Proteins can convert to β -sheet in single crystals

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Abstract

Raman microscopy was used to follow conformational changes in single protein crystals. Crystals of native insulin and of the 5S and 12S subunits of the enzyme transcarboxylase showed a mixture of Raman marker bands signifying α -helix, β -sheet and nonordered secondary structure. However, by reducing the S–S bonds in the insulin crystal, or by lowering the pH for the 5S crystal, or by soaking substrates into the 12S crystal, the secondary structure in each crystal became predominantly β -sheet. The β -form crystals could be dissolved only with difficulty and yielded high-molecular weight protein aggregates, indicating that the β -sheet formation involves intermolecular contacts. Although their morphology appeared unchanged, the crystals no longer diffracted X-rays. Using crystals that had not been exposed to laser light, the dye thioflavin T formed highly fluorescent complexes with the “ β -transformed” crystals but not with the native crystals.

Keywords: Raman microscopy; protein secondary structure; “ β -transformed” crystals; thioflavin

Supplemental material: see www.protein.science.org

Protein unfolding and misfolding events, leading eventually to extensive β -sheet structure, are central to amyloidogenic diseases (Booth et al. 1997; Horwich and Weissman 1997; Mihara et al. 1998). Thus, native soluble proteins partially unfold and aggregate, possibly using a residual “ β -sheet” domain as a nucleation motif. This leads to amyloid fibrillogenesis and the formation of highly insoluble fibrils. The core structure of this consists of β -sheets, with the strand perpendicular to the long axis of the fiber. Much of the process of β -sheet formation occurs in the aqueous phase. In this article, we show that native proteins in the crystalline phase can also undergo large-scale conversion to β -sheet. Although minor changes in protein conformation have been observed in crystals, for example, a localized pocket in carbonmonoxy myoglobin (Zhu et al. 1992), to the best of our knowledge, this is the first report of whole-scale changes in secondary structure.

Although the first extensive studies of protein crystals by Raman spectroscopy were undertaken in the 1970s, the scope of these experiments was limited by technical considerations (Yu and Jo 1973; Yu et al. 1974). Recently it has become apparent that with the benefit of modern technology, protein crystals often make an ideal platform for studying protein–ligand interactions (and even enzyme–substrate reactions) by Raman difference spectroscopy (Altose et al. 2001; Dong et al. 2001a; Zheng et al. 2002; Helfand et al. 2003). Among the crystal systems we have worked with are insulin (containing 50% α -helix; Dong et al. 2001b, 2003a) and the 12S and 5S subunits of the enzyme transcarboxylase. Monomers of these subunits have molecular weights (MWs) of 56 kD and 60 kD, respectively, and perform the decarboxylation and carboxylation half-reactions of transcarboxylase (Wood and Zwolinski 1976). Their structures have been solved recently and contain 32% α -helix and 21% β -sheet for 12S (Hall et al. 2003) and 42% α -helix and 13% β -sheet structure for 5S (Hall et al. 2004). During Raman studies with crystals of these three proteins, we found, adventitiously, that each protein could be transformed into predominantly β -sheet structures. The method relies on Raman microscopy to provide a Raman spectrum, and thence a conformational monitor, of the protein within the crystal. A powerful advantage of Raman microscopy is

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Abbreviations: TC: transcarboxylase; 5S: a subunit of TC that carboxylates pyruvate; 12S: a subunit of TC that transfers carboxylate from methylmalonyl-CoA to biotin; Ni-NTA: a nickel-charged agarose resin.

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that we are able to bring about and follow changes in a single crystal in situ in a hanging drop (Dong et al. 2001a; Zheng et al. 2002). The Raman spectrum recorded from the crystal is caused only by the focal volume of the laser beam within the crystal, which is $\sim 20\ \mu\text{m}$ in diameter and $50\ \mu\text{m}$ in depth. Within the volume, the protein concentration is typically 20–60 mM, thus providing very high quality data. Any Raman scattering collected from surrounding mother liquor makes a negligible contribution because protein concentrations there are less than 1 mM. Similarly, Raman scattering from the crystal surface does not contribute significantly to the spectral data.

