Biophysical and Structural Characterization of Site Specific β Recombinase and RelBE Toxin –Antitoxin Systems

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Table of Contents

Table of Contents i

List of Figures iv

List of Tables vi

List of Abbreviations vii

Abstract viii

Zusammenfassung xii

Acknowledgements xvi

1. Introduction 1

RECOMBINATION AND PLASMID MAINTENANCE 1

1. (A) Site-specific recombination systems: *3*

1. Resolvases 7

2. DNA invertases 7

3. Resolvo-invertases 8

1. (B) Plasmid addiction systems: *10*

1. The *ccd* locus of the F plasmid 14

2. The *relBE* loci of *Escherichia coli* 15

3. The *higBA* locus of the Rts1 plasmid 16

4. The *parDE* locus of the RK2 plasmid 16

5. The *pem (parD)* and *mazEF (chp)* loci 17

6. The *phd/doc* locus of the P1 plasmid 17

7. The *vapBC* loci 18

8. The ω-ε-ξ locus of plasmid *pSM19035* 18

PROTEIN FOLDING 26

The origin of the question *26*

BACKGROUND *29*

Solvent denaturations 30

Tryptophan fluorescence 31

Far-UV circular dichroism 34

Thermal denaturations 38

Differential scanning calorimetry 39

Multiple unfolding transitions 40

2. Materials and Methods 42

2.1. BACTERIAL STRAINS AND PLASMIDS 42

2.1.1. Streptococcus pyogenes β recombinase *42*

2.1.2. Escherichia coli RelB, RelE and RelBE Complex *42*

2.1.3. Methanococcus jannaschii RelBE complex *43*

2.2. *E. COLI* CULTURE MEDIUM 44

2.3. STANDARD BUFFERS COMPOSITION USED IN PURIFICATION AND EXPERIMENTS 45

2.4. PREPARATION OF DENATURANT STOCK SOLUTIONS 45

2.5. FPLC DEVICES AND COLUMNS 46

2.6. EXPRESSION AND PURIFICATION PROTOCOLS 47

2.6.1. Streptococcus pyogenes β recombinase *47*

2.6.2. Escherichia coli antitoxin RelB *48*

2.6.3. Escherichia coli RelBE toxin -antitoxin complex *49*

2.6.4. Escherichia coli toxin RelE *50*

2.6.5. Methanococcus jannaschii RelBE toxin -antitoxin complex *51*

2.7. DETERMINATION OF PURITY 53

2.8. DETERMINATION OF PROTEIN CONCENTRATION 53

2.9. CIRCULAR DICHROISM SPECTROPOLARIMETRY 54

2.9.1. Secondary structure prediction and determination. 55
2.10. UREA AND GUANIDINE HYDROCHLORIDE DENATURATION 56
2.11. THERMAL DENATURATION 57
2.11. (A) Far-UV CD monitored thermal denaturation 57
2.11. (B) Differential scanning calorimetry (DSC) 58
2.12. FLUORESCENCE SPECTROSCOPY 59
2.13. SIZE EXCLUSION CHROMATOGRAPHY 60
2.14. ANALYTICAL ULTRACENTRIFUGATION 60

3. Site specific recombination system *Streptococcus pyogenes* β recombinase 61

3.1. RESULTS 61
3.1.1. Circular dichroism spectropolarimetry 61
3.1.2. Examination of β recombinase unfolding by intrinsic tyrosine fluorescence 61
3.1.3. Hydrodynamic properties and stoichiometry 63
3.1.4. Denaturant-induced unfolding 65
3.1.5. Temperature-induced denaturation 67
3.1.5.1. Circular dichroism 67
3.1.5.2. Differential scanning calorimetry 70
3.2. DISCUSSION 72

