2. Materials and Methods

2.1. Bacterial strains and plasmids

2.1.1. *Streptococcus pyogenes* β recombinase

The β recombinase gene is from *Streptococcus pyogenes* broad-host-range low-copy-number plasmid pSM19035 (*42, 43, 157*). β recombinase protein (wild type) was expressed in *Escherichia coli* BL21 (DE3) (*158*) containing plasmid pBT241 (*35, 159*). The host strain containing plasmid was kindly provided by Prof. J.C. Alonso, Centro Nacional de Biotecnología, Madrid, Spain. β recombinase protein was prepared by Anshul Bhardwaj at MDC, Berlin, Germany.

2.1.2. *Escherichia coli* RelB, RelE and RelBE Complex

Escherichia coli K-12 MC1000 (*relBE*$^{+}$) were used as standard strain for hosting *RelB* and *RelBE* overexpression plasmids pSC302 and pSC2524$_{HE}$, respectively (*55-57, 105, 160-162*). RelB and RelBE overexpression plasmids and expression vector have been described by Christensen *et al* (*57*).

- The plasmid called pSC302 produces a fusion between RelB and Intein (*163*). ER2566 cells were used for purification of a RelB-intein fusion protein which carries a chromosomal copy of the T7 RNA polymerase gene inserted into *lac*Z (*57*).

- The plasmid pSC2524$_{HE}$ contains *relBE* gene, where *relE* is tagged with six histidines residues for purification convenience. Expected molecular mass for RelB and 6x His-RelE proteins are 9071.4 Da and 12048.1Da, respectively. Mass spectroscopy and sequencing analysis resulted in molecular mass of 8923

Da for RelB (First amino acid *i.e.* N-terminal methionine is missing, and one point mutation possibly lysine, K at 13th position from N-terminal) and 11664 Da for 6x His-RelE protein. Mutated or missing amino acids at C-terminal end of RelE could be a reason for molecular mass discrepancy.

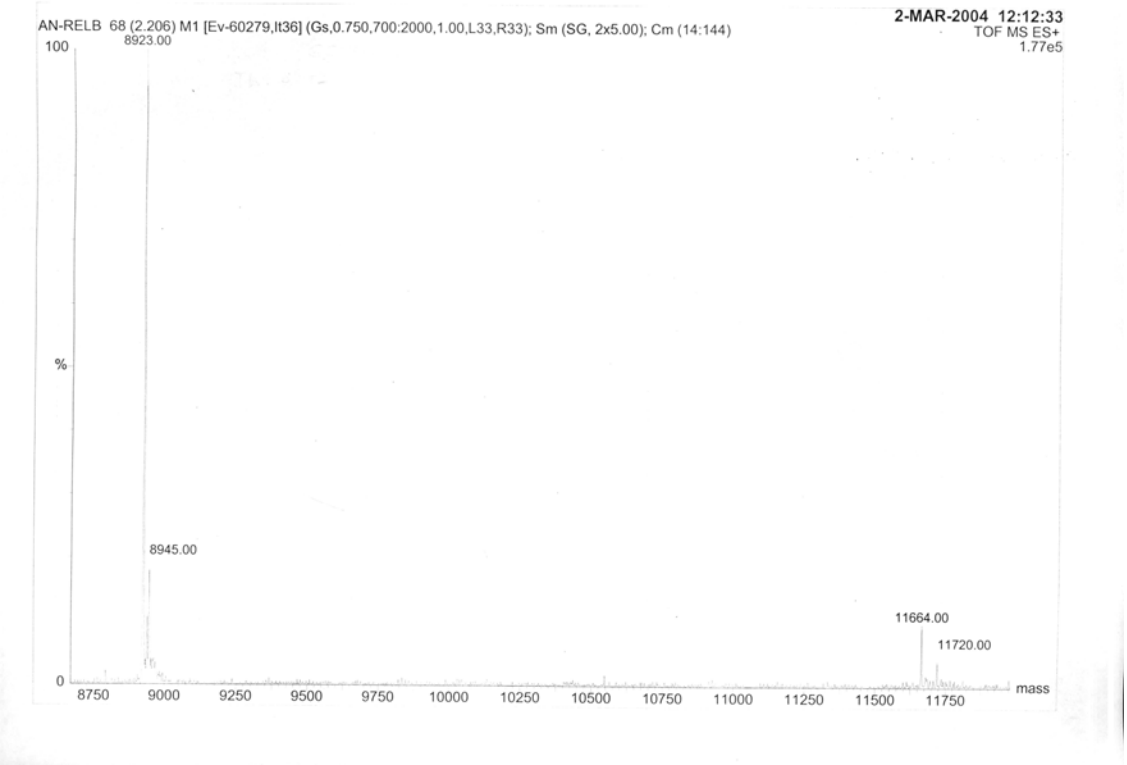

Figure 2.1: Mass spectroscopy analysis of *Escherichia coli* RelBE complex.

