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Alignment media for studying protein structures by NMR

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Abstract

By using an anisotropic medium, residual dipolar couplings can be measured in biomolecules. In this project, two different media have been studied and residual dipolar couplings have been measured by NMR spectroscopy in the E140Q mutant of the C-terminal domain of calmodulin. The mutant domain undergoes spontaneous conformational changes and the recorded data will be used to calculate theses structures.

Keywords

bicelles, strained polyacrylamide gels, dynamics, IPAP-HSQC

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Alignment media for studying protein structures by NMR

Annasara Dahlström

Sammanfattning

I kroppens alla celler finns proteinet calmodulin (CaM). Det binder kalcium och är viktigt för många typer av processer i cellen t.ex. nervtillväxt och muskelsammandragning. CaM består av två domäner eller delar. Varje domän kan binda två kalciumjoner. I det här projektet har en muterade form av en av dessa domäner studerats. Mutanten kallas E140Q, eftersom aminosyra 140 i proteinet har ändrats från glutaminsyra (E) till glutamin (Q). E140Q har en underlig egenskap när det har bundit till två kalciumjoner. Molekylen byter spontant mellan två olika konformationer.

Målet med det här projektet är att studera dessa två konformationer med hjälp av sk residuala dipolkopplingar. För att mäta dipolkopplingarna används NMR (kärnmagnetisk resonans), en fysikalisk-kemisk metod som används för att studera molekylers struktur och dynamik. Dessa dipolkopplingar talar om hur långt det är mellan två atomer och vilken riktning det är från den ena till den andra. Det är viktig information för att kunna beräkna strukturen hos stora molekyler. Dipolkopplingarna kan mätas när proteinet tvingas att ordna sig litet grann, så att en rikting på molekylen blir vanligare än alla andra. Detta kan göras på flera sätt, men här har flytande kristaller och geler med avlånga porer använts.

Jag har visat att mediet av flytande kristaller fungerar för att ordna proteinet och att det till en början har kvar sin nativa struktur. Tyvärr denaturerar proteinet långsamt i de flytande kristallerna och provet förstörs inom en dag. Trots detta har jag lyckats mäta dipolkopplingarna. Dessutom har jag visat att proteinets helixar finns kvar, men inte har samma orientering som i vildtypsproteinet. Mina data kommer att användas för att beräkna strukturen av E140Q.

Examensarbete 20 p i Molekylär bioteknik

Uppsala universitet februari 2004

1 Introduction

1.1 Calmodulin

One of the calcium-binding proteins in our cells is calmodulin (CaM). It is a vital protein, active in a number of cellular processes. CaM is 100% conserved in vertebrates, i.e. identical in all animals with a spinal column, and is found in all eukaryotic cells. The importance of calmodulin is also demonstrated by the fact that the protein is coded by multiple gene copies; in man with three copies [1].

As a signal-mediator CaM is involved in many different processes such as inflammation, metabolism, apoptosis, muscle contraction, cytoskeletal movement, short-term and long-term memory, nerve growth and immune response. CaM can bind to more than one hundred different proteins, making it possible to mediate all these processes. Although ubiquitously expressed, the protein is most abundant in brain and testis [1].

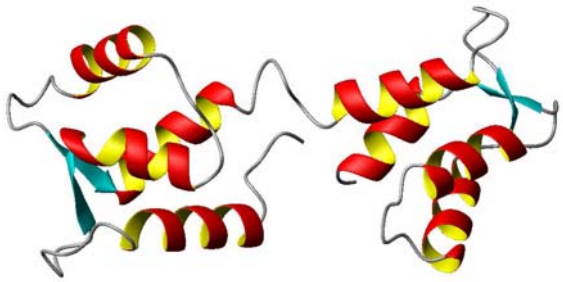

Figure 1: Calmodulin with its two domains.

The protein has two calcium-binding domains (Tr_1C and Tr_2C) with two binding sites each (Figure 1). These calcium-binding domains bind the calcium ions co-operatively, i.e. the binding of one ion increases the binding affinity for the second one. This means that, in the hypothetical case of infinite co-operativity, at half saturation, 50% of the domains have two calcium ions bound and 50% have no ions bound. However, it should be noted that there is no or very little co-operativity between the two domains. They are connected by a long hinge, but they function separately. In crystal structures, you often see the connection as an α-helix, but this is an artefact not seen in NMR solution structures [2]. The connecting amino acid residues are very flexible [3]. It has been shown that it is an advantage to have the two domains connected when the protein binds to other proteins in order to activate them.

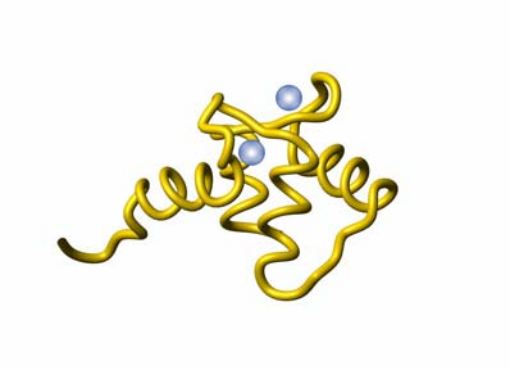

Figure 2: The two conformations of Tr_2C. Apo-form on the left and the calcium-bound form on the right with the blue spheres representing the calcium-ions.

As binding of calcium occurs, each protein domain undergoes large conformational changes, from the apo-form to the calcium-form (Figure 2). In this process, the angles and distances between the α-helices change. At the same time, the hydrophobic core of the protein becomes exposed to the surrounding solvent. Due to this, calmodulin-binding proteins typically have the ability to bind to one of the conformations, but not both. Most calmodulin-binding proteins bind to the calcium-form. Among these, you find kinases, phosphatases, signalling proteins and cytoskeletal and muscle proteins [4].

Calcium is the most abundant mineral in the human body. Most of it is found as hydroxyapatite in our bones. The extra-cellular concentration of free calcium ions is about 1.2 mM. Inside the cell, calcium is stored in the endoplasmic reticulum and the flow is regulated by different membrane channels. In a resting cell, the Ca^{2+} concentration is less than 0.1 μM, while it is 1–10 μM in an activated one. Since the calcium-binding constant ranges between 10^5–10^6 M^{-1} [5], calmodulin changes from the apo-state to the calcium-loaded state when the cell is activated. The rise in Ca^{2+} concentration can therefore be regarded as a switch that turns on many processes in the cell.

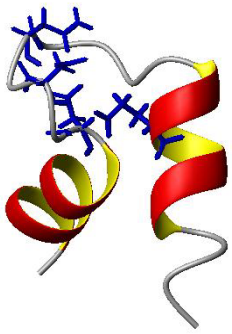

Figure 3: An EF-hand with its calcium-coordinating side-chains.

