# 3. Materials and Methods

This chapter describes the preparation of samples for the spectroscopic measurements. The spectroscopic devices and techniques are described in the following chapter.

## 3.1. Bacterial Strains and Plasmids

ω protein was overexpressed in *E. coli* BL21(DE3) cells carrying plasmid pT712ω (*35*). All the host strains with plasmids overexpressing *ω* or derivatives of *ω* were kindly provided by Dr. J. C. Alonso (Centro Nacional de Biotecnología, Madrid, Spain).

Arc protein was overexpressed in *E. coli* X90(DE3) cells carrying plasmid pSA300 (*24*). All the host strains with plasmids overexpressing *Arc* or derivatives of *Arc* were kindly provided by Dr. R. T. Sauer (MIT, Cambridge, MA, USA).

## 3.2. LB Medium for *E. coli*

| | |
|---|---|
| 10 g/l | NaCl |
| 10 g/l | Bacto-Trypton |
| 5 g/l | Bacto-Yeast Extract |

pH was adjusted with 2 N NaOH (pH = 7.4) and further supplemented with

| | |
|---|---|
| 100 µg/ml | Ampicilin (sodium salt) (only ω protein) |
| 120 µg/ml | Ampicilin (sodium salt) (only Arc protein) |
| 20 µg/ml | Chloramphenicol (only ω protein) |
| 150 µg/ml | Rifampicin (only ω protein) |
| 1 mM | IPTG (for induction) |

## 3.3. FPLC columns and devices

FPLC® System Amersham-Pharmacia (Freiburg, Germany)

Phospho-Cellulose column (diameter 40 mm/ length 25 cm)

SP- Sepharose column (12/40)

Heparin- Sepharose (12/20)

HiLoad™ (16/60) Superdex™ 200

Sephadex G10 (5/2)

## 3.4. Buffers

| | |
|---|---|
| PI | 100 mM NaCl, 50 mM TRIS-HCl, pH 7.5. |
| PII | 50 mM NaCl, 50 mM TRIS-HCl, pH 7.5. |
| PIV | 50 mM NaCl, 10 mM $MgCl_2$, 50 mM TRIS-HCl, pH 7.5. |
| PAI | 200 mM KCl, 1 mM EDTA, 2 mM $CaCl_2$, 10 mM $MgCl_2$, 100 mM TRIS-HCl, pH 8.0. |
| PAII | 200 mM KCl, 0.1 mM EDTA, 5 % glycerol, 50 mM TRIS-HCl, pH 8.0. |
| PAIII | 100 mM KCl, 0.1 mM EDTA, 5 % glycerol, 50 mM TRIS-HCl, pH 8.0. |
| PAIV | 100 mM KCl, 10 mM $MgCl_2$, 50 mM TRIS-HCl, pH 7.5. |

## 3.5. Determination of Protein Purity

The protein purity is an essential factor in Raman difference spectroscopy experiments. Protein impurities might contribute to the Raman spectra and cause unintended artifacts. Therefore, proteins used for spectroscopic measurements were as pure and homogeneous as possible. The purity of the protein samples was controlled by polyacrylamide gel electrophoresis (PAGE) on 15 % sodium dodecyl sulphate (SDS) gels stained with Coomassie blue according to a standard protocol (*74*).

## 3.6. Expression and Purification of ω and Arc Proteins

In this paragraph, the procedure of ω protein purification is described. Conditions used for the Arc protein preparation will be given in square brackets ([]). *E. coli* BL21(DE3) [X90(DE3)] cells carrying plasmid pT712ω [pSA300] were grown in a shaker at 37°C in LB medium. Gene expression was induced at $OD_{600}$ ~ 0.8 [1] by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture. The shaking continued for ~ 2.5 hours in the case of Arc protein. For ω protein, Rifampicin was added to a total concentration of 150 μg/ml after 30 min of shaking and then shaking continued for ~ 1.5 hour. Cells were harvested (8000 rpm, 15 min) and resuspended in ~ 60 ml of buffer PI [PAI]. After that, the cells were lysed with a French Press and cell debris was removed by centrifugation (19000 rpm, 60 min).