We have shown earlier that the time course of ligand-crystal soaking experiments can be followed by monitoring the intensities of Raman bands characteristic of the ligands (Zheng et al. 2002; Helfand et al. 2003). Here we follow changes in the Raman spectrum of the protein itself as perturbants are added to the hanging drop. The Raman spectrum of the crystal was recorded, the perturbant added to the mother liquor and dramatic changes were observed in the Raman spectra as the perturbant soaked into the crystal. Each Raman data set was recorded in ~ 100 sec, allowing a time course to be recorded. The transformations were catalyzed in separate ways: viz, by reduction of insulin's three S–S bridges; by lowering the pH of the mother liquor surrounding the 5S crystal; and by adding the substrates biotin and methylmalonyl-CoA to the mother liquor containing a 12S crystal. The changes in protein conformation in the crystal were evidenced by the well-known Raman marker bands (Miura and Thomas 1995; Krimm and Bandekar 1986); for example, a Raman marker band for α -helix occurs near $940\ \text{cm}^{-1}$ in a spectral "window" uncluttered by other features (Rodriguez-Casado et al. 2001). This band is clearly apparent in the native crystals but disappears when the crystals go into their β -form and is replaced by characteristic β -sheet markers.

Results and Discussion

Raman data for a single insulin crystal indicate that the protein within can undergo a transformation from a predominantly α -helical to predominantly β -sheet structure. The partial Raman spectrum of a single T_6 insulin crystal in its native conformation, shown in Figures 1A and 1C, agrees with published data (Yu and Jo 1973; Yu et al. 1974). The feature at $1657\ \text{cm}^{-1}$ is due to the amide I mode from α -helices, whereas the shoulder at $1680\ \text{cm}^{-1}$ is the corresponding motion from insulin's β -strand peptide bonds (Dong et al. 2001b). Peaks at 1615 and $643\ \text{cm}^{-1}$ are due to tyrosine ring modes, and peaks at 1604 and $621\ \text{cm}^{-1}$ are due to phenylalanine ring modes. The ring modes are expected to only undergo small changes in intensity on secondary structure change and, thus, act as internal intensity standards. Bands in the 490 – $570\ \text{cm}^{-1}$ region (Fig. 1C) are

Figure 1. Partial Raman spectra of an insulin (T_6) single crystal obtained by a Raman microscope. (Top) Native crystal. (Bottom) β -Transformed crystal after reducing agent tris(2-carboxyethyl)phosphine added to mother liquor.

caused by –S–S– stretching modes with a possible contribution from an α -helical amide IV mode. Dramatic changes occur when the reducing agent tris(2-carboxyethyl)phosphine is added to the mother liquor containing the crystal. The reductant diffuses into the crystal and reduces the S–S bonds to S–H (as evidenced by the appearance of a new band at $2573\ \text{cm}^{-1}$ because of S–H stretch; data not shown). Consequently, the region around $520\ \text{cm}^{-1}$ becomes featureless (Fig. 1D). Concurrently, the α -helical $1657\ \text{cm}^{-1}$ band (Fig. 1A) disappears and is replaced by a single β -sheet marker band at $1669\ \text{cm}^{-1}$ (Fig. 1B). These changes occur in about 1 hr. In the amide III region, major changes occur in band profile, with a β -sheet marker becoming predominant at $1236\ \text{cm}^{-1}$ on –S–S– bond reduction. Similarly, a distinctive α -helical marker at $946\ \text{cm}^{-1}$ disappears. The time course of these effects can be seen in Figure 2, where complete Raman spectra are shown with exposure to reducing agent from 1 to 57 min. The Raman data for the fully reduced crystal show no evidence of α -helical markers. The new protein form has a major contribution from β -sheet, with only a small contribution from disordered backbone (a shoulder near $1260\ \text{cm}^{-1}$ in the amide III region) and a narrowed amide I band with a width at half height of $24\ \text{cm}^{-1}$ (Table 1). We showed recently (Dong et al. 2003a) that insulin's amide I band width narrows with progressive self-assembly and that it narrows from approximately $40\ \text{cm}^{-1}$ for crystalline R_6 or T_6 insulin to $20\ \text{cm}^{-1}$ in insulin

Figure 2. Raman spectra of T₆insulin crystal between 1800 and 400 cm⁻¹ during the time course of S-S reduction. At t = 0 the reductant, tris(2-carboxyethyl)phosphine was added to a final concentration of 80 mM in the hanging drop.

fibrils. Thus, the present bandwidth at half height of 24 cm⁻¹ compares quite closely to that found for the fibrils. No change in the crystal morphology could be observed following reduction, but the crystals no longer diffracted X-rays.