The CaM domains consist of two so-called EF hands (Figure 3), a very common helix-loop-helix motif. The motif often occurs in pairs with a short anti-parallel β-

sheet between the calcium-binding loops. The consensus EF hand is 29 residues long with a twelve-residue calcium-binding loop. The calcium ion is coordinated in a pentagonal bipyramide arrangement by seven oxygen atoms, one of which belongs to a water molecule. The six other ligands are the side-chains of loop residues 1, 3 and 5, and the backbone carbonyl oxygen of residue 7. The last two ligands are the carboxylate oxygens of the glutamic acid in position 12, which provides bi-dentate coordination of the calcium ion.

1.1.1 E140Q

In this project, a mutant form of the C-terminal domain (Tr_2C-E140Q) has been studied. The domain consists of residues 76 to 148 of the wild-type protein, but with residue 140 changed from glutamic acid to glutamine. The mutant was originally designed for studies of the half saturated state. Due to the co-operativity, this cannot be done in the wild-type protein. The calcium affinity for the two loops of wt-Tr_2C is log $K_{1,wt}$ = 6.45 and log $K_{2,wt}$ = 7.45 respectively. When the second loop is mutated, also the first loop is affected and the affinity is decreased by one order of magnitude to log $K_{1,E140Q}$ = 5.2. The mutated loop, on the other hand, has an affinity 1000 times weaker than in the wild-type protein (log $K_{2,E140Q}$ = 3.15) [6]. During these studies, it became apparent that the calcium-saturated Tr_2C-E140Q undergoes rapid exchange between two conformations under equilibrium conditions [6-8]. Studies have shown that the time scale of this conformational switch is about the same as the off-rate of calcium from the wild-type protein, with a time constant of 20 µs at 28ºC. It has also been shown that the populations of the two conformations are approximately equal in size. In terms of energy this corresponds to two minima with approximately the same energy, separated by a barrier of 50 kJ/mol (Figure 4) [7, 9]. The hypothesis is that the conformations have a strong resemblance with the wild-type apo (closed) and calcium-saturated (open) forms. Due to the rapid exchange, only the average structure is observed in the NMR spectra.

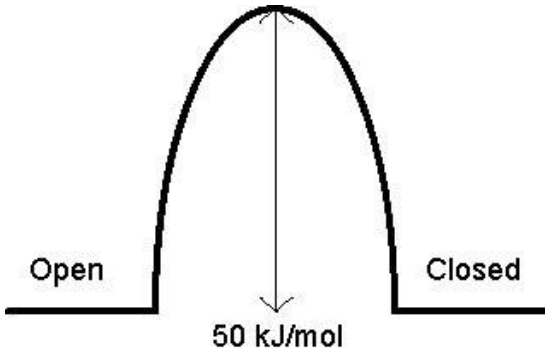

Figure 4: Energy diagram for the conformation transition between the open and closed forms of E140Q.

To calculate the NMR structure, large sets of structural constraints, such as inter-atomic distances, are measured and a number of possible structures are calculated. In the present case the distances correspond to a distance in one of the two structures or, if both of the distances are measurable, an average. The chemical shifts of the mutant are more or less an average of the shifts in the two separate structures. Apo-E140Q has the same structure as the wt apo-form.

1.2 Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) is a powerful tool in chemical analysis, both for structural and dynamic studies. NMR spectroscopy measures the resonance frequencies of nuclear spins, which reflect the magnetic (and indirectly the chemical) surroundings of the atomic nuclei. The sample is placed in a very strong magnetic field, around which the nuclear spins precess. The frequency of the precession depends on the magnetic field strength and the nucleus studied. By disturbing the system with a radio signal, you can detect the resonance frequency.

1.2.1 NMR theory

Basic NMR theory has been described extensively in a large number of NMR textbooks, e.g. *Spin dynamics* by Malcolm Levitt and *Protein NMR spectroscopy* by Cavanagh et al. [10, 11]. Spin is a strictly quantum mechanical property of the fundamental particle. The nuclear spin angular momentum, **I**, is given by

$$\left|\mathbf{I}^2\right| = \mathbf{I}\cdot\mathbf{I} = \hbar^2\left[I(I+1)\right]$$

where I is the spin quantum number. Nuclei with an odd number of particles have a half-integer spin (e.g. ^{1}H, ^{13}C and ^{15}N), and those with an odd number of neutrons and an odd number of protons have an integer spin (e.g. ^{2}H and ^{14}N). Those with an even number of protons and neutrons lack a net spin (e.g. ^{12}C). As spins larger than ½ have their drawbacks (see below), you often choose to work with half-integer spin. Thus, not all nuclei are detectable, or suitable, and you are forced to introduce specific isotopes into your samples, increasing the sample cost dramatically. Quantum mechanics restricts the number of the specified Cartesians components of **I** to one. The value of the z-component of **I** is specified as

$$I_z = m_I\hbar$$

with $m_I = I, I-1, \ldots, -I$. This gives $2I+1$ possible values of I_z. The vector is therefore quantised, as the magnitude of the vector is constant and the z-component has a set of discrete values. Protons have $I = \frac{1}{2}$ and therefore will have two possibilities of spin direction. These different directions are occasionally denoted ↑, "spin up" or ↓, "spin down", but more often $|\alpha\rangle$ or $|\beta\rangle$. Nuclei with a non-zero spin also have a nuclear magnetic moment, **μ**, defined as

$$\boldsymbol{\mu} = \gamma\mathbf{I}$$
$$\mu_z = \gamma I_z = \gamma m_I\hbar$$

where γ is the gyromagnetic ratio. Depending on the sign of γ, **μ** is parallel or anti-parallel with the angular momentum. In the presence of a magnetic field, B_0, along the z-axis, nuclei in the states $|\alpha\rangle$ and $|\beta\rangle$ will have different energies. These are given by

$$E_{m_I} = -\mu_z B_0 = -\gamma m_I\hbar B_0$$

where E is the energy. For spin-½ nuclei the energy difference between the spin states is

$$\Delta E = E_\beta - E_\alpha = \tfrac{1}{2}\gamma\hbar B_0 - \left(-\tfrac{1}{2}\gamma\hbar B_0\right) = \gamma\hbar B_0$$

For most nuclei γ is positive, causing the β-level to be higher in energy than the α-level. When applying an oscillating electromagnetic field with a frequency corresponding to ΔE, the magnetisation in the sample is excited and the spins can make the transition from $|\alpha\rangle$ to $|\beta\rangle$. The frequency required is given by Planck's law

$$\hbar\omega_L = \Delta E = \gamma\hbar B_0$$

where ω_L is the so-called Larmor frequency. During an NMR experiment, the nuclear magnetic moments interact with the local magnetic field, which depends on the surrounding atoms and the distribution of electrons surrounding the nucleus. This phenomenon is called shielding. The local magnetic field and the Larmor frequency can therefore be expressed as

$$B_{loc} = (1-\sigma)B_0$$
$$\omega_L = \gamma B_{loc} = (1-\sigma)\gamma B_0$$

where σ the shielding constant. This gives rise to small shifts in the spectrum and gives you the possibility to separate the different resonances. This is called chemical shift and is expressed

$$\delta = \frac{\omega - \omega^\circ}{\omega^\circ} \times 10^6 = (\sigma^\circ - \sigma) \times 10^6$$

where ω is the frequency of the measured signal, ω° is the resonance frequency of a reference signal and σ° is the shielding constants of the reference signals. The factor of 10^6 indicates that δ is given in ppm.

