Protein Folding

Circular Dichroism Spectroscopy of Folding in a Protein Monolayer**

*Neil Keegan, Nicholas G. Wright, and Jeremy H. Lakey**

DNA arrays on surfaces have caused a revolution in nucleic acid analysis, and a similar revolution is required for proteins if high-throughput screening of their functions is to be realized.[1,2] In addition, proteins can also form the basis of self-assembling nanostructures.[3,4] One important obstacle is the assembly of functional protein monolayers on useful surfaces. Unlike DNA, proteins must retain an intricate three-dimensional structure to function, and measurement of these conformations under changing solution conditions is difficult in monomolecular films. However, our increasing awareness of natively unfolded proteins, which adopt defined conformations only upon interaction with ligands or receptors, means that structural change is also an important readout for protein–protein and protein–DNA interactions.[5] Finally, measurement of changes in protein secondary structure that lead to insoluble protein aggregates is crucial to the study and identification of conditions such as Alzheimer's disease and Creutzfeldt–Jacob disease (CJD).[6]

Fourier-transform infrared spectroscopy in either attenuated total internal reflection or grazing incidence modes is very useful for measuring protein structure in monolayers,[3,7] but its use is limited by its sensitivity to bulk water. Fluorescence spectroscopy is a sensitive but not a generic assay for protein folding, as it relies upon the presence of aromatic amino acids in suitable positions. Circular dichroism (CD) spectroscopy in the ultraviolet range ($\lambda = 180$–250 nm) measures the conformation of the core peptide backbone and is insensitive to the presence of bulk water.[8] The characteristic spectra for helix, sheet, or random structures, which arise from the π–π^* and n–π transitions, make this the spectroscopic method of choice for protein scientists who wish to monitor folding, stability, and conformational change.[9] Small sample size, accessible concentrations, and relatively inexpensive instrumentation mean that CD spectroscopy is the

[*] N. Keegan, Prof. J. H. Lakey
Institute of Cell and Molecular Biosciences and
Institute for Nanoscale Science and Technology
University of Newcastle upon Tyne
Newcastle NE2 4HH (UK)
Fax: (+44) 191-222-7424
E-mail: j.h.lakey@ncl.ac.uk
Prof. N. G. Wright
School of Electrical, Electronic, & Computer Engineering and
Institute for Nanoscale Science and Technology
University of Newcastle upon Tyne
Newcastle NE1 7RU (UK)

[**] This work was supported by the Medical Research Council, the Biological and Biotechnological Research Council, and the Wellcome Trust.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

 DOI: 10.1002/ange.200462977

most commonly used noncrystallographic method for protein structure characterization. It is also the only method that can measure the changing structure of proteins exposed to different aqueous bulk solutions. In the past, sensitivity has been a problem, but modern spectrometers easily resolve signals as small as $10^{-5}\,\Delta A$ (0.3 mdeg), and synchrotron sources now allow us to access data at wavelengths shorter than 180 nm.[10] Recently, CD spectra have been collected for proteins on gold films using extended acquisition times (10^3 scans).[11] With this in mind we investigated the practical limits for CD spectroscopy on a single protein monolayer, and we found that high-quality data can be collected with reasonable acquisition times on an unmodified commercial spectrometer.

The C-terminal, pore-forming (P) domains of colicins have been extensively studied by CD spectroscopy,[12,13] and we chose the 22.4-kDa colicin N P-domain (ColN-P) to test the sensitivity of CD to a protein monolayer. It is helix rich with a globin fold found in many protein families.[14] The exposed alanine residue at position 351 was replaced by a single cysteine (A351C) moiety by site-directed mutagenesis (Figure 1). The mutant protein ColN-P-Cys and the cysteine-free wild-type ColN-P were purified as described[15] and gave the expected helix-rich CD spectrum.

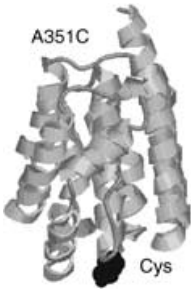

Figure 1. Structure of the pore-forming domain of colicin N (PDB:1A84) showing the position of alanine replacement by cysteine. The shape of the observed CD spectrum (Figure 3A) results from the 60% α-helix content.

If one treates ColN-P as a sphere of 3-nm diameter, a 1-mm^2 area of a *perfectly packed* protein monolayer should contain 1.1×10^{11} protein molecules (2.7 $ng\,mm^{-2}$). In a normal solution experiment using a 0.2-mm-pathlength CD cuvette and a protein concentration of 0.5 $mg\,mL^{-1}$ (2.2×10^{-5} M), an equivalent 1-mm^2 light beam will traverse a volume of 0.2 mm^3 or 0.2 μL containing 4.4×10^{-12} mol of protein or 2.6×10^{12} protein molecules. Thus the densest monolayer will provide a signal about 20-fold smaller than normal.