4. *Escherichia coli* RelBE toxin -antitoxin system 77

4.1. RESULTS 77
4.1.1. Circular dichroism spectropolarimetry of Escherichia coli RelB, RelE and RelBE complex 77
4.1.2. Hydrodynamic properties and stoichiometry 79
4.1.2.1. Size exclusion chromatography 79
4.1.2.1.1. Antitoxin RelB mass determination 79
4.1.2.1.2. Toxin RelE mass determination 80
4.1.2.1.3. Toxin -antitoxin RelBE complex mass determination 81
4.1.2.2. Analytical ultracentrifugation 82
4.1.2.2.1. Antitoxin RelB mass determination 82
4.1.2.2.2. Toxin RelE mass determination 83
4.1.2.2.3. Toxin -antitoxin RelBE complex mass determination 84
4.1.3. Denaturant-induced unfolding 85
4.1.3.1. Circular dichroism 85
4.1.3.1.1. Antitoxin RelB Gdn-HCl induced unfolding 86
4.1.3.1.2. Toxin RelE Gdn-HCl induced unfolding 88
4.1.3.1.3. Toxin -antitoxin RelBE complex Gdn-HCl induced unfolding 91
4.1.3.2. Fluorescence spectroscopy 92
4.1.3.2.1. Antitoxin RelB Gdn-HCl induced unfolding 92
4.1.3.2.2. Toxin RelE Gdn-HCl induced unfolding 93
4.1.3.2.3. Toxin -antitoxin RelBE complex Gdn-HCl induced unfolding 98
4.1.3.3. Comparitive analysis of Gdn-HCl unfolding curves obtained from CD and fluorescence measurements. 101
4.1.3.3.1. Toxin RelE Gdn-HCl induced unfolding 101
4.1.3.3.2. Toxin -antitoxin RelBE complex Gdn-HCl induced unfolding 102
4.1.4. Temperature-induced unfolding 105
4.1.4.1. Circular dichroism 105
4.1.4.1.1. Antitoxin RelB unfolding 105
4.1.4.1.2. Toxin RelE and toxin -antitoxin RelBE complex unfolding 107
4.1.4.2 Differential scanning calorimetry (DSC) 109
4.2. DISCUSSION 110
Is antitoxin RelB a natively unfolded protein??? 111
Energetics of the RelBE structure 113

5. *Methanococcus jannaschii* RelBE toxin -antitoxin system 119

5.1. RESULTS 119

5.1.1. Circular dichroism spectropolarimetry of Methanococcus jannaschii RelBE complex *119*
5.1.2. Hydrodynamic properties and stoichiometry *120*
5.1.2.1. Size exclusion chromatography 120
5.1.3. Denaturant-induced unfolding *121*
5.1.3.1. RelBE Gdn-HCl unfolding as monitored by circular dichroism 122
5.1.4. Temperature-induced unfolding *123*
5.1.4.1. Circular dichroism 123
5.1.4.1.1. Native buffer conditions 123
5.1.4.1.2. Denaturing buffer conditions 125
5.1.4.2. Differential scanning calorimetry 127
5.1.4.2.1. *Native buffer conditions* 127
5.1.4.2.2. *Denaturing buffer conditions* 128
5.2. DISCUSSION 133
Stability profile *134*

6. Outlook 136

IMPLICATIONS 138

7. Bibliography 139

8. Publications 153

9. Appendices 154

9.1. MODEL ANALYSIS OF DENATURANT-INDUCED TRANSITIONS 154
9.1.1. Denaturation of monomer *154*
9.1.1. (A) *Two state monomer unfolding* 154
9.1.1. (B) *Three state monomer unfolding* 157
9.1.2. Denaturation of dimer *159*
9.1.2. (A) *Two-state dimer denaturation* 159
9.1.2. (B) *Three-state dimer denaturation* 161
9.1.2. (B) (I) *Three state dimer denaturation assuming monomeric intermediates* 161
9.1.2. (B) (II) *Three state dimer denaturation with dimeric intermediate* 164
9.2. AMINO ACID SEQUENCES (WILD TYPE) 167
9.2.1. Streptococcus pyogenes β recombinase *167*
9.2.2. Escherichia coli RelB *167*
9.2.3. Escherichia coli RelE *167*
9.2.4. Methanococcus jannaschii RelB *167*
9.2.5. Methanococcus jannaschii RelE *167*