The energy levels of a nuclear spin are modified by interactions with other spins or external magnetic or electric fields. There are four types of spin couplings: Zeeman, quadrupolar, dipolar and scalar couplings. The Zeeman coupling is the interaction with the static magnetic field giving rise to the "up" and "down" spin states. The quadrupolar coupling arises when the studied nucleus has a spin larger than ½ and is due to the interaction between the quadrupole moment and the electric field gradient. It can have a size comparable to the Zeeman coupling. The third type of spin coupling is the dipole-dipole coupling, a direct interaction between magnetic dipoles. In isotropic media, the effect is averaged out, but in anisotropic media (discussed later in this section), you can measure a residual dipolar coupling (RDC), a downscaled dipolar coupling. The scalar coupling is also known as the J-coupling or the indirect dipolar coupling. It is an indirect interaction between dipoles, mediated by the electrons in the bonds of the molecule and not through space as the dipolar coupling.

Because of these additional couplings, the energy transitions in a coupled spin system are not the same in all cases. The energy level is modified with extra terms, due to the couplings. This gives rise to extra peaks in the spectrum forming multiplet patterns. For a spin system with two spins, two peaks are detected. For larger spin systems, the

number and height of the individual peaks can be derived from Pascal's triangle and the frequency difference between them corresponds to the energy of the coupling. As an example, the J-coupling is approx. 92 Hz for the amide ^{1}H and ^{15}N spins in a peptide. In an isotropic medium, a split signal (doublet) can therefore be detected with a peak separation of 92 Hz.

Couplings can be described with their respective Hamiltonian. For N scalar and dipolar coupled ½-spins the total spin Hamiltonian is (in units of $\hbar$)

$$\begin{aligned}\mathcal{H} &= \mathcal{H}_z + \mathcal{H}_J + \mathcal{H}_D \\ &= \sum_{i=1}^{N} \omega_i I_{iz} + 2\pi \sum_{i=2}^{N} \sum_{j=1}^{i-1} J_{ij} \mathbf{I}_i \cdot \mathbf{I}_j + \sum_{i=2}^{N} \sum_{j=1}^{i-1} b_{ij} \left(3(\mathbf{I}_i \cdot \mathbf{e}_{ij})(\mathbf{I}_j \cdot \mathbf{e}_{ij}) - \mathbf{I}_i \cdot \mathbf{I}_j\right)\end{aligned}$$

$$b_{ij} = -\frac{\mu_0}{4\pi} \frac{\gamma_i \gamma_j}{r_{ij}^3}$$

The first term is the Zeeman coupling with ω_i being the Larmor frequency of the ith spin. The second term is the scalar coupling with J_{ij} being the scalar-coupling constant between the ith and the jth spins, and the third term is the dipolar coupling with the unity vector $\mathbf{e}_{ij}$ parallel to the line joining the centres of the two nuclei. The dipolar coupling constant, b_{ij}, is dependent on the gyromagnetic ratio of the two spins and inversely proportional to the cube of the distance and r_{ij} the inter-nuclear distance. For protons and other spins with a high gyromagnetic ratio the dipolar coupling is large. The dipolar coupling rapidly decreases, however, as the inter-nuclear distance increases. The J- and dipole-dipole interaction may be decoupled by a suitable choice of radio-frequency pulses, saturating one of the spins.

For two coupled spins in high magnetic fields, the dipolar coupling can be reduced to

$$\begin{aligned}\mathcal{H}_D(\Theta_{ij}) &= d_{ij}\left(3I_{iz}I_{jz} - \mathbf{I}_i \cdot \mathbf{I}_j\right) \\ d_{ij} &= b_{ij}\,\tfrac{1}{2}\left(3\cos^2\Theta_{ij} - 1\right)\end{aligned}$$

with the angle Θ_{ij} between the vector $\mathbf{e}_{ij}$ and the magnetic field B_0. If the dipolar coupled spins are heteronuclear, the Hamiltonian may be further simplified to

$$\mathcal{H}_D(\Theta_{ij}) = d_{ij}\, 2I_{iz}I_{jz}$$

The equation shows that the dipolar coupling between two spins i and j, depend on the angle Θ_{ij} and hence on the molecular orientation. In an isotropic medium, the dipolar couplings average out, since the integral of d_{ij} over all angles equals zero. However, in an anisotropic medium, there is a preferred molecular orientation and certain angles will give a contribution to the Hamiltonian. By measuring several dipolar couplings in a molecule, the relative angles between pairs of dipolar-coupled spins can be calculated. This provides important structural information.

The orientation of the molecule can be described with an alignment tensor. The tensor can be reduced to five independent components. These can be further reduced to an axial and a rhombic component, which together with the three Euler angles determine the orientation.

1.2.2 Different types of spectra

When analysing an NMR sample, you modify the experiment based on what you want to measure. The easiest experiment is the one-pulse experiment. With small molecules, this can be enough to analyse the sample. Another very common experiment is the two-dimensional NOESY. With the recorded NOESY spectrum, you can measure so-called NOEs (nuclear Overhauser effect), which can be easily converted into approximate interatomic distances. This is the traditional way to calculate NMR-structures in proteins and other large molecules. When you work with proteins, the standard experiment is a ^{15}N-HSQC (hetronuclear single quantum correlation) experiment to correlate each backbone amide nitrogen atom with its attached proton. You normally get one signal for every residue, except for proline, which has no amide proton. The chemical shifts of the signals are the fingerprint of the protein.

In an HSQC experiment, the proton magnetisation is first excited. Then the magnetisation is transferred to nitrogen. Here the magnetisation is kept for a certain time (t_1) before being transferred back to the proton and detected during t_2. The data is then Fourier transformed in the t_1 and t_2 dimensions, which yields the corresponding frequencies F_1 and F_2. The resulting peaks in the two-dimensional spectrum give the chemical shifts of the covalently bound nitrogen and proton spins in the F_1 and F_2 dimensions. In a very crude HSQC experiment, no attempt is made to decouple the scalar and dipolar interactions. Therefore, every peak will be split into four, i.e. a doublet in each dimension. Since the spectrum usually is quite crowded, this is not a desirable feature. To prevent this, decoupling is used. Two strategies are available, either by destroying the scalar coupling or by reversing its effect for half of the time. Usually the first approach is used in t_2 and the latter in t_1. The HSQC experiment can easily be modified to correlate other types of atoms, e.g. ^{13}C and protons.