Both ω and Arc proteins were purified in four steps as described below and shown in Figure 3.1.

*1st step – Phospho-Cellulose Chromatography*

The supernatant was collected, passed trough a 0.2 [0.45] μm filter, and loaded on the Phospho-Cellulose column equilibrated with buffer PII [PAII]. The column was washed with ~ 0.8 l of buffer PII [PAII], then the elution of bound material was performed with 500 ml of a salt gradient of 50 to 2000 mM NaCl [200 to 1000 mM KCl] at a flow rate of 2.5 ml/min and 10 ml fractions were collected. The ω protein eluted at about 800 mM NaCl [600 KCl].

*2nd step – SP-Sepharose Chromatography*

Fractions containing ω [Arc] protein were pooled, dialyzed against the buffer PII [PAII] and loaded on the SP-Sepharose column. The column was washed with 250 ml of buffer PII [PAII] and then eluted with 200 ml of a salt gradient of 50 to 1000 mM NaCl [200 to 1000 mM KCl] at a flow rate of 1.5 ml/min. Four minute fractions were collected and the ω protein eluted at around 600 mM NaCl [400 KCl].

*3rd step – Heparin-Sepharose*

Pooled fractions containing omega protein [Arc] were dialyzed against buffer PII [PAIII]. The column was washed with 200 ml of buffer PII [PAIII] and the protein was eluted with 200 ml of a linear gradient of 100 to 1000 mM NaCl [KCl] at a flow rate of 1 ml/min. 4 ml fractions were collected and protein eluted at ~ 400 mM NaCl [250 mM KCl]. In some cases, this step was omitted and the protein directly purified by gel filtration after the SP-Sepharose chromatography (Figure 3.1).

*4th step – Gel filtration chromatography*

As final purification step samples were gel filtrated on a HiLoad (16/60) Superdex 200 column equilibrated with buffer PIV [PAIV]. Pooled fractions from the 3rd purification step were dialyzed against buffer PIV [PAIV] and loaded on the column. Proteins eluted as single peaks. Peak fractions of the ω protein that does not contain tryptophan were controlled by measuring fluorescence spectra. Fractions characterized by the tyrosine spectrum only were pooled and used for sample preparation.