2.1.3. *Methanococcus jannaschii* RelBE complex

The *Methanococcus jannaschii* RelBE complex protein was expressed in *Escherichia coli* BL21 cells containing pET21b RelBE(R62S) (*104, 164*). Mass spectrometry and sequencing analysis revealed molecular mass discrepancy in RelE component of the complex, where R (arginine) at 62nd position was replaced by S (serine). The purified protein was generously provided by Djordje Francuski, Prof. W. Saenger, FU, Berlin, Germany.

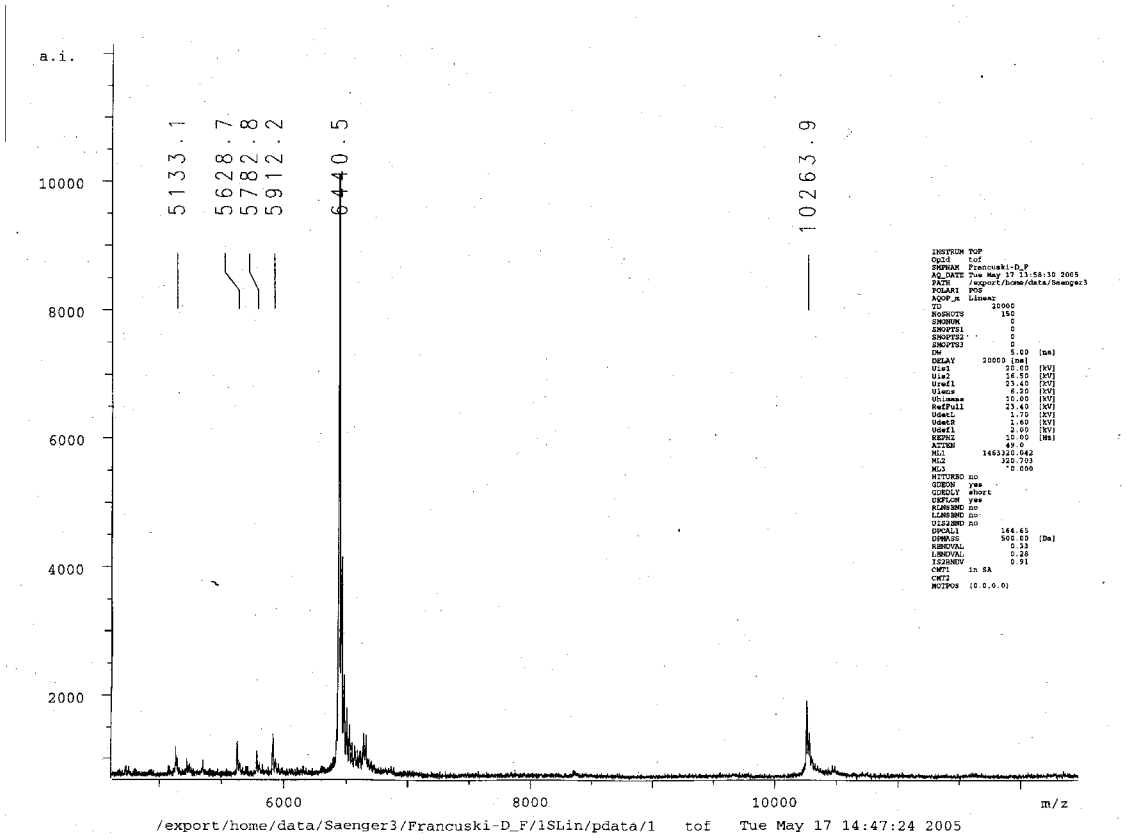

Figure 2.2: Mass spectroscopy of *Methanococcus jannaschii* RelBE complex

2.2. *E. coli* culture medium

Luria-Bertani (LB) Broth

10 g/L	Yeast extract
5 g/L	Tryptone
5 g/L	Sodium Chloride

2xYT medium (Yeast extract tryptone)