We created a reproducible protein monolayer by sulfur–gold chemisorption. All experiments were performed in buffer A, which comprised sodium phosphate (50 mM, pH 7.0) and NaCl (300 mM), with additions as specified. The ColN-P-Cys mutant was reduced with β-mercaptoethanol (2-sulfanylethanol, 10 mM), which was subsequently removed by gel filtration (Pharmacia PD-10 column or equivalent). Surface plasmon resonance (SPR; Biacore model X, Biacore Ltd., St. Albans, UK) was used to measure monolayer formation. Bare gold surfaces (Biacore AU-Chip) bound > 2200 resonance units (RU; 2.2 $ng\,mm^{-2}$ [16]) of both ColN-P and ColN-P-Cys proteins (Figure 2A). A wash step with sodium dodecyl sulfate (Buffer A + 0.5% w/v SDS) removed only some of the nonspecifically bound ColN-P from the surface, thus leaving 1200 RU. Under the same conditions 1900 RU of ColN-P-Cys protein remained, of which much is likely to be nonspecifically bound. Pretreatment of the gold

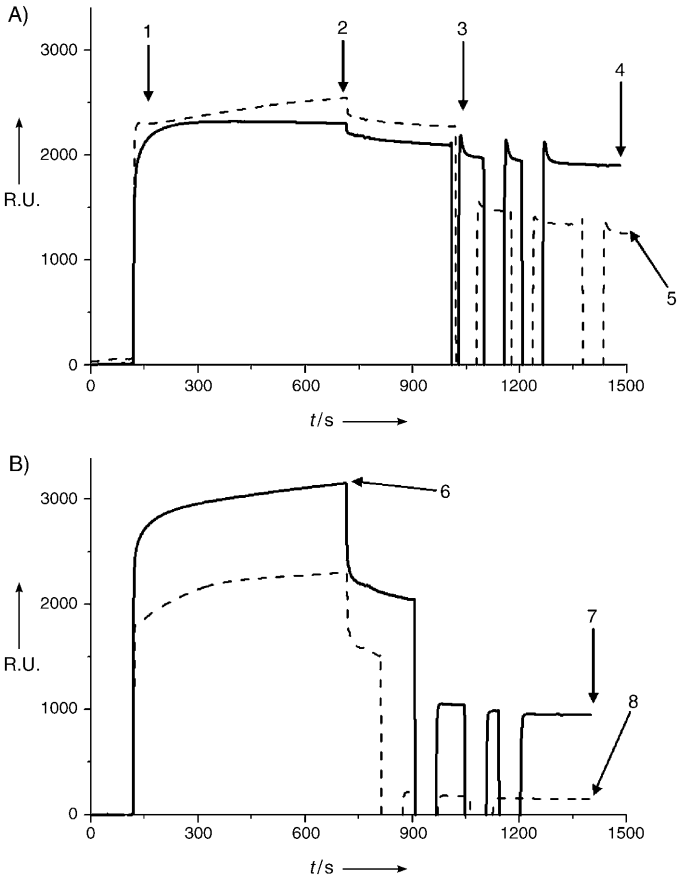

Figure 2. Surface plasmon resonance. A) Injection of ColN-P (2.5 $mg\,mL^{-1}$; -----) and ColN-P-Cys (——) in buffer A over a bare gold surface starting at point 1 (5 μL min^{-1}). Both proteins bind strongly to the surface and do not desorb upon washing with buffer (point 2). After washes with 0.5% SDS, indicated by regions with a negative baseline (point 3), the cysteine-free wild-type protein shows significant nonspecific binding (1200 RU (point 5)). ColN-P-Cys shows additional binding (1900 RU (point 4)). B) Pretreatment of the gold surface with β-mercaptoethanol (5 mM) increases the specificity of binding during identical injections both before, and especially after, the SDS washes. The upward slope on the ColN-P-Cys trace during injection indicates that the protein monolayer has not saturated during this short assembly period (point 6). ColN-P-Cys shows 1000 RU (point 7) final binding whereas ColN-P only binds 150 RU (point 8). All solutions contain phosphate (50 mM)/NaCl (300 mM) as buffer (pH 7.0).