Curriculum Vitae 169

Declaration 171

List of Figures

Figure 1.1: The formation and resolution of a Holliday junction in homologous recombination in *E. coli*……………………………………………………………………………………………………4
Figure 1.2: Schematic representation of homologous DNA recombination and recombinase function………………………………………………………………………………………………...5
Figure 1.3: Pictorial representation of functioning of Integrases and Resolvases group of recombinases…………………………………………………………………………………….....6
Figure 1.4: Schematic representation of cell death induced by plasmid-located type II TA modules…………………………………………………………………………………………………11
Figure 1.5: Genetic organization and components of TA loci……………………………………....14
Figure 1.6: Location of known toxin -antitoxin modules on the *E. coli* genome……..……………15
Figure 1.7: Genetic organization and components of the *E. coli relBE* operon……………………20
Figure 1.8: Structures of archaeal *P. horikoshii* RelBE complex……………………………………21
Figure 1.9: Comparison of RelBE from *pyrococcus horikoshii* with MazEF and EF-G…….…….…23
Figure 1.10: The interplay between RelB, RelE and the translational apparatus…………….…..24
Figure 1.11: Pictorial representation of folded and unfolded protein………………………….....29
Figure 1.12: Representative circular dichroism spectra of a folded and unfolded protein.………30
Figure 1.13: Representative urea denaturation fluorescence spectra of protein……………..…..32
Figure 1.14: Representative urea denaturation curve……..…………………………………….……35
Figure 1.15: Fraction of unfolded protein as a function of urea denaturant..…….………………..…36
Figure 1.16: The linear extrapolation method…….…………………….……….…………..….…37
Figure 2.1: Mass spectroscopy analysis of *Escherichia coli* RelBE complex……….……………….43
Figure 2.2: Mass spectroscopy of *Methanococcus jannaschii* RelBE complex…………………...44
Figure 2.3: SDS-PAGE analysis of purified *Streptococcus pyogenes* β recombinase protein..…48
Figure 2.4: SDS-PAGE analysis of purified Escherichia coli RelB protein………………………..…49
Figure 2.5: SDS-PAGE analysis of purified *Escherichia coli* RelBE complex protein…………....50
Figure 2.6: SDS-PAGE analysis of purified *Escherichia coli* RelE protein…………………….….51
Figure 2.7: 15% Tricine-SDS-PAGE analysis of purified *Methanococcus jannaschii* RelBE complex………………………………………………………………………………………………53
Figure 3.1: Far-UV CD Spectra of β recombinase………………………..…………..….……..62
Figure 3.2: Fluorescence emission spectra at varying urea concentrations…………………..…..62
Figure 3.3: Size exclusion chromatography of β recombinase…………………………...……..63
Figure 3.4: Analytical ultracentrifugation of β recombinase………………………………......….64
Figure 3.5: Denaturant-induced unfolding of β recombinase……………………..............….66
Figure 3.6: Thermal unfolding of β recombinase, spectra at selected temperatures……………..68
Figure 3.7: Thermal unfolding of β recombinase.……………………..………….……………..…69
Figure 3.8: Thermal unfolding of β recombinase including renaturation spectra…...................…69
Figure 3.9: Fitted DSC curves of β recombinase……………………………..…….…………...70
Figure 4.1: Far-UV CD spectra of RelB……………………………..……………………77
Figure 4.2: Far-UV CD spectra of RelE…………………………………….……………………77
Figure 4.3: Far-UV CD spectra of RelBE complex…………………………….………...............78
Figure 4.4: Molecular mass determination of RelB by size exclusion chromatography…………80
Figure 4.5: Molecular mass determination of RelE by size exclusion chromatography. ………...81
Figure 4.6: Molecular mass determination of RelBE complex by size exclusion chromatography………………………………………………………………………………..82
Figure 4.7: Molecular mass determination of RelB by analytical ultracentrifugation…................83
Figure 4.8: Molecular mass determination of RelE by analytical ultracentrifugation…………..…84
Figure 4.9: Molecular mass determination of RelBE complex by analytical ultracentrifugation…85
Figure 4.10: Gdn-HCl induced unfolding curve of RelB as monitored by circular dichroism….....87
Figure 4.11: Fit for the Gdn-HCl induced unfolding curve of 0.056 g/L RelB as monitored by circular dichroism………………………………………………………………………………87
Figure 4.12: Fit for the Gdn-HCl induced unfolding curve of 0.4 g/L RelB as monitored by circular dichroism…………………………………………………………..................................88