Insulin is a small protein, (MW) 5.8 kD, but we also observed the transformation into “β-forms” for two larger proteins in the 55-kD range. These are the carboxylation (5S) and decarboxylation (12S) subunits of the enzyme transcarboxylase (Wood and Zwolinski 1976). The 12S subunit transfers a carboxylate group, -COO⁻, from methylmalonyl-CoA to a biotin found on the “swinging arm” of transcarboxylase’s 1.3S subunit. Subsequently, the biotin

delivers the carboxylate to pyruvate on the 5S subunit to form oxaloacetate. Remarkably, the β-transformations of the 5S and 12S subunits are brought about by quite separate and distinctive means. A crystal of 5S transforms from its

Table 1. Amide I band width at “half-height”

Protein crystal	Native crystal (Amide I band width at half height [cm ⁻¹])	β-transformed crystal (Amide I band width at half height [cm ⁻¹])
Insulin	44	24
5S	49	40
12S	44	36

Figure 3. Partial Raman spectra of single crystals of the 5S and 12S subunits of transcarboxylase. (Top) 5S crystals, solid trace: native crystal, dotted trace: β -transformed crystal (by lowering pH from 6.5 to 4.5). (Bottom) 12S crystals, solid trace: native, dotted trace: β -transformed (by adding biotin and methylmalonyl-CoA to the mother liquor).

native mixed α/β conformational state by reducing the pH in the surrounding mother liquor from 6.5 to 4.5. In contrast, the 12S crystal undergoes transformation to the β -form by adding both its substrates biotin and methylmalonyl-CoA to the mother liquor; addition of a single substrate has no effect, (we have repeated this latter finding about 50 times in other experiments; e.g., Zheng et al. 2002). Some of the Raman evidence for these transformations is shown in Figure 3. Complete Raman spectra are given in the Supplemental Material, Figure 1. On lowering pH (5S crystal) or adding substrates (12S crystal), the α -helical markers near 937 and 900 cm^{-1} decrease or disappear. In addition, there is a loss of intensity in the 1340 cm^{-1} region where α -helices also contribute (Overman and Thomas 1998). The disappearance of the α -helices is mirrored by the increase of an intense amide III β -sheet band near 1240 cm^{-1} . In Figure 3 the aromatic ring modes (Phe at 1003, 1032, and 1208 cm^{-1} and Tyr at 852 and 1208 cm^{-1}) can be taken as internal intensity standards. In addition, the amide I profiles shift to

1668 cm^{-1} for 12S and to 1669 cm^{-1} for 5S. The amide I bands for the insulin (1669 cm^{-1}) and 12S and 5S crystals converted to the β -forms indicate that β -sheet formation has occurred. β -sheet markers occur at 1667 cm^{-1} for silk fibers (Trabbic and Yager 1998; Shao and Vollrath 1999; Shao et al. 1999) and at 1667–1668 cm^{-1} in β -amyloid fibrils (Dong et al. 2003b), whereas isotope editing in insulin crystals showed that β -strand features occur near 1680 cm^{-1} (Dong et al. 2001b). For a 5S crystal, taken from pH 6.5 to 4.5, the 940 cm^{-1} band decreases monotonically in intensity from $t = 0$ to 4 hr, after which the residual intensity at 940 cm^{-1} (Figure 3) remains constant. The time course is shown in Figure 4.

For the native crystals the amide I profile, 1620–1700 cm^{-1} , contains contributions at different positions from α -helices, β -sheets, and unordered regions of polypeptide. It can be seen in Table 1 the amide band width at half height is reduced by 20%–40% for all three proteins on forming the β -form crystal. As discussed above, amide I bandwidth is a good probe for the degree of heterogeneity in polypeptide chain conformation (Dong et al. 2003a). Thus, the observed narrowing in Table 1 is further evidence for the disappearance of α -helical structure and the appearance of more uniform secondary structure.

It is apparent that there are quantitative differences for the β -transformations among the three samples: 12S appears to form the most homogenous β -structure, with no evidence for α -helical marker at 940 cm^{-1} , and no shoulder on the 1240 cm^{-1} amide III β -sheet band. 5S does have a shoulder in the amide III region near 1260 cm^{-1} indicating significant

Figure 4. 5S subunit demonstrates a time-dependent change of Raman intensity at 940 cm^{-1} after pH change (the graph is plotted from Supplemental Material, Fig. 1A). The y-axis is the ratio of Raman intensity of α -helix peak at 940 cm^{-1} to the intensity of peak at 1450 cm^{-1} (CH_2 deformation, which should have constant intensity in both native and “ β -transformed” crystals).

disordered structure and 5S does have some remaining α -helix structure—shown by the residual intensity at 936 cm^{-1} (Figs. 3, 4). β -transformed insulin shows no evidence for α -helices remaining, but a shoulder of the 1237 cm^{-1} β -sheet band at 1260 cm^{-1} (amide III) suggests significant unordered structure (Fig. 2).