with short-chain thiols inhibits nonspecific protein interactions with gold,[3] and therefore the surface was pretreated with β-mercaptoethanol (5 mM) in buffer A. When ColN-P was applied to this thiol-functionalized surface, which was then washed with 0.5% SDS, only 150 RU remained bound compared to 1000 RU (equivalent to 1 $ng\,mm^{-2}$) of ColN-P-Cys[16] (Figure 2B). Thus, 85% is likely to be bound through the cysteine residue, and the continuing upward slope of the SPR signal during the injection phase (Figure 2B) indicates that higher densities would result from longer incubation times.[3]

CD spectroscopy was performed in a standard demountable 0.2-mm-pathlength quartz-glass cuvette (106-QS, Hellma) and, as calculated above, a far-UV spectrum of a 20-fold diluted ColN-P solution (0.025 $mg\,mL^{-1}$ or $1.1\times$

10^{-6} M) provided a clearly resolvable signal (Figure 3A). For monolayer measurements the flat cover was coated by evaporation on one side with 2 nm of chromium followed by 10 nm of gold. This layer gave a characteristic absorption spectrum (not shown[11]), a CD spectrum (accumulated mean of 50 scans) which deviated by less than ± 1 mdeg from air baseline values (Figure 3A), and a photomultiplier voltage that only exceeded the empirical limit of >600 V at wavelengths below 196 nm (Figure 3B). The gold-layer baselines differed slightly between samples, possibly as a result of small variations in gold thickness, so the corresponding baseline for the cuvette containing the relevant buffer was subtracted from each set of data. Correct repositioning of the cuvette each time was also important. After baseline acquisition the gold was incubated with ColN-P-Cys (0.7 mg mL^{-1}, buffer A) for 3 h. Without pretreatment with β-mercaptoethanol, the protein bound strongly to the surface, and after a buffer wash it gave a signal larger (2 mdeg at 208 nm; Figure 3A) than the theoretical maximum, which indicated that, as in the SPR experiment, large amounts of nonspecific binding had occurred. A subsequent wash with 0.5% SDS reduced the value to the theoretical maximum seen in the diluted standard (1.5 mdeg at 208 nm). However, the SPR data indicate that this is still likely to contain nonspecifically bound protein.

To overcome this problem and ensure that data from a single monolayer of thiol-immobilized protein was collected, the gold surface was pretreated with β-mercaptoethanol (5 mM) in ethanol for 1 h. Incubation of this surface with ColN-P-Cys followed by washing steps with 0.5% SDS resulted in a lower intensity of 0.6 mdeg at 208 nm (Figure 3C). This value, which resulted from a longer assembly period than in the SPR experiment, equates to a protein density of 1.6 ng mm^{-2} (60% higher than that recorded by SPR). The signal corresponds to 62% of the maximum calculated packing and compares well with the 50% of maximum packing observed for OmpF (*Escherichia coli* outer membrane protein F) on gold surfaces.[3] Thus, the CD arises from a less than fully packed monolayer and could be further improved.

The utility of immobilized proteins was demonstrated by an unfolding–refolding cycle commonly used in protein stability measurements. This cycle would normally require a series of protein samples pre-equilibrated at a range of increasing denaturant concentrations.[17] A spectrum of the ColN-P-Cys monolayer was measured in phosphate buffer. This buffer was replaced by 2 M urea, which partially unfolded the secondary structure. Then the same sample was equilibrated in 8 M urea causing a complete loss of secondary