Figure 4.13: Gdn-HCl induced unfolding of RelE for comparison as monitored by circular dichroism……………………………………………………………………………………………….89
Figure 4.14: Fit for the Gdn-HCl induced unfolding curve of 0.082 g/L RelE as monitored by circular dichroism………………………………………………………………………………………90
Figure 4.15: Fit for the Gdn-HCl induced unfolding curve of 0.4 g/L RelE as monitored by circular dichroism……………………………………………………………………………………………….90
Figure 4.16: Gdn-HCl induced unfolding curves of RelBE as monitored by circular dichroism……………………………………………………………………………………………….91
Figure 4.17: Normalized Gdn-HCl induced unfolding curves of RelBE as monitored by circular dichroism……………………………………………………………………………………………….92
Figure 4.18: Fluorescence emission spectra of RelB at varying Gdn-HCl concentration………..93
Figure 4.19: Fluorescence emission spectra of RelE in the presence of Gdn-HCl denaturant….94
Figure 4.20: Fluorescence emission spectra of RelE at varying Gdn-HCl concentration………..95
Figure 4.21: Gdn-HCl denaturation curve of RelE as monitored by fluorescence………………..96
Figure 4.22: Fit for Gdn-HCl induced unfolding curve of 0.082 g/L RelE as monitored by fluorescence……………………………………………………………………………………………97
Figure 4.23: Fit for Gdn-HCl induced unfolding curve of 0.4 g/L RelE as monitored by fluoreoscence…………………………………………………………………………………………..97
Figure 4.24: Fluorescence emission spectra of RelBE complex in buffer and Gdn-HCl containing buffer…………………………………………………………………………………………………….99
Figure 4.25: Fluorescence emission spectra of RelBE at varying Gdn-HCl concentration in buffer…………………………………………………………………………………………………….99
Figure 4.26: Gdn-HCl denaturation curve of RelBE as monitored by fluorescence……………..100
Figure 4.27: Plot of equilibrium unfolding data monitored by circular dichroism and fluorescence for 0.082 g/L RelE………………………………………………………………………………………101
Figure 4.28: Plot of equilibrium unfolding data monitored by circular dichroism and fluorescence for 0.4 g/L RelE…………………………………………………………………………………………..102
Figure 4.29: Plot of equilibrium unfolding data monitored by circular dichroism and fluorescence for ~0.08 g/L RelBE complex……………………………………………………………………….103
Figure 4.30: Plot of equilibrium unfolding data monitored by circular dichroism and fluorescence for 0.4 g/L RelBE………………………………………………………………………………………..104
Figure 4.31: Plot of equilibrium unfolding data monitored by circular dichroism and fluorescence for 0.8 g/L RelBE……………………………………………………………………......................104
Figure 4.32: Thermal unfolding spectrums of RelB as measured by circular dichroism………………………………………………………………………………………………105
Figure 4.33: Thermal unfolding curve of 0.084 g/L RelB as monitored by change in ellipticity at selective wavelength of 220 nm by CD………………………………………..…………………...106
Figure 4.34: Thermal unfolding curve of 0.0084 g/L RelB as monitored by change in ellipticity at selective wavelength of 220 nm by CD……………………………………………………………106
Figure 4.35: Selected thermal unfolding spectra of RelB (including renaturation spectrum)…..107
Figure 4.36: Selected thermal unfolding spectra of RelE (including back to 20 °C spectrum)....108
Figure 4.37: Selected thermal unfolding spectra of RelBE (including back to 20 °C spectrum).108
Figure 4.38: Raw and fit DSC curves of RelB………………………………………….............109
Figure 5.1: Far-UV CD spectra of *Methanococcus jannaschii* RelBE complex………..............119
Figure 5.2: Molecular mass determination of *Methanococcus jannaschii* RelBE complex by size exclusion chromatography………………………………………………………………………..120
Figure 5.3: Gdn-HCl induced unfolding of *Methanococcus jannaschii* RelBE complex as monitored by circular dichroism…………………………………………….…………………122
Figure 5.4: Thermal unfolding spectra of *Methanococcus jannaschii* RelBE complex as measured by circular dichroism…………………………………………………………..……………..123
Figure 5.5: Thermal unfolding curve of *Methanococcus jannaschii* RelBE complex as monitored by change in ellipticity at selective wavelength of 222 nm by CD…………….…………………124
Figure 5.6: Thermal unfolding spectra of *Methanococcus jannaschii* RelBE complex (including renaturation spectra) ……………………………………………………………….……………..125

Figure 5.7: Smoothed thermal unfolding curve of *Methanococcus jannaschii* RelBE complex in the presence of Gdn-HCl as monitored by change in ellipticity at selective wavelength of 222 nm by CD……………………………………………………………………………………………….**126**
Figure 5.8: Smoothed thermal unfolding curve of *Methanococcus jannaschii* RelBE complex in the presence of Gdn-HCl as monitored by change in ellipticity at selective wavelength of 222 nm by CD…………………………………………………………………………….…………………**127**
Figure 5.9: Differential scanning calorimetry of *Methanococcus jannaschii* RelBE complex…..**128**
Figure 5.10: Differential scanning calorimetry of *Methanococcus jannaschii* RelBE complex in the presence of 1.78 M Gdn-HCl in buffer…………………………………………………….…….**129**
Figure 5.11: Differential scanning calorimetry of *Methanococcus jannaschii* RelBE complex in the presence of 2.78 M Gdn-HCl in buffer……………………………………………………….….**131**
Figure 5.12: Differential scanning calorimetry of *Methanococcus jannaschii* RelBE complex in the presence of 3.85 M Gdn-HCl in buffer……………………………………………………..………**131**
Figure 5.13: Deconvoluted excessive heat capacity curves of *Methanococcus jannaschii* RelBE complex in the presence of Gdn-HCl in buffer………………………………………………………**132**
Figure 5.14: Plot of melting temperatures (T_m) and unfolding enthalpies (ΔH^0) of *Methanococcus jannaschii* RelBE complex *versus* Gdn-HCl concentrations, determined from differential scanning calorimetry measurements……………………………………………………………………….**132**