In all three cases the transformation to the β -form crystals could not be reversed; for example, raising the pH from 4.5 to 6.5 in the mother liquor containing a β -transformed 5S crystal did not change the dotted trace in Figure 3. Moreover, each β -transformed crystal lost its ability to diffract X-rays, although it retained its original appearance. The crystals could be taken up into solution, though with more difficulty than the native crystals. However, we were unable to perform gel electrophoresis under denaturing or non-denaturing conditions, indicating the presence of large aggregates of each protein that did not run in the electrophoresis lanes. These findings show that significant intermolecular β -sheet contacts have been formed. Attempts at obtaining Raman data from these solutions were frustrated by high spectral backgrounds due to quasi-elastic scattering or luminescence.

Attempts to examine the infrared profiles of the crystals using a Fourier transform infrared (FTIR) microscope were thwarted by the high optical densities of, for example, the

amide I region of the crystals. However, we were able to obtain qualitative evidence for the onset of extensive β -structure using the dye thioflavin T. The latter is used to detect β -structure in pathological samples, for example, in the brains of deceased Alzheimer's patients, as well as for in vitro samples (LeVine III 1999). As Figure 5 shows, native crystals of 12S showed no staining with thioflavin and virtually no green fluorescence. However, the β -transformed crystal showed yellow staining and bright green fluorescence on treatment with the dye. Similar effects were seen for the 5S and insulin crystals for the native and β -transformed forms. These data are given in Supplemental Material, Figures 2 and 3. In addition, for 5S protein, the native and β -transformed crystals could be stained by the dye Congo Red, but only the β -transformed crystals evidenced birefringence. All the experiments using thioflavin T or Congo Red were carried out with newly grown crystals that had not been used for Raman data collection. This supports our conclusion that protein conformational changes in the crystals are brought about by the added perturbants and are not caused by the conditions used for the Raman experiment; for example, the effects of the laser beam.

Many proteins have the property of misfolding and forming aggregates in solution that contain extensive β -sheet secondary structure (Kelly 1997). Here we show that mis-

Figure 5. Staining 12S-methylmalonyl-CoA-biotin crystals with thioflavin T. (A) Native crystal stained with thioflavin T solution. (B) β -Form crystal (soaked with 5 mM MMCoA and 1 mM biotin) same treatment as (A). (C) No fluorescence can be detected from native crystal from (A); exposure time 15 sec. (D) β -Transformed crystal from (B) shows intense fluorescence; exposure time 1 sec.

folding leading to β -sheet formation can be brought about in single crystals. Moreover, we demonstrate that for different proteins, the process can be caused by three very different means: in insulin by reducing the S–S bonds in the crystal, in transcarboxylase's (TC's) 5S subunit by lowering the pH of the surrounding mother liquor; and in TC's 12S subunit by soaking its two substrates into the crystal. That such drastic conformational changes occur for protein crystals, with no apparent change in the crystal morphology, seems remarkable. Because the crystals we examined were 50–500 μm in average dimension, the laser focal volume ($20 \times 50 \mu\text{m}$) can represent a significant portion of the crystal. Moreover, the Raman data for the β -transformed crystals were independent of the position of the focused laser beam in the crystal. These factors enable us to state that the β -transformations occur throughout the crystal in an apparently uniform manner. Small molecules usually diffuse into protein crystals and equilibrate in a few minutes (Helfand et al. 2003). The time scale of the change to the β -sheet forms is from tens of minutes to 4 hr (Figs. 2, 4). Thus, conformational changes in the crystals occur slowly compared to “soak in” times, and it is likely that the transformation occurs in a homogeneous fashion starting at nucleation points distributed throughout the crystal.

It is worth emphasizing that our findings may represent a general phenomenon. It is likely that in many crystallization trials, undertaken earlier by X-ray crystallographers, some of the crystal samples underwent a “ β -transformation”. However, this could have gone undetected because the crystals no longer diffracted X-rays and were thus discarded. In another context, our findings may be relevant to the mechanism of fibril formation. Thus, during fibrillogenesis, part of the formation of the β -sheet core structure may occur in the nascent fibrils themselves, in an environment that resembles a protein crystal more than a protein solution.

Materials and methods

Materials

Reagents were purchased from Sigma. 5S-His₆ was incorporated into a pETBluse-2 expression plasmid and expressed using Tuner(DE3)pLacI *Escherichia coli* cells (Novogen). The 5S protein was purified using a Ni-NTA column and a size-exclusion column. Crystals of 5S were prepared by the hanging-drop vapor diffusion method. The composition of the precipitation buffer was as follows: 0.1 M Tris pH 7.0, 18%–32% PEG 4K. Insulin and 12S crystals were prepared as described (Dong et al. 2001b; Hall et al. 2003), respectively.