16 g/L	Pancreatic Digest of Casein
10 g/L	Yeast extract
5 g/L	Sodium Chloride

2.3. Standard buffers composition used in purification and experiments

Stock solutions

1 M Na_2HPO_4; At 25°C

1 M NaH_2PO_4; At 25°C

Phosphate Buffers (Gomori Buffers) (*165*)

Above stock solutions of Na_2HPO_4 and NaH_2PO_4 are combined, to compute the ratio of the base form to acid form at the desired pH is calculated according to the Henderson-Hasselbalch equation:

$$pH = pK_a + \log\left[\frac{proton - acceptor}{proton - donor}\right] \qquad \text{Equation 2.1}$$

Phosphate-buffered Saline (PBS), pH 7.4; 25°C

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4

2 mM KH_2PO_4

2.4. Preparation of denaturant stock solutions

Urea stock solutions were prepared by weight and the molarities of the solutions were calculated using the equation (*166*):

$$\frac{d}{d_0} = 1 + 0.2658 \times W + 0.0330 \times W^2 \qquad \text{Equation 2.2}$$

where d is the urea solution density, d_0 is the density of water, and W is the weight fraction of denaturant in the solution. The molarities, M, were calculated using the equation:

$$M = 117.66(\Delta N) + 29.753(\Delta N)^2 + 185.56(\Delta N)^3 \quad \text{Equation 2.3}$$

where ΔN is the difference between the refractive indices of the denaturant and buffer solutions; measured at the sodium D line (*167*). All refractive indices were measured with optical refractometer (A.Krüss Optronic, Hamburg, Germany). Urea stock solutions were used if the molarities from the three approaches agreed to within 1% standard error range. Urea stock solutions were used within 24 hours of preparation (*168*). Guanidine hydrochloride stock solutions were prepared similarly, except using the following equation (*169*):

$$\frac{d}{d_0} = 1 + 0.2710 \times W + 0.0330 \times W^2 \quad \text{Equation 2.4}$$

$$M = 57.147(\Delta N) + 38.68(\Delta N)^2 - 91.6(\Delta N)^3 \quad \text{Equation 2.5}$$

2.5. FPLC devices and columns
(Pharmacia/ Amersham Biosciences)

LKB pump-500
LKB controller LCC-500 plus (connected to a LKB REC 102 recorder and LKB FRAC-100 fraction collector)
LKB UV M II detector
Tricorn 10/300 GL Superose™ 12 column
HiLoad™ 16/60 Superdex™ 75 column
Heparin HE20, Cation exchange column

2.6. Expression and purification protocols

2.6.1. *Streptococcus pyogenes* β recombinase

The β recombinase protein was expressed in *E. coli* BL21 (DE3) containing plasmid pBT241. The bacterial cultures were grown from 1:20 diluted overnight culture in a shaker at 37 °C in LB Broth supplemented with 0.1 g/l ampicillin. At the optical density of 0.8 at 600 nm gene expression was induced by 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (Roche GmbH, Mannheim, Germany). The culture was harvested (5,000 rpm, 10 min) 2 h after induction; and cells were resuspended in the lysis buffer containing 1 M Gdn-HCl, 50 mM Tris-HCl, 1 M NaCl pH 7.5 and 5% glycerol in the presence of pefabloc (Roche GmbH) and protease inhibitor cocktail (Roche GmbH). Cells were disrupted in a French press cell at 12,000 psi and resulting extract was centrifuged at 14,000 rpm, 60 min, 4 °C to remove cell membrane and other insoluble material. The supernatant was then dialyzed in step-wise manner against dialysis buffer 50 mM Tris-HCl, 1 M NaCl pH 7.5 and 5% glycerol to remove Gdn-HCl. The dialyzed sample was treated at an optical density of 40 at 260 nm with 0.1% Polyethyleneimine (Serva GmbH, Heidelberg, Germany). After 30 min constant stirring at 4 °C, precipitated DNA was removed by centrifugation. The supernatant was dialyzed against 50 mM Tris-HCl, 0.3 M NaCl and 5% glycerol pH 7.5 and further loaded onto a 100 ml phosphocellulose column (Whatman, VWR International GmbH) pre-equilibrated with dialysis buffer. The column has been washed with dialysis buffer till the UV absorption at 260 nm of the eluant reduced to zero. Protein was eluted by 50 mM sodium phosphate buffer pH 7.5, 1 M NaCl and 5% glycerol and gel filtrated on a Superose 12 column (Amersham Biosciences, Germany). The purified material was stored at -70 °C in the presence of 50% glycerol. With time purified protein tended to aggregate in buffer containing less than 1 M NaCl. Protein concentrations were determined from the

UV absorption at 278 nm in 50 mM sodium phosphate buffer pH 7.5, 1 M NaCl, and 5% glycerol.