List of Tables

Table 1.1: Assorted TA systems and targets of their respective toxins……………………….....…**19**
Table 1.2: Aromatic amino acids, their residue volumes, mean percent buried in proteins, hydrophobicity, absorbance and fluorescence properties…………………………………………**32**
Table 1.3: λ_{max} values for tryptophan and two tryptophan models in various solvents…………...**33**
Table 3.1: Thermodynamic parameters for the fit of β recombinase equilibrium unfolding data to a three-state dimer denaturation model with a monomeric intermediate……………………………..**67**
Table 3.2: Thermodynamic parameters for the β recombinase obtained by the model analysis of DSC thermograms………………………………………………………………………………..**71**
Table 4.1: Secondary structure contents of RelB, RelE and RelBE complex……………….……**78**
Table 4.2: Calculated thermodynamic parameters for RelB and RelE by circular dichroism and fluorescence…………………………………………………………………….....................**98**
Table 5.1: Thermodynamic parameters for the *Methanococcus jannaschii* RelBE complex obtained by the model analysis of CD and DSC thermograms…….………………….................**130**
Table 9.1: Equilibrium unfolding of dimeric proteins monitored by spectrometry as expressed in dimeric equivalents…………………………………………………………………...………**166**
Table 9.2: Calculated physico-chemical parameters and available details for proteins…………**168**

List of Abbreviations

Abbreviation	Full expression
aa	amino acid
au	arbitrary unit(s)
BSA	bovine serum albumin
CD	circular dichroism
Da	Dalton
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetra acetic acid
FPLC	fast performance liquid chromatography
G	Gibbs free energy
Gdn-HCl	guanidine hydrochloride
H	enthalpy
I	intermediate state
IPTG	isopropyl-β-D-thiogalactopyranoside
LB medium	Luria -Bertani medium
M	Molar
N	native state
$(protein)_2$	(protein) dimer state
OD	optical density
PBS	phosphate buffered saline
pI	isoelectric point
R	gas constant
RPM	rotation per minute
SDS-PAGE	sodium dodecyl sulphate poly- acrylamide gel electrophoresis
sec	second(s)
T	temperature
TA	toxin -antitoxin
Tris	Trishydroxy-methylaminomethane
U	unfolded state
UV	ultraviolet

Abstract

Recombination and plasmid maintenance are highly coordinated phenomena required for stable plasmid inheritance. To gain biophysical and structural insight in these phenomena, we tried to evaluate the properties of proteins of three important systems, namely β recombinase and the toxin – antitoxin complexes RelBE from *Escherichia coli* and *Methanococcus jannaschii*.

β recombinase, a *Streptococcus pyogenes* pSM19035 –encoded β gene product is a site-specific recombinase (23.8 kDa) that catalyzes resolution between two directly oriented recombination sites (*six* sites), and both resolution and DNA inversion between two inversely oriented *six* sites. Assembly of the synaptic complex requires binding of the β recombinase to the *six* sites and the presence of Hbsu.

In size exclusion chromatography and analytical ultracentrifugation we observed the existence of a stable β recombinase dimer in solution. To better understand the role of β recombinase in recombination, their unfolding mechanisms were monitored by spectropolarimetry, fluorimetry and differential scanning calorimetry. The changes in spectral and thermodynamic properties accompanying β recombinase dimer denaturation under denaturant equilibrium were surprisingly different from the thermal melting. Measured denaturant (Gdn-HCl/urea) induced transitions unfolding curves were biphasic with a bend at 5 M urea and 2.2 M Gdn-HCl characteristic of transient intermediates. Therefore thermodynamic parameters were calculated by fitting experimental curve assuming a three-state dimer denaturation model with monomeric intermediates; this result was further supported by analytical ultracentrifugation experiments which point to molecular mass of monomers in the presence of 5 M urea. Calculated ΔG value; 17.9 kcal/mol from fits were found to be in reasonable agreement, irrespective of denaturant used either urea or Gdn-HCl for unfolding.

Interestingly, thermal unfolding of dimeric β recombinase by differential scanning calorimetry (DSC) with 4.2 μM and 18.5 μM did not reveal the formation of intermediates or different melting domains. The melting curves were characterized by symmetrical peaks and a fit directed the formation of denatured dimers in accordance with model ($D_F \leftrightarrow D_U$). Transition temperature; T_m of ~67.7 °C and calorimetric enthalphy; ΔH^{cal} of ~110 kcal/mol were measured. Therefore, we can define the unfolding of β recombinase in terms of thermodynamic parameters (ΔG, ΔH, C½, and *m*), monitored secondary structure changes, molecular mass changes, and thermal melting in our experimental conditions.