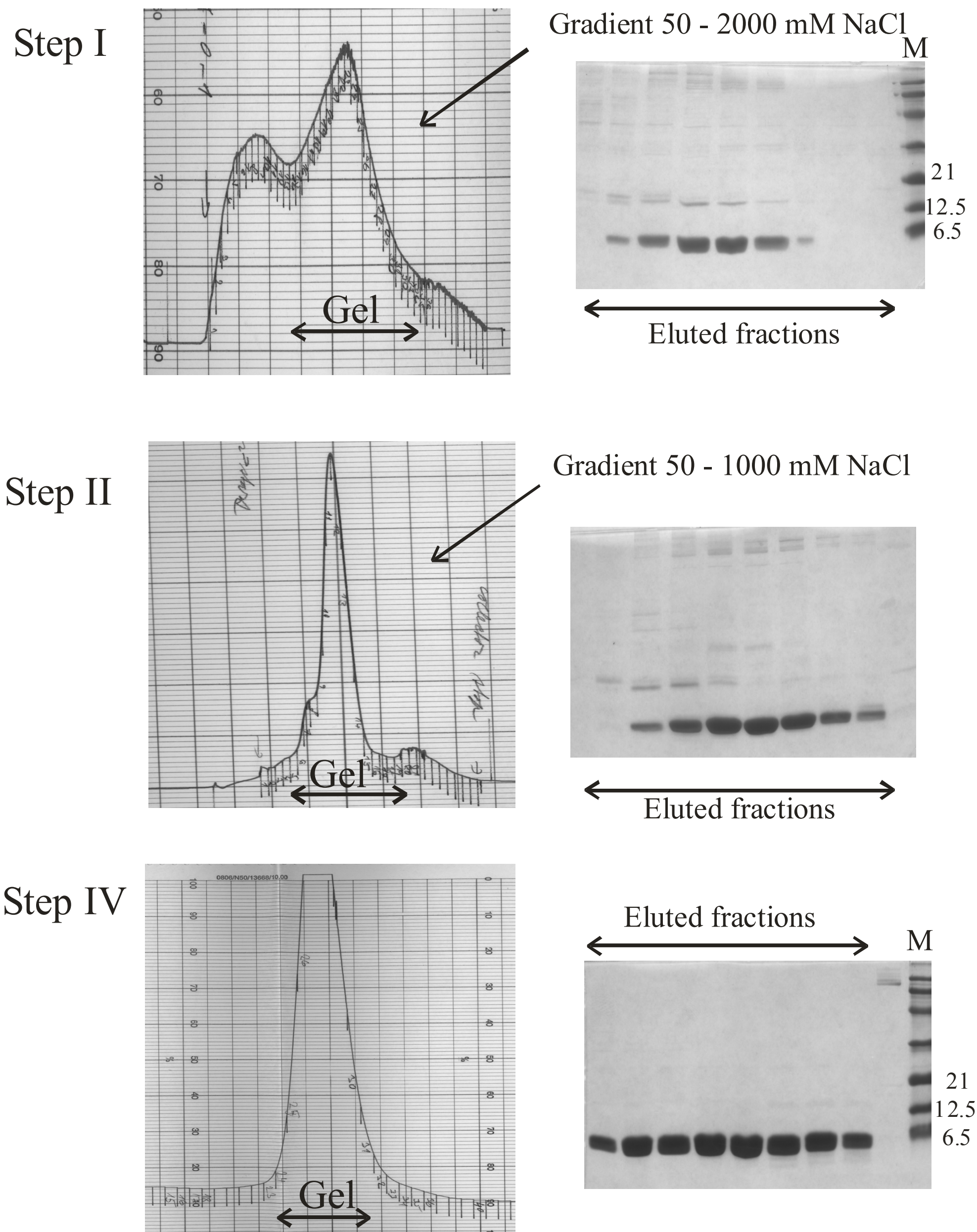


Figure 3.1: Purification of ω protein. Elution profiles of the three purification steps and SDS PAGE gels of the eluted fractions are shown (M: marker). Step I: phosphocellulose chromatography; Step II: SP-Sepharose chromatography; Step IV: gelfiltration.

## 3.7. Oligonucleotide Preparation

Single stranded oligonucleotides were purchased from the Department of Functional Genomics and Proteomics of the Masaryk University Brno, Czech Republic. Alternatively, they were synthesized by Biotez, Berlin, Germany. Equimolar amounts of each strand were mixed, heated to 95°C for 10 min, and annealed by slow cooling. Duplex formation was controlled by gel filtration using a HiLoad (16/60) Superdex 200 column equilibrated in buffer PIV or in PAIV (for Arc operator DNA). The double stranded oligonucleotides

eluted first in peaks of very high intensity followed by small peaks of the single stranded oligonucleotides. The top fractions of the duplex peak were pooled and used for sample preparation.

## 3.8. Sample Preparation

### 3.8.1. ω and Arc–DNA Complexes

Proteins, operator and non-operator DNA were concentrated in a 5K cutoff Ultrafree Millipore membrane (Millipore, Bedford, MA, USA) to final concentrations of over 100 mg/mL and ~60 mg/mL, respectively. The concentration of proteins were estimated from the absorbance at 278 nm using for both proteins an absorption coefficient of $\varepsilon_{278nm}$ = 7000 $M^{-1}\cdot cm^{-1}$ calculated from the amino acid sequence with the Expasy server. DNA concentrations were determined in the same way at 260 nm. Absorption coefficients were calculated from the base composition according to *(75)*. Complexes of Arc proteins with operator and non-operator DNA were prepared by mixing proteins and DNA in 2:1 molar ratio. Concentrations of free DNA and Arc protein samples that were used for complex preparation were then adjusted with buffer PAIV to concentrations that were identical to the components in the complex. For example, 7 µl of DNA and 8 µl of protein were used to prepare the complex. Then, the DNA and protein samples used for measurements were diluted with 8 µl and 7 µl of buffer, respectively.