The conversion of native crystals to β -transformed crystals

Insulin β -transformed crystals were formed by adding the reducing agent tris(2-carboxyethyl)phosphine to mother liquor around the

native crystals, final concentration 80 mM, for 1 hr (Fig. 1). 5S β -transformed crystals were formed by lowering pH in the mother liquor from 6.5 to 4.5. 12S β -transformed crystals were formed by adding the substrates methylmalonyl-CoA (5 mM) and biotin (1 mM) to the mother liquor and waiting 1.5 hr. No pH change occurred during substrate addition. Raman features from substrates are not detected (Fig. 3).

Raman data collection

Raman data were obtained from crystals in hanging drops (Altose et al. 2001). In these experiments a plastic tray containing the crystallization wells is placed on the microscope stage. Crystals in a hanging drop are viewed by a video camera mounted on the microscope. A laser beam is directed along the optic axis of the microscope and is focused within the crystal. This can be viewed using the microscope and provides the control necessary when using crystals with dimension of tens to hundreds of microns. The back-scattered light from the focal volume in the crystal is collected by the microscope objective and is directed to a Raman spectrograph to provide the Raman spectrum associated with the focal volume. As mentioned in the introduction, the recorded Raman spectrum contains contributions only from the focal volume (approximately a cylinder, 20 μm in diameter, 50 μm deep) within the crystal. Data are collected in 60 sec with approximately 80 mW of 647.1-nm laser excitation.

α -Helical-to- β -sheet transitions have been reported for bacteriophage coat protein, brought about by heating the proteins in aqueous solution (Thomas et al. 1981; Thomas and Day 1981). Thus, the question arises as to whether the conditions of the Raman experiments, especially any heating effects of the laser beam, bring about the observed changes in protein conformation within the crystals. Several control experiments demonstrate that the β -transformations do not depend on the laser beam. First, we have many hundreds of data sets for a variety of protein crystals in hanging drops, carried out over 3 yr under conditions similar to those used in the present work. These data include the crystals used here prior to the addition of perturbants. For all samples, no changes in protein conformation are observed when the crystals are merely exposed to the laser beam. In a second set of control experiments, the 12S crystals were exposed to their methylmalonyl CoA substrate in the hanging drop, Raman spectra were recorded at 10-min intervals for 90 min, and no change was observed in the protein in Raman profile. However, on further addition of biotin to the hanging drop, the Raman spectrum changed progressively to that of β -sheet over the next 90 min. Third, the effect of laser beam heating was explored by placing a thermocouple in the hanging drop. On focusing the laser beam (120 mW) into the hanging drop, the temperature rose by about 3°C in 1 min, with no further increase over time. Given the rapid equilibration between the crystal and its “bath,” it is likely that similar changes occur in the crystal. However, such a modest amount of heating itself does not trigger detectable conformational changes. The key point remains that we see no evidence for protein conformational change in hundreds of control samples in the absence of perturbants.

For the 12S, 5S, and insulin single crystals, the results for transformation into the β -forms were completely reproducible. For 5S, more than 30 identical sets of results were obtained on more than 30 crystals over a period of 12 mo; for 12S more than 20 crystals gave identical results, and for insulin, about 10 crystals were tried. For both native and “ β -transformed” crystals, the Raman data were independent of the position of the laser focal volume in the crystal and of the orientation of the crystal with respect to the direction of the laser beam.

Treating crystals with thioflavin T and S

Native crystals of 12S and insulin were stained with 1% thioflavin T solution for 10 min and washed three times with well solution. The β -transformed crystals were stained with 1% thioflavin T for 10 min followed by a 10-min rinse with 70% ethanol and then washed with water three times. Intense fluorescence (excitation band 450–490 nm) was detected only for β -transformed crystals; exposure times are shown in the legend to Figure 5. Crystals of 5S were stained with thioflavin S (instead of thioflavin T because of the material availability), following the same steps as above.

Electronic supplemental material

Supplemental Figure 1. Time-dependent Raman spectra of 5S protein subunit after changing pH from 6.5 to 4.5 in the mother liquor and Raman spectra for 12S subunit before (red color) and after (purple color) adding the two substrates methylmalonyl-CoA and biotin to the mother liquor.

Supplemental Figure 2. Staining 5S crystals with thioflavin S.

Supplemental Figure 3. Staining insulin crystals with thioflavin T.

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