

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

# **Intrinsic Disorder and Coiled Coil formation in Prostate Apoptosis Response factor-4 (Par-4)**

Submitted in fulfilment of the requirements  
of the degree of Doctor of Philosophy

Institute of Fundamental Sciences  
Massey University  
New Zealand

Martin Schwalbe

2010



## Abstract

Prostate apoptosis response factor-4 (Par-4) is a ubiquitously expressed pro-apoptotic and tumour suppressive protein. Par-4 contains a highly conserved coiled coil (CC) region at the C-terminus, particularly the distal 40 residues fulfil the criteria for a leucine zipper (LZ). This C-terminal domain serves as the primary recognition domain for a large number of binding partners. Par-4 is tightly regulated by the aforementioned binding partners and also by post-translational modifications. Biophysical data presented here describe Par-4 as primarily an intrinsically disordered protein (IDP). Bioinformatic analysis of the highly conserved Par-4 reveals low sequence complexity and enrichment in polar and charged amino acids. High proteolytic susceptibility and increased hydrodynamic radii are consistent with largely extended structures in solution. Spectroscopic measurements using circular dichroism (CD) and nuclear magnetic resonance (NMR) also reveal characteristic features of intrinsic disorder. Under physiological conditions, data show that Par-4 self-associates via the C-terminal domain possibly through coiled coil formation. Analysis of various constructs comprising the Par-4 LZ domain by NMR, CD, light scattering and other techniques reveals an environment-dependent conformational equilibrium between primarily disordered monomers and predominantly coiled coil dimers. Whereas the disordered monomers are easily observed by NMR, the coiled coil fraction is not amenable to NMR studies possibly due to intermediate exchange processes. Mutational approaches that stabilise the coiled coil fraction result in NMR spectra of lower quality compared to the wild-type form. The high degree of sequence conservation suggest that coiled coil formation and intrinsic disorder are essential for Par-4 to function as an effective regulator of apoptosis.



## Acknowledgements

I would like to thank my Supervisor Dr. Steve Pascal for his patience with me. I further like to thank him and my Co-Supervisor Dr. Andrew Sutherland-Smith for invaluable advice on how to study Par-4, but also for never-ending ideas of what I could do next. I also wish to thank Dr. Pat Edwards for his brilliant assistance with the acquisition and analysis of NMR spectra. In this context I also like to thank Dr. Stephen Headey and Dr. Jo Claridge. Dr. David Libich for pushing us on to the intrinsic disorder track and the amount of time he spent on helping to finish the publication. Trevor Loo for a phenomenal assistance with cloning and protein purification. Dr. Alice Clark, Dr. Greg Sawyer, Dr. Matt Bennett and Jan Richter for their help in teaching an NMR spectroscopist how to get crystals and everything related to crystallisation. Professor Geoff Jameson, Dr. Gill Norris and Dr. Mark Patchett for interesting discussions during the lab meetings. I wish to thank Andrew and Alice again for turning a German grammar inspired thesis into proper English (at least I hope so). All members of the Centre for Structural Biology I forgot to mention for a great atmosphere in the group, for their assistance and for their advice. As I was not working all the time in the lab, I would like to thank all people I regularly met outside Massey for their friendship, encouragement and distraction. This includes the staff of the Celtic for the “Happy Hour” and all people of the Celtic Crowd, as enjoying a pint with friends is more fun than drinking it alone. In particular I have to say thanks to my golf flight, Nick Bennett, Jo Claridge, Greg Sawyer, Craig van Stratum and Raymond Illston for entertainment on the course (even when I enforced playing in the rain) and on the 19th hole. Finally, I wish to thank my family for their never-ending support, even though I was on the other side of the world.



# Glossary of Abbreviations

A <sub>xxx</sub>	Absorption at <b>XXX</b> nm
AATF	Apoptosis- <b>antagonising</b> transcription factor
Apaf-1	Apoptotic <b>protease activating</b> factor-1
Amp	<b>Ampicillin</b>
A $\beta$	Amyloid <b><math>\beta</math></b> -peptide
aPKC	<b>atypical protein kinase C</b>
APP	Amyloid <b>precursor protein</b>
AR	Androgen <b>receptor</b>
AU	Analytical <b>Ultracentrifugation</b>
BACE-1	<b><math>\beta</math></b> -site APP cleaving enzyme-1
BCA	<b>Bicinchoninacid</b>
CAD	Caspase <b>activated DNase</b>
Cam	<b>Chloramphenicol</b>
CARD	Caspase <b>activation and recruitment domain</b>
CC	Coiled <b>coil</b>
CD	Circular <b>dichroism</b>
cFLIP	cellular <b>FLICE-like inhibitory protein</b>
CHT1	<b>Choline transporter 1</b>
CM	Carboxy <b>methyl</b>
D2DR	<b>Dopamine D2 receptor</b>
DAP	<b>Death-activated protein kinase</b>
DD	<b>Death domain</b>
DED	<b>Death effector domains</b>
DIABLO	<b>Direct IAP-binding protein with low pI</b>
DISC	<b>Death-inducing signalling complex</b>
Dlk	<b>DAP like kinase</b>
DLS	<b>Dynamic light scattering</b>
DOM	<b>Disordered monomer</b>
DTT	<b>Dithiothreitol</b>
EDC	1-ethyl-3-(3- <b>dimethylaminopropyl</b> )carbodiimide
EDTA	Ethylene <b>diaminetetraacetic acid</b>
ER	Endoplasmatic <b>reticulum</b>
ERK	Extracellular signal <b>regulated kinase</b>
FADD	<b>Fas associated death domain</b>
FLICE	<b>FADD-like interleukin 1<math>\beta</math> converting enzyme</b> (= caspase 8)
GRP78	<b>Glucose-regulated protein-78</b>
GST	<b>Glutathione S-Transferase</b>