In this project, a version of the HSQC spectrum has been used to measure residual dipolar couplings. It is called in-phase/anti-phase (IPAP) HSQC [12]. In these spectra, the sum of the J-couplings and dipolar couplings is measurable. For every experiment, two spectra are recorded. The first is the in-phase spectrum, which really is a partially decoupled HSQC with two positive peaks for each shift, split by the scalar and, if measuring on an anisotropic sample, the dipolar coupling in the ^{15}N dimension. In the anti-phase version of the partially decoupled HSQC, one of the peaks is inverted and thereby negative. By adding and subtracting the spectra, two HSQC-like spectra are obtained, one with the “upper” component of the doublet and one with the “lower” (Figure 5). The magnitude of the coupling is measured, by comparison of the chemical shifts of the peaks in the two spectra. When made under isotropic conditions, the scalar coupling for each residue is obtained. By subtracting those couplings from the coupling data in the anisotropic case, you get the residual dipolar coupling of each residue. This approach gives us an opportunity to study couplings in well-resolved spectra.

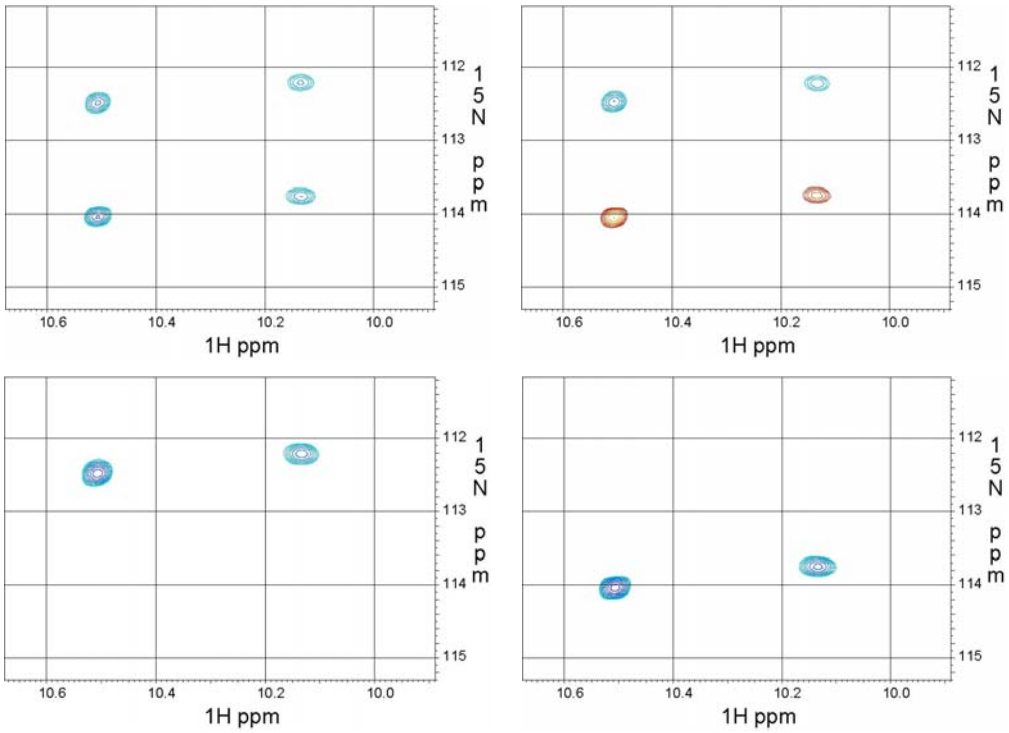

Figure 5: Examples of IPAP spectra. In-phase spectrum (top left), anti-phase spectrum (top right), summation spectrum (bottom left) and subtraction spectrum (bottom right). Blue and red indicates positive and negative intensities respectively.

1.3 Partially ordered media

In this project, different kinds of ordered media have been used. The reason for using ordered media is that the dipolar couplings cannot be measured in an isotropic medium like water. Crystallising the protein is not ideal either, as the NMR signals then become severely broadened and the spectra become difficult to work with. Therefore, you use a partially ordered medium. Thereby you gain in information content, without losing the clarity of the spectra. There are a number of different alternatives for aligning proteins. In this project, two of them have been tested: liquid crystalline media and polyacrylamide gels, described further below.

1.3.1 Liquid crystalline media

Liquid crystals can be formed by bicelles or lamellas. They consist of a hydrophobic core and a hydrophilic surface and spontaneously align in the magnetic field. Steric hindrance, caused by the presence of bicelles, causes the ordering of protein molecules dissolved in the liquid crystalline phase. The liquid crystals are arranged with their surface normal orthogonal to the magnetic field (Figure 6) [13]. Many different media have been developed, but none can be used in all applications. In this study, a non-ionic phase composed of a mixture of *n*-alkyl-poly(ethylene glycol) and *n*-alkyl-alcohol has been used. Previous studies by Rückert and Otting have shown that this particular phase is stable in temperatures ranging from approximately 10 to 40°C depending on the alkyl chains chosen [14]. This medium will henceforth be referred to as the Otting phase. Since it is non-ionic, it is not pH-sensitive and only

slightly sensitive to salt concentration. It has been reported to work with more or less any protein without causing denaturation [14].

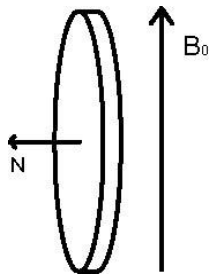

Figure 6: Model for the alignment of the bicelles in the magnetic field.

1.3.2 Polyacrylamide gels

Porous gels can be used to order proteins [15]. In ordinary polyacrylamide gels, the pores are generally spherical, resulting in an isotropic phase. The pores can however be modified to ellipsoids by stretching or compressing the gel. Tycko and co-workers have shown that this gives a preferred orientation [15]. Our efforts have been concentrated on stretching the gels. Two approaches have been suggested for stretching the gels. For both methods, you need a gel with a diameter somewhat larger than the NMR tube. The first is to force the gel through a funnel into the NMR tube [16]. The alternative is to shrink the gel by dehydration and re-swell it in the tube [17]. In this project, the latter method has been used.

1.3.3 Other systems

Filamentous phages are a third category of partially ordered media. The main problem with this method is a tendency of phage aggregation at low pH and low temperature, which makes measurements of dipolar couplings difficult [18].