Free ω protein and DNAs were prepared in the same way as the components of Arc–DNA complex. The complexes of ω proteins with several operator and non-operator DNA models (see Figure 5.1) were prepared by mixing appropriate amounts of the components dissolved in buffer PIV, to produce a 1:1 molar ratio of ω dimer ($\omega_2$) to DNA-heptad. For example, $\omega_2$ protein was mixed with operator DNA composed of tetraheptads in 4:1 molar ratio in order to cover all four omega binding sites on the oligonucleotide. A mixture of $\omega_2$ with the non-operator DNA was prepared in 2:1 molar ratio. The precise concentrations of particular complexes are given in the figure legends of chapter 5.1. Final concentrations of complex samples were in the range of 80-90 mg/mL. All solutions were incubated for at least 12 h before any measurement.

### 3.8.2. KorB–operator DNA complexes

All KorB protein, operator DNA and complex samples were provided by D. Khare (MDC, Berlin, Germany). The samples were prepared in following way:

A gene encoding the two proteins KorB-O and KorB was overexpressed in *E. coli*, after induction with IPTG, and its product was purified similar to the preparation of wild-type KorB described elsewhere (*44*). For spectroscopic measurements the samples were finally purified by gel filtration through a superdex 75 column, equilibrated with 20 mM Tris-HCl pH 7.6 and 50 mM NaCl. The protein samples were further concentrated in 5K MWCO Ultrafree Millipore tubes. Protein concentrations were determined from the absorbance at 280 nm using an extinction coefficient of 13,940 $M^{-1}\cdot cm^{-1}$ for KorB-O and for wild type KorB protein at 278 nm using an extinction coefficient of 23,800 $M^{-1}\cdot cm^{-1}$ (calculated using the Expasy server).

Oligonucleotides were purchased from Biotez, Berlin, Germany. The sequences of both strands are (5'-A$^{Br}$UTTTAGCGGCTAAAAG-3' / 5'-C$^{Br}$UTTTAGCCGCTAAAA$^{Br}$U -3'). Concentrations of DNA were determined spectrophotometrically using the extinction coefficient $\varepsilon_{260nm}$ = 271,222 $M^{-1}\cdot cm^{-1}$. Equimolar amounts of each strand were mixed, heated to 95°C, annealed by slow cooling and purified by gel filtration using a superdex 75 column equilibrated in 20 mM Tris-HCl buffer pH 7.6 and 50 mM NaCl. Peak fractions were concentrated in 5K MWCO Ultrafree Millipore tubes.

The KorB-O–DNA complex was prepared by mixing KorB-O and 17-bp $O_B$ DNA in 2:1 molar ratio and subsequent gel filtration. Wild type KorB–DNA complex was prepared by mixing KorB and DNA in 1:1 molar ratio. The column and the buffer used were the same as in the final purification steps described above for the individual components. The samples were concentrated in a Millipore Ultrafree centrifugal filter device. The concentration of the complex was determined by measuring the absorbance at 260 nm and using the DNA extinction coefficient.

Concentrations of the samples used for Raman measurements were 15.4 mg/mL for DNA, 22 and 34.2 mg/mL for KorB-O, and 62 and 59 mg/mL for the KorB-O–DNA complex. In the measurement of KorB–DNA complex, concentrations were 15 mg/mL for DNA, 102 mg/mL for KorB, and 72 mg/ml for KorB–DNA complex.

### 3.8.3. Sac7d–DNA Complex

Sac7d protein was received from Dr. A. H.-J. Wang (Academia Sinica, Taipei, Taiwan) and prepared in his laboratory in the following way. Recombinant Sac7d was

overexpressed in *E. coli*, after induction with IPTG, and purified as described elsewhere (*61*). The purified protein was dialyzed against deionized water and lyophylized. The lyophilized Sac7d protein was dissolved in buffer PIV and dialyzed against this buffer. After that, the protein was filtered through a Superdex 200 column, equilibrated with buffer PVI. Both Sac7d protein and d(GAGGCGCCTC)$_2$ were concentrated in Centricon YM-3, 3000 MWCO Millipore tubes up to 60 and 40 mg/mL, respectively. Complexes were prepared by mixing appropriate amounts of both components in 1:1 molar ratio. Final concentrations were 11 and 12 (13) mg/ml for DNA, 12 and 22 (17) mg/ml for Sac7d, and 11 and 50 (36) mg/ml for the Sac7d–d(GAGGCGCCTC)$_2$ complex. Concentrations given in parentheses are for samples in $D_2O$ buffer.