The *Escherichia coli* and *Methanococcus jannaschii* RelBE addiction modules play a crucial role in the cell death program that is triggered under various stress conditions. It codes for toxin RelE and the antitoxin RelB, which interferes with the lethal action of the toxin by direct protein-protein interaction. Additionally, RelE is a very efficient inhibitor of translation, both *in vivo* and *in vitro*. Furthermore RelB autoregulates transcription of *relBE*, and the RelB-RelE complex yields even better repression than RelB alone. Thus RelE is a co-repressor of *relBE* transcription. Considering cellular importance and to shed more light on the system, biophysical studies were made on the RelBE complex, RelB, and RelE from *E. coli* and RelBE complex from *M. jannaschii*.

Secondary structure measurements for *E. coli* proteins RelB, RelE and RelBE complex monitored by circular dichroism yielded highly defined secondary structural elements in our experimental setup. This is a noteworthy point in the view of RelB in solution from the theoretical considerations and due to crystal structure data, which was published for the RelBE complex from *Pyrococcus horikoshii* where an unfolded structure was suggested. The size exclusion chromatography and analytical ultracentrifugation results presented in this report revealed that RelB and RelE in the lowest measured concentration range (4.8 μM RelBE in size exclusion chromatography) interact directly to form a heterodimeric complex in solution. The complex stoichometry found to be a highly concentration dependent

phenomenon. In our size exclusion chromatography experiments RelBE heterodimeric and heterotetrameric complexes were observed. Analytical ultracentrifugation experiments also revealed equilibrium between RelBE heterodimeric and heterotetrameric complexes pointing to a dissociation constant roughly in µM range. RelB and RelE in solution are found to be dimeric at high concentrations, only at low concentration of 0.07 g/L equilibrium molar masses between dimer and monomer have been observed by size exclusion chromatography. Overall, these observations point towards a 1:1 ratio of RelB and RelE proteins to form a stable complex in solution. Dimerization of toxin and antitoxin were found to be a common denominator of toxin –antitoxin systems.

The denaturation unfolding stability curves obtained for RelB and RelE irrespective of method applied to monitor (Circuar dichroism or fluorescence spectroscopy) point towards a concentration dependent unfolding process. However, at low concentration curves direct towards presence of intermediates. With increase in protein concentration denaturant stability of dimer protein increases and leads to coupled dissociation-unfolding process. The experimental curves were fitted with the model assuming “Two-state monomer” and “Two state dimer” unfolding mechanism. Thermodynamic parameters, ΔG, C½, and *m* were calculated from the applied models for RelB and RelE. Unfolding studies monitoring circular dichroism showed that the antitoxin RelB has a significantly lower stability than the toxin RelE. For RelB a surprising reversibility of thermal unfolding (in DSC and CD experiments) was found, unlikely to RelE and RelBE complex which exhibited a strong aggregation tendency at temperatures of ~50 °C and showed no reversibility. A fit assuming “two-state" model for folded to unfolded dimers ($D_F \leftrightarrow D_U$) resulted in a transition temperature, T_m of 60.7 °C with an unfolding enthalpy, ΔH^{cal} of 41.6 kcal/mol.

Unfolding of RelBE complex found to be a complicated mechanism in which the unfolding atleast at high concentrations is a closely coupled dissociation-unfolding process. Under our experimental conditions, analysis of

RelB, RelE and RelBE complex revealed a high stability contribution of RelE in RelBE complex.

Size exclusion chromatography of RelBE complex derived from *M. jannaschii* pointed that complex stoichometry is a highly concentration dependent phenomenon. Denaturant induced unfolding was monitored by circular dichroism and differential scanning calorimetry. Unfolding in the presence of denaturant (Gdn-HCl) was found to be a cooperative process with protein concentration dependence. Similar to *E. coli* RelBE, dissociation of the complex is closely associated with the unfolding of the components. To evaluate thermal melting, excessive heat capacity curves from differential scanning calorimetry had to be measured at increasing Gdn-HCl concentrations (1.78, 2.78 and 3.85 M). Deconvolution revealed apparent values of thermodynamic parameters; enthalpy change, ΔH = 110.3 kcal/mol and melting temperature, T_m = 116 °C obtained by linear extrapolation to 0 M Gdn-HCl.

Zusammenfassung

Rekombination und Plasmiderhaltung sind in hohem Grade koordinierte Phänomene. Um biophysikalische und strukturelle Einblicke in diese Phänomene zu gewinnen, versuchten wir, die Eigenschaften der Proteine von drei wichtigen Systemen zu ermitteln, nämlich von β Rekombinase und den Toxin –Antitoxin-Komplexen RelBE von *Escherichia coli* und *Methanococcus jannaschii*.