Purple membrane fragments from bacteriorhodopsin can also be used [19]. However, if the salt concentration in the sample is low, they tend to bind to the protein and the NMR data is affected negatively as the peaks are broadened and the conformation of the protein may be altered [20].

1.4 Aim of the project

The aim of the project was to produce the data necessary to calculate the two separate structures of E140Q. This included measuring dipolar couplings from NMR in ordered phases and also expressing and purifying the protein.

2 Material and methods

2.1 Expression and purification of calmodulin E140Q

Tr_2C E140Q was grown in *Escherichia coli* strain MM294 with the temperature- and IPTG-induced plasmid PICBWR amp^R in minimal medium containing $^{15}NH_4Cl$ as the sole nitrogen source. Purification was performed in a few chromatography steps, as follows. The cell suspension (20 mM imidazole, 20 mM NaCl, 1 mM EDTA at pH 7.0) was sonicated and heated to 70°C, before the protein containing solution was pumped onto a DEAE-cellulose column and eluted with a salt gradient (0.1–0.5 M NaCl). The protein-containing fractions (identified with UV spectroscopy and agarose electrophoresis) were freeze-dried. After solvation, the protein was applied onto a Sephadex® G50 superfine column in 50 mM NH_4Ac at pH 6.0. The protein fractions were put on a DEAE-Sephadel® column and eluted with a salt gradient (0.05–0.4 M NaCl). The pure fractions (identified with UV spectroscopy and electrophoresis) were pooled and dialysed before final freeze-drying. The expression system and purification procedure is further described by Evenäs et al. [6].

2.2 Partially ordered media

2.2.1 The Otting phase

In this study, a 5% solution of *n*-dodecyl-penta(ethylene glycol) and hexanol was used. NMR samples contained approx. 600 µl, 570 µl 90% H_2O/10% D_2O (or protein solution) and 31.5 µl *n*-dodecyl-penta(ethylene glycol). During shaking, 8 µl of hexanol were added in microlitre steps. For the protein samples, a fraction of the water was exchanged with a protein stock solution. The stock solution was made with 0.2 mM NaN_3, 0.1 mM DSS and approx. 20 equivalents $CaCl_2$. The pH of the final solution was adjusted to 6.0.

2.2.2 Strained polyacrylamide gels

The gels were made from 6% acryl amide (standard 30:0.8 mixture) with 0.1% ammonium persulfate and 0.5% TEMED in water. The gels were dried and thereby shrunk, put into the NMR tube and re-swollen with the solution of interest (protein or D_2O). Dichlorodimethylsilane was used to reduce the friction between the glass and the gel.

2.3 NMR spectroscopy

All measurements were made on a Varian Inova spectrometer operating at a proton Larmor frequency of 599.89 MHz. Except for the temperature dependent measurements, all data was recorded at 28ºC. The ordering of the different media was estimated by measuring the quadrupolar coupling of deuterium in D_2O with a simple one-pulse experiment.

2.3.1 Protein NMR

Protein concentrations ranged from 0.5 to 1.5 mM. To study the influence of the medium on the protein, an unlabeled protein sample was prepared. When the quadrupolar coupling of the D_2O was stable, a NOESY experiment was performed. The experiment was repeated after two weeks with the same sample. To verify further

the integrity of the protein in the phase, a ^{15}N-HSQC spectrum was recorded on a uniformly labelled sample.

IPAP spectra were performed on the ^{15}N-labelled sample in the Otting phase. For comparison, IPAP spectra were also recorded under isotropic conditions. HSQC and IPAP spectra were recorded with 128×1024 complex point and spectral widths of 1500 and 8000 in the ^{15}N and ^{1}H dimensions, respectively.

2.3.2 Data processing and analysis

All NMR data were processed with nmrPipe [21]. Prior to Fourier transformation, the FIDs were multiplied with a Lorentzian to Gaussian window function in the direct dimension and with a phase-shifted sine bell function in the indirect dimension to enhance resolution, i.e. to reduce peak overlap. For the IPAP experiments, the in-phase and anti-phase spectra were added and subtracted as described above. The spectra were visualised with nmrDraw [21]. The peaks of the spectra were picked and assigned according to previous studies [6]. For the "add" and "subtract" spectra of the IPAP experiment, the shift differences of the corresponding peaks were calculated. The process was automated by a home-written script. The RDCs were calculated by subtracting the scalar couplings measured in the isotropic phase from the total coupling in the aligned media.

The measured RDCs were compared with the calculated dipolar couplings in the two forms of wt-Tr_2C. To calculate these dipolar couplings, the software PALES was used [22]. The software calculates the dipolar couplings as they should be in a liquid crystal medium. The correlation coefficients for the various structure elements were calculated.

The dipolar couplings were also used to calculate "average structures" in Module [23] from the PDB coordinates from the wt-apo and wt-Ca^{2+} structures. The input data consisted of a coordinate file and a table of dipolar couplings. The molecule was divided up in six parts, corresponding to each helix, the linker between the two EF-hands and the loop region. The program calculates the alignment tensors for the different parts and aligns them accordingly. The different parts were subsequently manually translated and rotated in 180º steps to fit together.

3 Results and discussion

3.1 Protein expression and purification

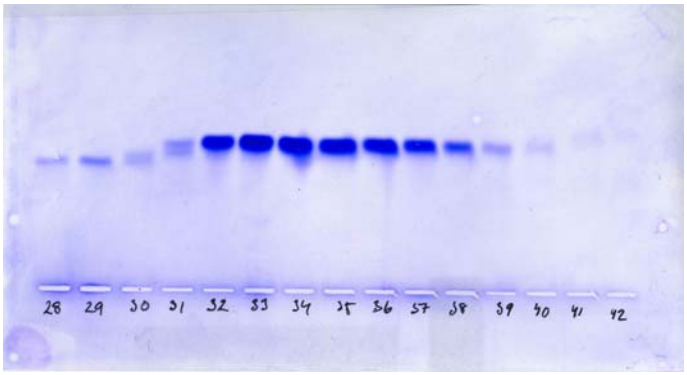

Figure 7: Agarose electrophoresis gel stained with Coomassie Blue. The different lanes represent fractions collected after the last chromatography step in the purification. Lanes 31–39 contain the correct form of E140Q.

The electrophoresis gel (Figure 7) after the last purification step showed that the protein was pure. The protein in lane 40 had travelled a bit further than in the earlier lanes due to deamidation and therefore excluded. The total protein yield from purification fractions 31–39 was 43.5 mg. Compared with previous studies, the HSQC spectrum (Figure 8) of this batch showed a correctly folded protein with all the expected chemical shifts and no extra peaks.

