collection was not composed of fully intact molecules, but entirely or partially of proteolytic products? The only way to know this for certain is to analyze the crystal actually used for data collection by SDS–PAGE or mass spectrometry, a procedure seldom if ever applied or even practical. A good estimate may,

however, be obtained by simply subjecting brother crystals from the same samples to the analyses and perhaps this should be considered standard practice.

The fact that many proteins crystallize in spite of proteolytic cleavage, because of proteolytic cleavage or even better because of proteolytic degradation would seem to have some significant implications for protein structure itself. Because crystallization depends on homogeneity, the failure of intact proteins to crystallize while their proteolytic products do so would seem to confirm that proteins do indeed inhabit a variety of structurally varied dynamic states and show structural fluidity. A significant reason that many proteins will not crystallize is likely to be a consequence of those dynamic and mobile polypeptide elements which assume transient structural states. Many proteins, even after they experience proteolytic cleavage, are nonetheless crystallizable. This suggests that cleavage or nicking of the polypeptide chain does not necessarily introduce serious structural perturbations to the molecule and in some cases even leads to increased structural stability and homogeneity.

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