β-Rekombinase (23,8 kDa) ist ein vom Plasmid pSM19035 aus *Streptococcus pyogenes* kodiertes Genprodukt. β-Rekombinase katalysiert ortsspezifisch die Trennung von Rekombinationsorten der DNA (six sites), wenn zwei solche Orte direkt orientiert sind, und sie bewirkt deren Trennung und Inversion, wenn die six sites inverse Orientierung haben. Der Aufbau des synaptischen Komplexes erfordert die Bindung der β-Rekombinase an die DNA und die Gegenwart des Proteins Hbsu.

Durch Gel-Permeations-Chromatographie (FPLC) und analytische Ultrazentrifugation konnten wir zeigen, daß β-Rekombinase in Lösung stabile Dimere bildet. Um das Verhalten von β-Rekombinase in der Rekombination besser zu verstehen, wurden einige physikochemische Eigenschaften, insbesondere ihre Denaturantien- und Hitze-induzierte Auffaltung, mittels Circulardichroismus-Messungen Fluoreszenzspektroskopie und Differenzieller Scanning-Kalorimetrie (DSC) analysiert. Die Änderungen der spektralen und thermodynamischen Eigenschaften, die die Denaturierung von Dimeren der β-Rekombinase unter Denaturant-Gleichgewicht begleiten, waren überraschend verschieden vomthermischen Schmelzen. Guanidiniumchlorid- bzw. Harnstoff-induzierte Auffaltungskurven waren biphasisch mit einem Knick bei 2.2 M Guanidiniumchlorid bzw. 5 M Harnstoff und zeigten das Auftreten von transienten Zwischenstufen an. Für die Berechnung von thermodynamischen Parametern wurden die experimentellen Kurven mit einem Drei-Zustands-Modell angepasst, für das die Denaturierung von Dimeren über eine Zwischenstufe aus gefalteten

Monomeren angenommen wurde. Die Ergebnisse wurden unterstützt durch Ergebnisse der analytischen Ultrazentrifugation, die in 5 M Harnstoff, am Knick der Auffaltungskurve, bereits das Vorliegen von Monomeren anzeigten. Die für Guanidiniumchlorid- und Harnstoff-Auffaltung berechneten ΔG-Werte (17.9 kcal/mol) stimmten weitgehend überein. Interessanterweise wurden für die thermische Auffaltung von dimerer β-Rekombinase mittels Differentieller Scanning Kalorimetrie (DSC) bei 4.2 μM und 18.5 μM keine Hinweise für das Auftreten von Zwischenstufen gefunden. Die Schmelzkurven waren durch symmetrische Peaks charakterisiert und legten die Bildung von denaturierten Dimeren gemäß ($D_F \leftrightarrow D_U$) nahe. Übergangstemperaturen T_m (66.7 °C) und kalorimetrische Enthalpy ΔH^{cal} (110 kcal/mol) wurden gemessen. Folglich können wir die Auffaltung von β-Rekombinase im Hinblick auf diese themodynamischen Parameter definieren, wie durch Sekundärstrukturänderungen überwacht, molekulare Masse ändert; und thermisches Schmelzen in unseren experimentellen Bedingungen.

Die sog. Addiction Module RelBE von *Escherichia coli* und *Methanococcus jannaschii* spielen eine entscheidende Rolle für den programmierten Zellentod, der unter verschiedenen Stressbedingungen ausgelöst werden kann. relBE kodiert für Toxin RelE und Antitoxin RelB. Protein RelB verhindert die toxische Wirkung von Protein RelE durch direkte Protein-Protein Wechselwirkung und Bildung eines stabilen Komplexes. RelE ist in vivo und in vitro ein sehr effizienter Inhibitor der Translation. RelB autoreguliert die Transkription von relBE, wobei der RelBE-Komplex sogar eine bessere Repression aufweist als RelB allein. Damit wird das Toxin RelE zum Korepressor der relBE Transkription. In Betracht der zellularen Bedeutung und zur Erhellung seiner Eigenschaften wurden biophysikalische Studien an den Proteinen RelB, RelE und dem RelBE Komplex vom *E. coli* und dem RelBE-Komplex von *M. jannaschii* durchgeführt.