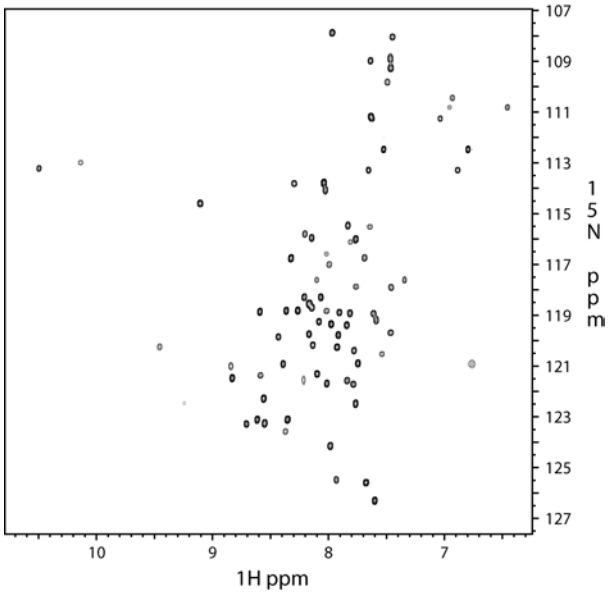

Figure 8: HSQC spectrum of calmodulin E140Q that was purified in this study.

3.2 Partially ordered media

The goal of this project was to measure dipolar couplings for E140Q, but first the alignment medium had to be studied. This was done by measuring the quadrupolar coupling of the deuterium signal in D_2O in the ordered media. The quadrupolar coupling is, as the dipolar coupling, affected by the order of alignment in the media and can thus be used as an indicator of ordering.

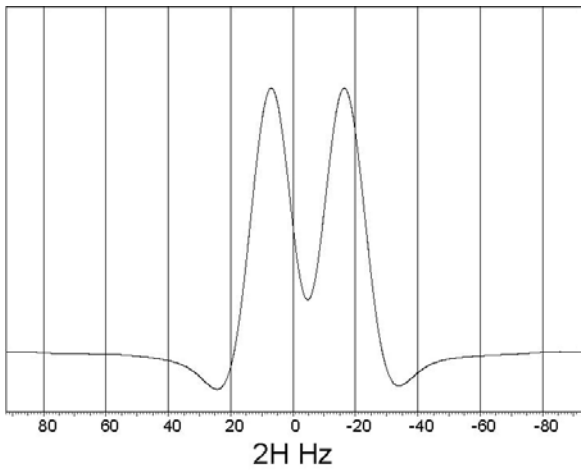

Figure 9: Quadrupolar coupling of the deuterium signal in D_2O in the ordered media.

The ordering of the Otting phase is dependent on the temperature. The temperature dependence of the ordering was minimal in the interval 10-28ºC. The average quadrupolar coupling in this temperature range was 31 Hz, varying only by 3 Hz (Figure 10). If the temperature is too high (approx 35-40ºC), the function of the phase is abruptly lost as the phase separates, if the temperature is too low, the phase does not orientate to the same extent as it does at higher temperatures [14]. These measurements were conducted as two series. This most likely explains the discontinuity of the coupling at 18ºC (Figure 10). If the medium had been allowed to re-orient for a longer time-period between the individual experiments, the spread had most certainly been reduced. At higher temperatures, the phase ordering is slower than it is at lower temperatures although the same maximum value is achieved.

A relatively large temperature span can be used for measuring dipolar couplings in this phase. The exact values of the temperature interval where the phase can be used depend on the alcoholic content. If more alcohol is added, the temperature range is shifted towards lower temperatures, but otherwise the alignment of the phase is the same [14]. Compared with the data published by Rückert and Otting, the present data is very similar [14]. The quadrupolar coupling is in the same range, even though the temperature range differ somewhat. This could be expected, as the *n*-dodecyl-penta(ethylene glycol) content was the same, but not the alcoholic content.

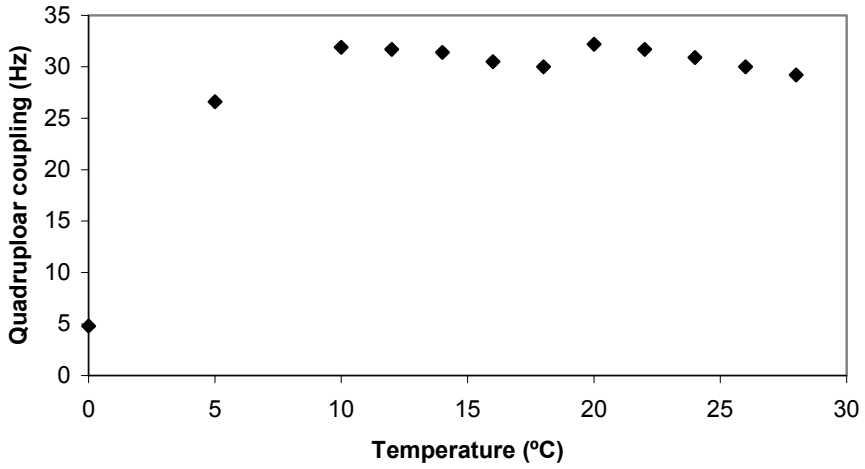

Figure 10: Temperature dependence of the Otting phase. The quadrupolar coupling measured at different temperatures.

As an alternative to the Otting phase, stretched polyacrylamide gels were also tried. The attempted method was that suggested by Sass et al. [17]. Our attempts to re-swell the gels in the NMR tube failed. No visible elongation was observed. This was confirmed by NMR experiments; no quadrupolar coupling was detected in the deuterium signal from D_2O. This was probably due to the friction between the swelling, rather sticky gel and the glass walls of the tube.

In the literature, other methods for swelling gels are available. Maybe one of the most interesting versions is to use a charged polyacrylamide/acrylate gel. This kind of gel swells much more than an uncharged gel due to electrostatic effects. After initial swelling, a pipette can be pushed through the gel. Thereafter it can be shrunk on the pipette, inserted into the NMR tube, re-swollen with the protein solution and the pipette can be extracted. This gives an ordering of the protein not only by steric means, but also by electrostatic [24]. This method is worth trying in the future, since it is very useful to measure dipolar couplings in different media, as all components of the alignment tensor can be determined. To determine fully the tensor, five different media have to be used. As the gel experiments were not successful, the following discussion concerns the Otting phase.

3.3 Characterisation of protein stability in ordered media

When the properties of the medium had been found to be satisfactory, the protein-media interaction was studied. Two factors are important to establish. First the medium must be unaffected by the presence of the protein, secondly the protein must be native and stable.

To verify that the medium remained intact in the presence of protein, the quadrupolar coupling was measured as in earlier experiments. No significant change was observed in either ordering or stability (data not shown). Thus, the medium was functional and unaffected by the protein. This confirmed the findings of Rückert and Otting in their study of the N-terminal domain of the *E. coli* arginine repressor [14].