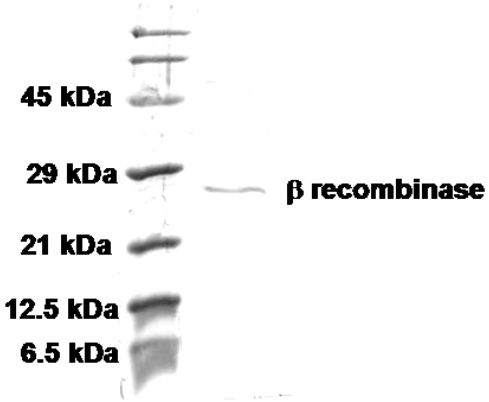

Figure 2.3: SDS-PAGE analysis of purified *Streptococcus pyogenes* β recombinase protein.

2.6.2. *Escherichia coli* antitoxin RelB

The RelB protein was expressed in *E. coli* ER2566 cells containing plasmid pSC302 (pT7::*relB*::*intein*). The cells were grown in Luria-Bertani (LB) Broth supplemented with 0.5 g/l ampicillin at 30°C from a 1:20 diluted overnight culture. At an optical density of ~0.6 at 450 nm; overproducing cells were induced by 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (Roche GmbH, Mannheim, Germany). The culture was harvested 4 h post-induction and cells were resuspended in column/lysis buffer containing 20 mM Tris-HCl pH 8.8, 500 mM NaCl, 0.1% Triton X-100, 0.1 mM EDTA and protease inhibitors (Roche GmbH). Cells were disrupted by French press cell. The disrupted cells were then centrifuged at 14,000 rpm for 30 min, 4 °C to remove cell membranes and other

insoluble material. The supernatant containing soluble protein extract was loaded onto a column of chitin beads (NEB, Germany) and washed with 10 volumes of column/lysis buffer. The cleavage of the fusion protein was induced by 3 column volumes of cleavage buffer containing 20 mM Tris-HCl pH 8.8, 500 mM NaCl, 0.1 mM EDTA, and 30 mM dithiothreitol (DTT, Fermentas, Germany). The column was left at 4 °C for 24 h. RelB protein was eluted with 2 to 3 volumes of cleavage buffer without DTT or protease inhibitors in a fraction of 1/10 column volume. To check the purity, fractions were run on a 15% SDS-PAGE and stained with Coomassie blue (Serva GmbH, Germany). The RelB protein was estimated to be 95% pure. The purified protein was dialyzed against 25 mM Tris-HCl pH 8.0, 100 mM KCl, 1 mM DTT, 1 mM EDTA, and 20% glycerol and stored at -70°C.

Figure 2.4: SDS-PAGE analysis of purified *Escherichia coli* RelB protein

2.6.3. *Escherichia coli* RelBE toxin -antitoxin complex

The RelBE complex was expressed in *E. coli* Top10 cells containing plasmid pSC2524$_{HE}$ (pA1/04/03::relBhis$_6$E). The cells were grown in 2XYT medium supplemented with 0.5 g/l ampicillin at 30 °C from 1:20 diluted overnight

culture. At the optical density of 0.4 at 600 nm; overproducing cells were induced by 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (Roche GmbH, Mannheim, Germany). The culture was harvested 3 h post-induction; and cells were resuspended in the lysis/washing buffer containing 20mM Tris-HCl, pH 8.0 and 100 mM NaCl. Cells were disrupted by French press cell. The disrupted cells were then centrifuged at 14,000 rpm for 30 min at 4 °C to remove cell membranes and other insoluble material. Filtered soluble cell extract was incubated with Talon for 12 h at 4°C as prescribed by manufacturer (Clontech, Germany) for native purification. Column was washed with 10 column volumes of lysis/washing buffer. The complex was eluted with elution buffer containing 20 mM Tris-HCl pH 8.0, 100 mM Imidazole, and 100 mM NaCl. RelBE complex was dialyzed with 50 mM sodium phosphate buffer pH 8.0 and 100 mM NaCl at 4 °C.