Circulardichroismus-Messungen an den *E. Coli* Proteinen RelB, RelE und

dem RelBE-Komplex, erbrachten unter unseren experimentellen Bedingungen in hohem Grade definierte strukturelle Sekundärelemente. Dieses Ergebnis ist insbesondere für RelB bemerkenswert. Für RelB in Lösung wurde aus theoretischen Erwägungen und auf Grund von Kristallstrukturdaten, die am RelBE-Komplex aus *Pyrococcus horikoshii* erhoben worden sind, eine entfaltete Struktur vorgeschlagen. Die mittels FPLC und analytischer Ultrazentrifugation erhaltenen Resultate zeigten, daß RelB und RelE bereits bei den niedrigsten gemessenen Konzentrationen (4.8 μM RelBE) einen heterodimeren RelBE-Komplex bilden. Die Stoichiometry des Komplexes ist ein stark konzentrationsabhängiges Phänomen. In FPLC-Experimenten wurden heterodimere und heterotetramere RelBE-Komplexe beobachtet. Auch mittels analytischer Ultrazentrifugation wurden Gleichgewichte zwischen heterodimerem RelBE und heterotetrameren Komplexen nachgewiesen, die auf eine Dissoziationskonstante in mikromolaren Bereich hinweisen. RelB und RelE in Lösung liegen bei hohen Konzentrationen als Dimere vor, nur bei niedriger Konzentration (0,07 g/l) wurden mittels FPLC für RelB molare Massen gefunden, die Gleichgewichte zwischen Dimeren und-Monomeren anzeigten. Insgesamt sprechen diese Beobachtungen für die Bildung eines stabilen RelBE-Komplexes in Lösung mit einem 1:1 Verhältnis von RelB und RelE. Die Dimerisation von Toxin und Antitoxin scheint eine allgemeineEigenschaft der Komponenten von Toxin –Antitoxin-Systemen zu bilden.

Unabhängig von der angewandten Analysenmethode (Circulardichroismus oder Fluoreszenzspektroskopie) zeigten die Denaturant-induzierten Auffaltungskurven von RelB und RelE einen von der Proteinkonzentration abhängigen Verlauf. Bei niedrigen Konzentrationen spricht der Verlauf der Kurven für das Auftreten von Zwischenstufen. Bei hohen Proteinkonzentrationen steigt die Stabilität der dimeren Proteine an, so daß es zu einer gekoppelten Dissoziations-Auffaltungsreaktion kommt. Die experimentellen Kurven wurden mit Modellen angepaßt, für die „Two-state-Monomer-“ und „Two-State-Dimer-“ Mechanismen angenommen wurden. Thermodynamische Parameter (ΔG, C½,

m) wurden für RelB und RelE berechnet. Antitoxin RelB hat eine erheblich niedrigere Stabilität als Toxin RelE. Für RelB wurde eine erstaunliche Reversibilität der thermischen Auffaltung (in DSC- und CD-Experimenten) gefunden, wogegen RelE und der RelBE-Komplex bei Temperaturen von ca. 50 °C eine starke Aggregationstendenz aufwiesen und kaum Rückfaltung zeigten. Eine Anpassung der RelB-Auffaltungskurve unter Annahme eines „two-state"-Modells für die Auffaltung von gefalteten in entfaltete Dimere ($D_F \leftrightarrow D_U$) ergab eine Übergangstemperatur, T_m = 60,7°C, und eine Auffaltungsenthalpy ΔH_{cal} = 41.6 kcal/mol. Die Auffaltung des RelBE-Komplexes folgte offensichtlich einem komplizierten Mechanismus, bei dem zumindestens bei hohen Konzentrationen ein gekoppelter Dissoziations-Auffaltungs-Mechanismus zugrunde liegt. Unter unseren experimentellen Bedingungen ergab die Analyse von RelB, RelE und RelBE einen hohen Stabilitätsbeitrag von RelE im RelBE-Komplex .

Die FPLC-Analyse des RelBE-Komplexes aus *M. jannaschii* zeigte, daß die Stoichometry des Komplexes ein in hohem Maße ein von der Konzentration abhängiges Phänomen ist. Die Denaturant-induzierte Auffaltung wurde mittels CD und DSC verfolgt. Die Auffaltung in Gegenwart von Denaturant (Guanidiniumchlorid) erwies sich als ein kooperativer Prozeß, der von der Proteinkonzentrationabhängig war. Ähnlich zu *E. coli* RelBE, ist die Auffaltung des Komplexes als gekoppelte Reaktion von Dissoziation des Komplexes und Auffaltung der Komponenten zu verstehen. Die hohe Thermostabilität des RelBE-Komplexes aus *M. jannaschii* machte Messungen in Guanidiniumchlorid-haltigen Puffern (1.78, 2.78, 3.85 M) erforderlich. Dekonvolution der experimentellen Kurven ergab nach linearer Extrapolation für 0 M Guanidiniumchlorid ΔH = 110.3 kcal/mol und T_m = 116 °C.

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