### 3.8.4. Hydrogen/Deuterium Exchange

Hydrogen-deuterium exchange was accomplished by incubation of protein and DNA in $D_2O$ based buffer PIV (50 mM Tris-DCl, pD 7.5, 50 mM NaCl and 10 mM $MgCl_2$) and passing the samples through a homemade Sephadex G10 (Pharmacia) column of 2 cm length. The column was equilibrated in $D_2O$ with buffer PIV. Protein and DNA samples elute in the void volume and were fully separated from the $H_2O$ solvent. This procedure accomplishes only H→D exchange (OH→OD and NH→ND) of hydrogen atoms that are not protected by the native fold of the protein (buried in the hydrophobic core) or by covalent bonds. Protein and DNA samples were H/D exchanged separately. Then, concentrations of the components were determined and finally, complex was prepared as described in Chapter 3.8.1.

## 3.9. Determination of Complex Formation

Under native PAGE conditions, polypeptides and DNA retain their higher-order structure and the ability to interact with other polypeptides or nucleic acids. The electrophoretic mobility of DNA and protein–DNA complexes depends on many factors, including size, shape, and surface charge. Complex formation was checked from the standard Laemmli 20 % SDS protocol but omitting the SDS from both gel and tank buffer solutions. Gels were stained with 0.001 % ethydiumbromide. Bands containing the DNA were then visualized by UV light using the Biometra system and photographed (Figure 3.2).

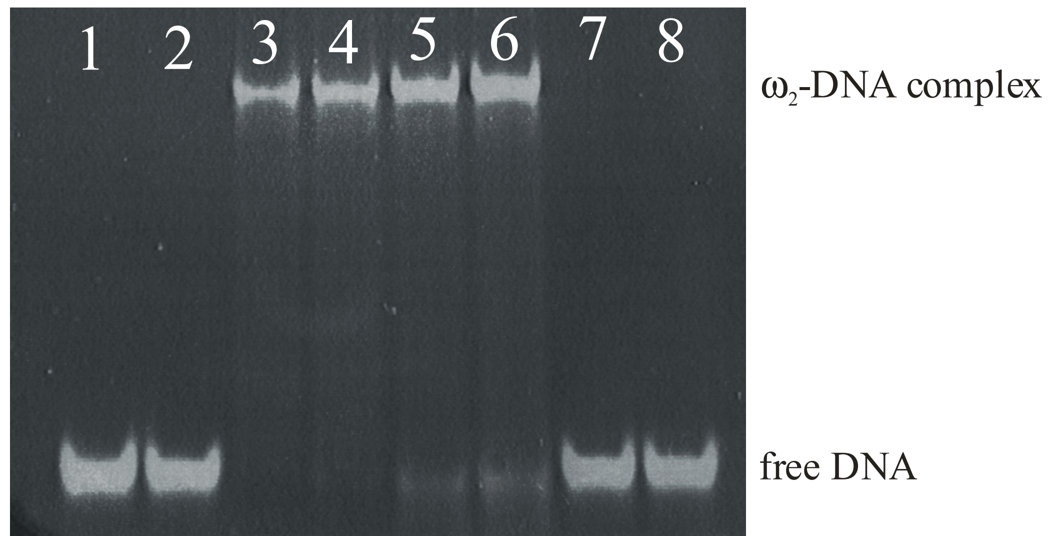


Figure 3.2: Native gel of $\omega_2$-DNA complexes. Lanes 1, 2 and 7, 8 show fast moving free DNA with two heptads in $\longrightarrow_2$ (lanes 1 and 2) and $\longleftrightarrow$ (lanes 7 and 8) orientation, respectively. Lanes 3, 4 and 5, 6 show the slow moving $\omega_2$-DNA complexes: $\omega_2$ - $\longrightarrow_2$ complex (lanes 3 and 4); $\omega_2$ - $\longleftrightarrow$ complex (lanes 5 and 6).