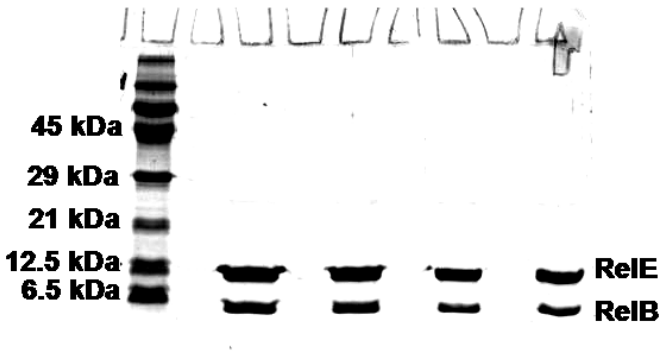

Figure 2.5: SDS-PAGE analysis of purified *Escherichia coli* RelBE complex protein.

2.6.4. *Escherichia coli* toxin RelE

The procedure for RelE purification was the same as for RelBE complex preparation except that RelB protein was washed out with the denaturing

washing buffer. Talon-bound RelBE complex was washed with washing buffer containing 8 M urea, 20 mM Tris-HCl pH 8.0 and 100 mM NaCl. Denaturant washing removes most of RelB protein leaving histidine tagged RelE bounded to Talon. His-RelE protein was eluted from column using elution buffer containing 8 M urea, 100 mM imidazole, 20 mM Tris-HCl pH 8.0 and 100 mM NaCl. The refolding of His-RelE was carried out at 4 °C by step wise dialysis reducing urea concentration from 8 to 0 M. His-RelE was dialyzed against 50 mM sodium phosphate buffer pH 8.0 and 100 mM NaCl; and stored at 4 °C.

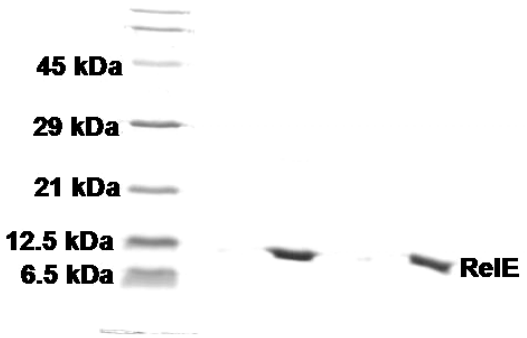

Figure 2.6: SDS-PAGE analysis of purified *Escherichia coli* RelE protein.

2.6.5. *Methanococcus jannaschii* RelBE toxin -antitoxin complex

The *Methanococcus jannaschii* RelBE complex protein was expressed in *E. coli* BL21 cells. The bacterial cultures were grown from 1:100 diluted overnight culture in a shaker at 37 °C in LB Broth supplemented with 0.1 g/L ampicillin and 0.02 g/L chloramphenicol. At the optical density of 0.6 – 0.8 at 600 nm gene expression was induced by 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (Roche GmbH, Mannheim, Germany). All down streaming purification steps were

managed at 4 °C. The culture was harvested (6,000 rpm, 10 min) 3.5 h after induction; and cells were resuspended in the lysis buffer containing 50 mM Tris-HCl buffer, 50 mM NaCl pH 8.0 (Pellet from 4L culture in 30 ml of lysis buffer). Cells were disrupted in a French press cell at 12,000 psi and resulting extract was centrifuged in UC T160 rotor at 30,000 rpm, 60 min, 4 °C to remove cell membranes and other insoluble material. The supernatant was then filtered using 0.22 µm filters (Millipore, Germany). Filtered supernatant was passed through ion exchange chromatography setup containing cation exchanger heparin column.

Running buffer (A): 50 mM Tris-HCl pH 8.0, 50 mM NaCl

Elution buffer (B): 50 mM Tris-HCl pH 8.0, 2 M NaCl

After loading sample, column was washed with 3 column volumes of running buffer. The RelBE Complex was eluted with 1 M NaCl using FPLC gradient of running (A) and elution buffer (B) as follows:

Step 1: 10% B; 7 column volume
Step 2: 40 % B; 1 column volume
Step 3: 55% B; 8 column volume

Step 3 results in RelBE complex elution as single peak. Eluted protein complex was concentrated to the volume less than 1% of the matrix volume of the Superdex S75 size exclusion chromatography column but not more than 10 g/L total protein concentration. Concentrated samples were filtered using 0.22 µm filter and loaded onto Superdex S75 column. 20 mM Tris-HCl pH 8.0, 500 mM NaCl was used as running buffer. Collected samples contain more than 99% pure protein as checked by Tricine-SDS gels.