To characterise the structure of the protein in the Otting phase, a NOESY spectrum and a ^{15}N-HSQC spectrum were recorded. The NOESY spectrum showed the characteristic cross-peaks, i.e. the protein was folded in its native form (data not shown). It can therefore be assumed that the protein was in its functional conformation and that the data collected are reliable. This was confirmed by the HSQC, having the peaks at the expected chemical shifts. The HSQC spectrum shows however that with time the protein denatures. A NOESY-spectrum of a two-week-old sample was recorded and it showed a completely denatured protein, with most of the peaks gathered in a small region of the spectrum (data not shown). This was probably due to hydrophobic interactions between the cleft of the protein when in the Ca^{2+}-like form and the un-charged surface of the bicelles. It should be taken into account that calmodulin has very special features, i.e. the hydrophobicity of the exposed surface is quite large. It would therefore be interesting to perform the same experiment on the apo and Ca^{2+}-forms of the wild-type protein. It could be expected to work well with the apo-form, which exposes a much smaller hydrophobic surface. With the calcium-form, denaturation would be expected. The time scale of the denaturation process was slow enough to allow 2D-HSQC studies, but unfortunately not 3D. In a 3D-spectrum, several couplings can be measured in the same experiment. The analysis of the spectra was also complicated by the fact that the peaks are slightly broadened. When the sample becomes more ordered, the spectrum takes on features from solid state NMR, such as broader peaks. Another possible contribution to the peak broadening could come from a reduction of the transition rate between the two states, due to the interactions with the media. Many peaks are situated in the middle of the spectrum and they tend to overlap if the signals are not narrow and well defined. Otherwise, the peaks had the same chemical shifts as they would have in an isotropic medium.

3.4 Dipolar couplings

The IPAP spectra of E140Q in the medium give the sum of the J- and dipole couplings. Measurements showed that they are not 92 Hz and hence the conclusion must be that the protein was partially aligned. Maybe more interestingly, the different parts of the molecule had different alignment tensors in both size and direction, indicating that the protein not had a single static structure, but mobile helices moving with respect to one another. This supported the hypothesis of two different conformations. The measured dipolar couplings were in the range of –9 to 9 Hz (Figure 11). In the study by Rückert and Otting the range was –10 to 18 Hz [14], but since the measured values were population-weighted averages of two conformations, the spread was reduced.

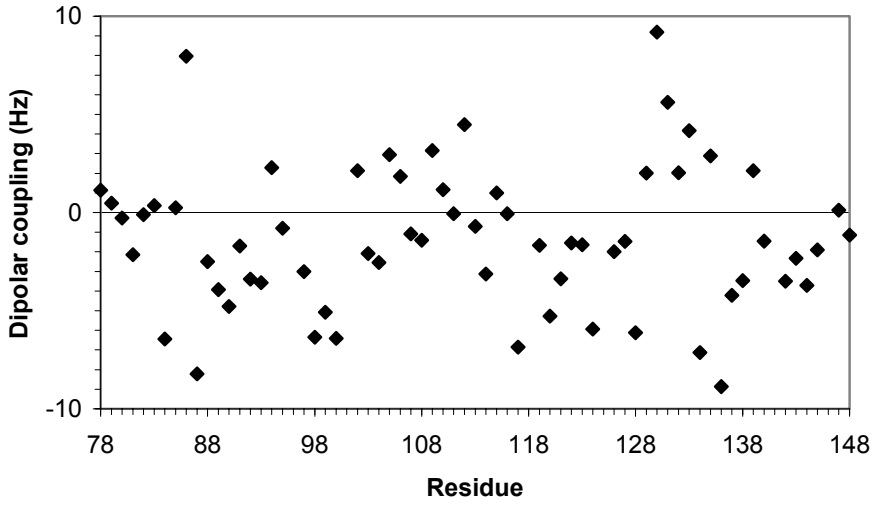

Figure 11: Measured residual dipolar couplings in E140Q.

The structure that can be calculated with these data will be an average structure, if extra measures are not taken to calculate two structures at the same time. When the dipolar couplings were compared with the calculated couplings of the wt-apo and wt-Ca^{2+} forms, a better correlation was seen for the helices, than for the linker and loops. For instance, helix H had a stronger correlation between the wt-apo-form and E140Q than between the wt-Ca^{2+}-form and E140Q. The opposite was demonstrated in helix G (Table 1, Figure 12). This suggested that the helices were partly ordered in the same way as the wt-helices would be in a liquid crystal. It should be emphasised that the calculated RDCs originate from NMR-structures, calculated from proton distances only. The resulting ^{1}H-^{15}N bond vectors can thus have a slightly incorrect direction. This gives an uncertainty to the calculated RDCs, which must be taken into account. If the same calculations had been made with better refined structures, the result would be easier to interpret.

Table1: Correlation coefficient between the measured dipolar couplings for the different structural elements in respect to those calculated from the different wt-structure.

Structural element	Ca^{2+}-Tr_2C	Apo-Tr_2C
Whole structure	0.15	0.05
Helix E	0.37	0.24
Helix F	-0.71	-0.75
Helix G	0.77	0.15
Helix H	-0.22	0.37