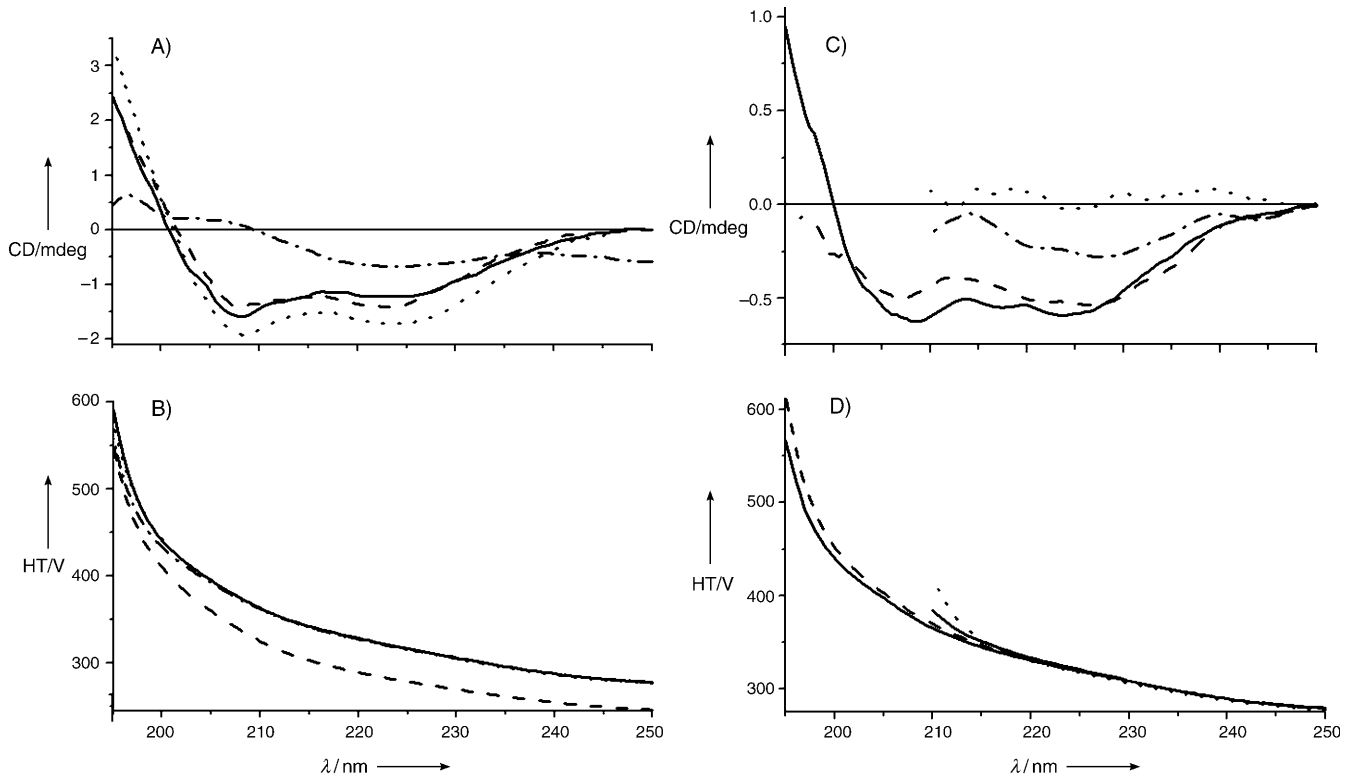

Figure 3. Circular dichroism. A) Solution measurement of ColN-P (0.025 mg mL^{-1}) in a 0.2-mm-pathlength cuvette (10 scans; ——). The gold baseline spectrum (–•–•) in the appropriate buffer was subtracted from all the following spectra. ColN-P-Cys bound to untreated gold (••••) and after SDS wash (-----; see Figure 2A). B) Photomultiplier voltage for the spectra in (A). C) Specific binding of ColN-P-Cys on β-mercaptoethanol-functionalized gold (see Figure 2B). Immobilized ColN-P-Cys in phosphate buffer (——) was then treated with 2 M urea (–•–•; absorption of urea limits data to >210 nm), 8 M urea (••••), and again in urea-free phosphate buffer (-----). D) Photomultiplier voltage for the spectra in (C); data from voltages >600 V were removed. Conditions: displayed data are means of 50 spectra collected at 20 nm min^{-1}, 4 s response time, 0.5 nm data pitch, bandwidth 2 nm, 25 °C, smoothed with a seven-point Savitzky–Golay[20] method (Jasco Spectra Analysis Software 1.53) on a Jasco J-810 spectrometer. All solutions contain phosphate (50 mM)/NaCl (300 mM) as buffer (pH 7.0). Further data showing untreated spectra, examples of data treatment, and Figure 3A, C with error bars added are to be found in the Supporting Information. HT is the photomultiplier voltage and is inversely related to light intensity passing through the sample.

structure. This finding repeats published solution data,[13] and refolding was achieved by simply incubating the sample again in buffer A.

The results demonstrate that even a crude system in which the protein is directly coupled to gold can be used to study the unfolding and refolding of the same protein sample rather than a series of different samples. The method could be improved by binding the proteins through a linker group to further reduce interaction with the gold surface.[2] Solutions can now flow over the immobilized proteins, so it is possible to add or remove ligands and denaturants easily, and the longer data collection times could be compensated for by designing overnight unfolding–refolding experiments where buffers are exchanged automatically on one fixed protein sample. Combined with the ability to assemble membrane proteins on gold and perform parallel SPR on these surfaces,[3,18] the use of immobilized protein monolayers in CD spectroscopy offers a way to extend the applications of this technique. Methods that increase the surface roughness of the gold[19] could further increase the sensitivity of the procedure.