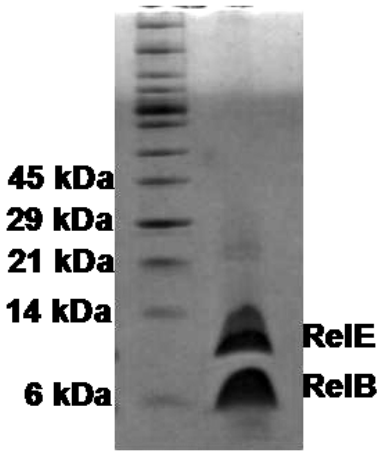

Figure 2.7: 15% Tricine-SDS-PAGE analysis of purified *Methanococcus jannaschii* RelBE complex.

2.7. Determination of purity

Purity of protein is must for the biophysical analysis of proteins. Protein used for analysis should be as pure and homogeneous as possible. The purity of proteins were controlled by single symmetrical elution peak as obtained by analytical runs on Tricorn 10/300 GL Superose^{TM} 12. At the same instance purity of protein was visually checked from 15 % SDS polyacrylamide gels (PAGE) stained with Coomassie blue according to the standard protocol (*170, 171*).

2.8. Determination of protein concentration

Protein concentration plays a very critical role in analysis. Protein concentrations were determined from the UV absorption (UV-Vis Spectrophotometer, Perkin Elmer, Germany) at 278 nm using the molar

absorbance of 9702, 16328, 3037, 13291, and 12739 $M^{-1} \cdot cm^{-1}$ for *Streptococcus pyogenes* β recombinase, *E. coli* RelBE complex, *E. coli* RelB, *E. coli* RelE and *Methanococcus jannaschii* RelBE complex, in monomer units respectively. Molar extinction coefficients of proteins considering their amino acid compositions were calculated using "Biopolymer calculator" on web link http://paris.chem.yale.edu/extinct.html.

2.9. Circular dichroism spectropolarimetry

Circular dichroism spectra in the far ultraviolet (UV) region were measured on a Jasco-J720 spectropolarimeter (Jasco, Germany) fitted with a thermostated cell holder and interfaced with a Neslab RTE-111 water bath.

Standard buffers used for CD measurements

Streptococcus pyogenes β recombinase: 50 mM sodium phosphate buffer pH 7.5, 1 M NaCl, and 5% glycerol.

E. coli RelB, RelE and RelBE complex: 1x PBS, pH 7.4

Methanococcus jannaschii RelBE complex: 20 mM sodium phosphate buffer pH 7.5, 0.5 M NaCl.

The sample concentration should ideally be chosen to give an Emax ~ 0.8, which occurs at protein concentration in the range of 10 - 100 μM. Emax includes absorption from all species in the measured solution including solvent and buffer salts. For this reason, it is important that the chloride concentration is kept at a minimum by dissolving the sample in water.

Molar mean residue ellipticity [θ] in degrees cm^2 $decimol^{-1}$ was obtained as per manufacturers recommendation from the measured raw data after

subtracting the buffer spectrum taking into account the measured ellipticity in millidegrees (θ), molar protein concentration in mol/L (*c*), optical pathlength in cm (*l*), number of amino acid residues (*N*), and molecular mass (M_w) through the relation

$$[\theta] = \theta/(10 \cdot c \cdot l) \qquad \text{Equation 2.6}$$

Mean residue molecular masses of 116.8, 117.15, 114.62, 119.28 and 120.75 Da were used for the *Streptococcus pyogenes* β Recombinase, *E. coli* RelBE complex, *E. coli* RelB, *E. coli* RelE and *Methanococcus jannaschii* RelBE complex, respectively. Typically, CD spectra were measured in the wavelength range of ~180 - 260 nm (scanning rate 50 nm / min). The 0.01 cm path length cylindrical quartz cuvettes were used. Low noise circular dichroism spectra were measured by averaging 5 scans. Data were collected at 0.5 nm intervals using a bandwidth of 2 nm, response time of 1 s and averaging time of 30 s for each measurement.