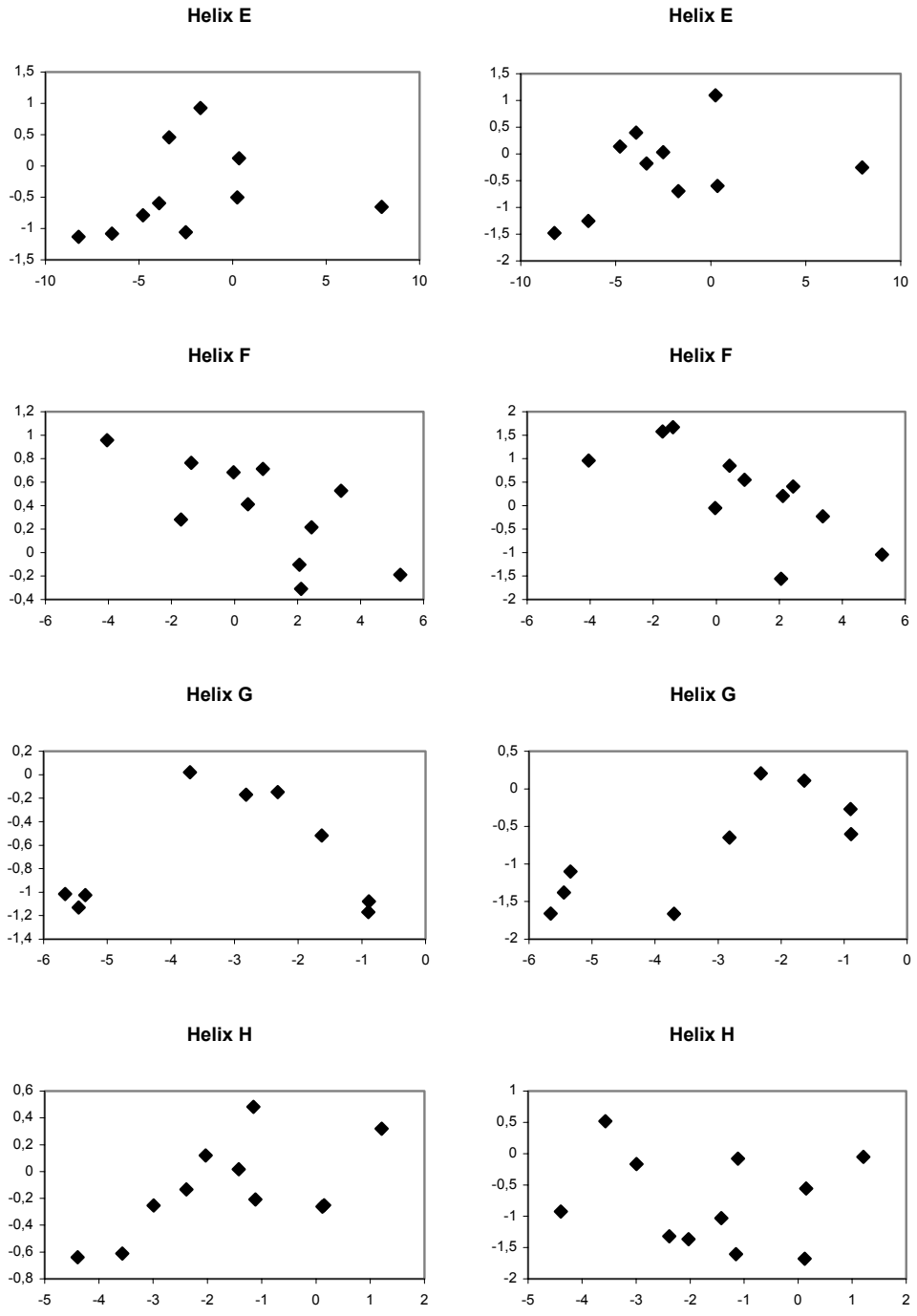

Figure 12: Calculated (y-axis) vs. measured (x-axis) residual dipolar couplings in different structural elements. Calculated couplings are from the apo-form (left column) and the Ca^{2+}-form (right column) respectively and made with PALES.

In order to verify the hypothesis that E140Q changes between the apo and the Ca^{2+} forms, a calculation was made to fit the two structures to the RDCs. This was made by rotating the molecular structures around the principal axis i.e. varying the Euler angles

and minimising the difference to the calculated RDCs. No significant improvement was seen between the results of the calculations with one of the two structures or with both of them. This suggested that the conformations of E140Q do not correspond exactly to the wild-type structures, even though a conformational change is present.

In the program Module, different parts of a molecule can be aligned with the alignment tensor. This gave a molecular structure consistent with the dipolar couplings (Figure 13). The structural models made with Module were well packed and they appeared to be "protein-like" except for the linker between the two EF-hands. This was caused by the fact that all structural elements were regarded as fixed, even though a linker per definition is flexible. The calculated alignment tensors were given with respect to the PDB-file with Euler angles and one term each for the axial and the rhombic component (Table 2). These were thereafter aligned. The result can vary depending on how the molecule is divided in the initial phase of the alignment process. Since the alignment tensors for the helices differ, the helices do not have the same order parameter. It thus indicates that a single-structure model is invalid.

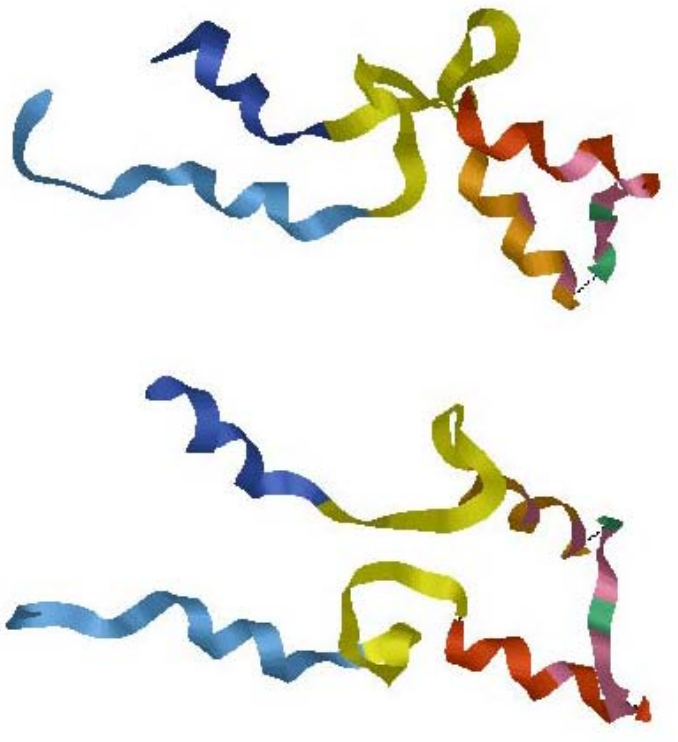

Figure 13: Protein structure of E140Q made in Module from the structure of wt-Tr_2C in its Ca^{2+} form and the measured RDCs. Bottom picture is a top-view. Pink indicates an overlap of side-chains. Additional colours indicate different structural elements.

Table 2: Alignment tensors for the different modules. Module 1 corresponds to the loop region, module 2–5 the helices and module 6 the linker.

	Alfa (º)	Beta (º)	Gamma (º)	Axial (10^{-4})	Rhombic (10^{-4})
Module 1	159.063	100.527	140.084	2.054	1.246
Module 2	-8.922	164.535	1.752	-0.822	-0.506
Module 3	23.783	132.178	105.049	2.264	1.077
Module 4	152.795	91.586	-142.451	3.603	1.772
Module 5	64.416	100.128	93.350	10.680	5.659
Module 6	84.277	61.501	-134.203	-6.731	-3.326

So far our initial attempts to separate the real conformations of E140Q have failed. This was due to the fact that the programs utilise fixed structures. By using only NOEs, no structure separation has been possible to make. In order to do that succesfully an unrealistically large number of NOEs have to be used. If a calculation with both NOEs and RDCs can be made, hopefully it will be possible to separate the structures. In the future perspective is also to measure RDCs in other media, such as gels, and to measure other additional RDCs in the backbone or the sidechains to further investigate the structures of E140Q.

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