Received: December 17, 2004

Keywords: circular dichroism · monolayers · nanostructures · protein folding · surface plasmon resonance

[1] a) C. M. Niemeyer, *Angew. Chem.* **2001**, *113*, 4254; *Angew. Chem. Int. Ed.* **2001**, *40*, 4128; b) G. MacBeath, S. L. Schreiber, *Science* **2000**, *289*, 1760; c) G. MacBeath, A. N. Koehler, S. L. Schreiber, *J. Am. Chem. Soc.* **1999**, *121*, 7967.
[2] B. H. Lee, T. Nagamune, *Biotechnol. Bioprocess Eng.* **2004**, *9*, 69.
[3] S. Terrettaz, W.-P. Ulrich, H. Vogel, Q. Hong, L. G. Dover, J. H. Lakey, *Protein Sci.* **2002**, *11*, 1917.
[4] U. B. Sleytr, P. Messner, D. Pum, M. Sara, *Angew. Chem.* **1999**, *111*, 1098; *Angew. Chem. Int. Ed.* **1999**, *38*, 1034.
[5] a) A. K. Dunker, C. J. Brown, J. D. Lawson, L. M. Iakoucheva, Z. Obradovic, *Biochemistry* **2002**, *41*, 6573; b) H. J. Dyson, P. E. Wright, *Curr. Opin. Struct. Biol.* **2002**, *12*, 54; c) G. Anderluh, I. Gokce, J. H. Lakey, *J. Biol. Chem.* **2004**, *279*, 22002.
[6] C. M. Dobson, *Nature* **2003**, *426*, 884.
[7] P. Rigler, W. P. Ulrich, H. Vogel, *Langmuir* **2004**, *20*, 7901.
[8] G. D. Fasman, *Circular Dichroism and the Conformational Analysis of Macromolecules*, Plenum, New York, **1996**, p. 738.
[9] R. W. Woody, *Energ. Biol. Macromol. E* **2004**, *380*, 242.
[10] a) B. A. Wallace, R. W. Janes, *Curr. Opin. Chem. Biol.* **2001**, *5*, 567; b) W. A. Stanley, A. Sokolova, A. Brown, M. Wilmanns, D. T. Clarke, D. I. Svergun, *J. Synchrotron Radiat.* **2004**, *11*, 490.
[11] M. Shimizu, K. Kazutoshi, H. Morii, K. Mitsui, W. Knoll, T. Nagamune, *Biochem. Biophys. Res. Commun.* **2003**, *310*, 606.
[12] a) F. G. van der Goot, J. M. González-Mañas, J. H. Lakey, F. Pattus, *Nature* **1991**, *354*, 408; b) S. L. Fridd, J. H. Lakey, *Biochemistry* **2002**, *41*, 1579.
[13] L. J. A. Evans, M. L. Goble, K. Hales, J. H. Lakey, *Biochemistry* **1996**, *35*, 13180.
[14] a) I. R. Vetter, M. W. Parker, A. D. Tucker, J. H. Lakey, F. Pattus, D. Tsernoglou, *Structure* **1998**, *6*, 863; b) L. Holm, C. Sander, *Febs Lett.* **1993**, *315*, 301.
[15] S. L. Fridd, I. Gokce, J. H. Lakey, *Biochimie* **2002**, *84*, 477.
[16] E. Stenberg, B. Persson, H. Roos, C. Urbaniczky, *J. Colloid Interface Sci.* **1991**, *143*, 513.
[17] C. N. Pace, B. A. Shirley, J. A. Thompson in *Protein Structure: A Practical Approach* (Ed.: T. E. Creighton), IRL, Oxford, **1989**, p. 311.
[18] a) B. Hu, D. Finsinger, K. Peter, Z. Guttenberg, M. Barmann, I. Kessler, A. Escherich, L. Moroder, J. Bohm, W. Baumeister, S. F. Sui, E. Sackmann, *Biochemistry* **2000**, *39*, 12284; b) R. Naumann, T. Baumgart, P. Graber, A. Jonczyk, A. Offenhausser, W. Knoll, *Biosens. Bioelectron.* **2002**, *17*, 25; c) S. Terrettaz, W. P. Ulrich, R. Guerrini, A. Verdini, H. Vogel, *Angew. Chem.* **2001**, *113*, 1790; *Angew. Chem. Int. Ed.* **2001**, *40*, 1740.
[19] H. Raether, *Springer Tracts Mod. Phys.* **1988**, *111*, 1.
[20] A. Savitzky, M. J. E. Golay, *Anal. Chem.* **1964**, *36*, 1627.