2.9.1. Secondary structure prediction and determination.

The Variable Selection Program, VARSLC1, was used to estimate the amounts of secondary structure from the far-ultraviolet circular dichroism data of *Streptococcus pyogenes* β recombinase, *E. coli* RelB, RelE and RelBE complex using a basis set of 33 proteins and singular value decomposition (*172*). Henceforth, the number of reference spectra was successively reduced in a search for the smallest set of reference spectra that provided the optimum fit to the protein circular dichroism spectrum under study. *K2d* algorithm has been used for the calculation of secondary structure for *Methanococcus jannaschii* RelBE complex (http://www.embl-heidelberg.de/~andrade/k2d/) (*173*). *K2d* algorithm uses a self-organising neural network to extract from a set of circular dichroism spectra ranging from 200 nm to 241 nm the secondary structure

features present in the data. The secondary structure was predicted from the amino acid sequence using either PHDsec program of the predictProtein package (http://cubic.bioc.columbia.edu/predictprotein/) or Consensus Secondary Structure Prediction (CSSP). CSSP method uses 8 different algorithms including SOPM, HNN, DPM, DSC, GOR IV, PHD, PREDATOR and SIMPA96 available on "Network Protein Sequence Analysis server"; http://npsa-pbil.ibcp.fr.

2.10. Urea and guanidine hydrochloride denaturation

It is well known that chaotropic agents such as urea or Gdn-HCl can disrupt a protein's native structure due to their non-specific interaction with the protein. This event is often referred as the "salt in" effect. This non-specific interaction is believed to migrate into the interior of the protein and form hydrogen bonds to atoms in the backbone (*174*). These bonds destabilize the intra-molecular interactions, which help to maintain the protein's secondary and tertiary structures, driving the protein to adopt a less compact, random coiled conformation. Other studies have shown that the non-covalent crosslinking between the denatured protein and the chaotropic agent reduced the conformational freedom of the protein (*175*).

Equilibrium unfolding studies were carried out by monitoring signal intensity changes by circular dichroism and fluorescence spectroscopy using urea or Gdn-HCl as a denaturant.

Standard buffers used for denaturant unfolding measurements

Streptococcus pyogenes β recombinase: 50 mM sodium phosphate buffer pH 7.5, 1 M NaCl, and 5% glycerol.

E. coli RelBE complex: 20 mM Tris-HCl, 150 mM NaCl, pH 8

E. coli RelB and RelE: 1x PBS, pH 7.4

Methanococcus jannaschii RelBE complex: 20 mM sodium phosphate buffer pH 7.5, 0.5 M NaCl.

Solutions of sample proteins in respective buffers were mixed stepwise with appropriate amounts of the same solution containing 9 M urea or 7.5 M Gdn-HCl to achieve the required concentration of protein and denaturant for the chemical denaturation. The molar concentrations for urea and Gdn-HCl stocks were verified using refractive index measurements as described above. Samples were incubated for 15 h at 4 °C before measurements to achieve equilibrium. The ellipticities were measured at 220 or 222 nm and a cell path length of 0.1 or 0.01 cm; bandwidth was 1 nm, averaging time was 20 s and 5 accumulations were performed per sample. Measurements were conducted at 20 °C.

2.11. Thermal denaturation

Thermal denaturation is one of the conventional protein denaturation methods. It is widely used to study thermal stability of proteins. Compared with other denaturation method, its disadvantage is that the process is irreversible most of the time. The most direct measurement used for protein thermal denaturation is the differential scanning calorimeter. Thermodynamic parameters such as partial specific heat capacity (ΔCp), enthalpy change (ΔH) and Gibb's free energy change (ΔG) can all be calculated from the measurements. Thermal denaturation of a protein can also be followed by its changes in the Far-UV CD spectra or other spectroscopic parameters (*176*).

2.11. (A) *Far-UV CD monitored thermal denaturation*

Thermal unfolding of the proteins was measured at a heating rate of 20 °C/h monitoring changes of the ellipticity at 220 or 222 nm. Temperature curves

were plotted from 5 °C to 90 °C. Slow cooling to 20 °C has been carried out to check the reversibility of unfolding. The upward shift in negative molar ellipticity at 222 nm was chosen as a selective marker for the loss of secondary structure.

2.11. (B) *Differential scanning calorimetry (DSC)*

Excessive heat capacity curves were measured using an ultrasensitive scanning microcalorimeter VP-DSC (MicroCal Inc., Northampton, Ma., USA). Protein solutions were dialyzed extensively against respective buffer. All samples and buffers were filtered through 0.2 µm HPLC membrane filters (Roth, Karlsruhe, Germany). The scans were conducted in a series, in order to reduce artefacts due to thermal history of the instrument. Protein solutions were degassed prior to the measurements and scans were performed from 15 to 90 or 120 °C employing scan rates of 1 K·min^{-1}. The thermodynamic parameters were calculated using the "Origin for DSC" software package supplied by the manufacturer (MicroCal Inc., Northampton, MA, USA). Package options involve subtraction of buffer baseline, defining a linear connect for the native and heat denatured state, and curve fitting of the processed data. The classical two-state model was applied for the deconvolution of the experimental curves. Each transition is characterized by two parameters, T_m and ΔH^{cal}. T_m is the thermal midpoint of a transition, ΔH^{cal} is the calorimetric heat change obtained by the deconvolution of measured excessive heat capacity curve. The basic equations used to deconvolute the DSC data are described in reference (*177, 178*). The van't Hoff enthalpy is related to the shape of the peak by the expression:

$$\Delta H^{vH} = C_{p,\max} \cdot \frac{4 \cdot R \cdot T_m^2}{\Delta H^{cal}} \qquad \text{Equation 2.7}$$

where ΔH^{cal} is the calorimetric enthalpy, $C_{p,max}$ the maximum in the heat-capacity function, R is the gas constant and T_m is the melting temperature.

To obtain the van't Hoff enthalpy, the non two-state curve fitting function of Origin is used. For two state transitions the quotient κ of calorimetric (ΔH^{cal}) and van't Hoff enthalpy changes (ΔH^{vH}), $\kappa = \Delta H^{cal}/\Delta H^{vH}$ should be close to unity. A ratio greater than unity implies that unfolding intermediates are present, and a ratio less than unity indicates that there might be intermolecular interactions, such as changes in the state of oligomerization. The calorimetric enthalpy (ΔH^{cal}) is the total integrated area under the thermogram peak which, after appropriate baseline correction represents the total heat energy uptake by the sample undergoing the transition. This heat uptake depends on the amount of the sample present in the active volume of the DSC cell and is, in principle at least, a model-free absolute measure of the absolute enthalpy of the process involved. On the other hand; the van't Hoff enthalpy (ΔH^{vH}) is an estimate of the enthalpy of the transition based on an assumed model for the process. Here one uses the area under the C_p peak at any temperature, divided by the total area, as a measure of the fraction or extent of unfolding that has occurred at that temperature. Since van't Hoff enthalpy (ΔH^{vH}) relies only on ratios of areas under the experimental curve, it does not require any information about concentration or purity of the sample. The comparison of calorimetric enthalpy (ΔH^{cal}) and van't Hoff enthalpy (ΔH^{vH}) can give information about the apparent mechanism of the process (*178, 179*).

2.12. Fluorescence spectroscopy

Fluorescence emission spectra's were recorded at 20 °C using a Shimadzu RF5001 PC spectrofluorimeter equipped with a thermal controlled cell holder and a cuvette with a 0.3 cm path length. Urea or Gdn-HCl induced denaturation was followed by measuring the changes in samples intrinsic emission fluorescence corresponding to tyrosine or tryptophan between 280 and 400 nm (λ_{ex} = 277 nm; λ_{ex} = 295 nm) at denaturant concentrations of 0 to 6 M.

Bandwidth of 5 nm for both excitation and emission has been used. The intensity of the Raman peak of water was used as an internal standard.

2.13. Size Exclusion Chromatography

Gel filtration experiments were performed using a Tricorn 10/300 GL Superose^{TM} 12 column (Amersham Biosciences, Germany) with a separation range of 2 to 200 kDa connected to an FPLC system (Amersham Biosciences, Germany). 0.1 ml volumes of different samples were analyzed using a flow rate of 0.5 ml/min. Absorbance was monitored at 280 nm. Previously, column was calibrated using commercially available gel filtration low molecular weight standards (Amersham Biosciences). A standard curve of elution volume *versus* log of molecular mass was determined as recommended by manufacturer (Amersham Biosciences).

2.14. Analytical Ultracentrifugation

Sedimentation equilibrium experiments were performed by Prof. J. Behlke at 10 °C on a Beckman Optima XL-A type analytical ultracentrifuge equipped with UV absorbance scanner optics. Proteins were analyzed in native buffers, and in denaturing buffer containing 5 or 8 M urea. Sedimentation equilibrium distribution was analyzed in six-channel cells with loaded centrepieces of 12 mm optical path length filled with ~ 0.07 ml solution. This type of cells allows the analysis of three solvent-solution pairs that were used to analyse simultaneously different samples in one and the same run. Sedimentation equilibrium was reached after 2 h run at over-speed of 34,000 rpm, followed by an overnight run at equilibrium speed of 30,000 rpm. Molecular masses were calculated by simultaneous fitting of the radical distribution curves using program POLYMOLE (*180*).