HCA	<b>H</b> ydrophobic cluster <b>a</b> nalysis
HMBP-3Cpro	<b>H</b> exa-histidine- <b>M</b> BP-tagged <b>3C</b> <b>p</b> rotease
HMQC	<b>H</b> eteronuclear <b>m</b> ultiple- <b>q</b> uantum <b>c</b> oherence
HSQC	<b>H</b> eteronuclear <b>s</b> ingle- <b>q</b> uantum <b>c</b> oherence
IAP	<b>I</b> nhibitors of <b>a</b> poptosis
ICAD	<b>I</b> nhibitor of <b>c</b> aspase <b>a</b> ctivated <b>D</b> Nase
ID	<b>I</b> ntrinsic <b>d</b> isorder
IDP	<b>I</b> ntrinsically <b>d</b> isordered <b>p</b> rotein
IEC	<b>I</b> on- <b>e</b> xchange chromatography
I $\kappa$ B	<b>I</b> nhibitor of <b><math>\kappa</math>B</b> family
IKK	<b>I</b> $\kappa$ <b>B</b> <b>k</b> inase
IMAC	<b>I</b> mmobilised <b>m</b> etal ion <b>a</b> ffinity chromatography
IPTG	<b>I</b> sopropyl- $\beta$ - <b>D</b> -thiogalactopyranoside
Kan	<b>K</b> anamycin
LB	<b>L</b> uria <b>B</b> ertani medium
LZ	<b>L</b> eucine <b>Z</b> ipper
MALLS	<b>M</b> ulti- <b>A</b> ngle <b>L</b> aser <b>L</b> ight <b>S</b> cattering
MAPK	<b>M</b> itogen- <b>a</b> ctivated <b>p</b> rotein <b>k</b> inases
MBP	<b>M</b> altose <b>b</b> inding <b>p</b> rotein
Mes	<b>2-M</b> orpholinoethanesulfonic acid
MOPS	<b>3-(N-M</b> orpholino) <b>p</b> ropanesulfonic acid
MS	<b>M</b> ass spectrometry
<i>MW</i>	<b>M</b> olecular <b>w</b> eight
<i>MWCO</i>	<b>M</b> olecular <b>w</b> eight <b>c</b> ut <b>o</b> ff
NF $\kappa$ B	<b>N</b> uclear <b>f</b> actor <b><math>\kappa</math>B</b>
NLS	<b>N</b> uclear <b>l</b> ocalisation <b>s</b> equence
NMR	<b>N</b> uclear <b>m</b> agnetic <b>r</b> esonance
NOESY	<b>N</b> uclear <b>O</b> verhauser <b>E</b> ffect <b>S</b> pectroscopy
NRMSD	<b>N</b> ormalised <b>r</b> oot <b>m</b> ean <b>s</b> quare <b>d</b> eviation
NTA	<b>N</b> itrilotriacetic acid
OD <sub>xxx</sub>	<b>O</b> ptical <b>d</b> ensity at <b>XXX</b> nm
Par-4	<b>P</b> rostate <b>a</b> poptosis <b>r</b> esponse <b>f</b> actor- <b>4</b>
PAGE	<b>P</b> olyacrylamide <b>g</b> el <b>e</b> lectrophoresis
PBS	<b>P</b> hosphate <b>b</b> uffered <b>s</b> aline
PCD	<b>P</b> rogrammed <b>c</b> ell <b>d</b> eath
PCR	<b>P</b> olymerase <b>c</b> hain <b>r</b> eaction
PI3K	<b>P</b> hosphoinositide <b>3</b> - <b>k</b> inase
PKA, PKB or PKC	<b>P</b> rotein <b>k</b> inase <b>A</b> , <b>B</b> or <b>C</b>
PML	<b>P</b> romyelocytic <b>l</b> eukemia
POD	<b>P</b> redominantly <b>o</b> rded <b>d</b> imer

PTEN	<b>Phosphatase and tensin</b> homolog deleted on chromosome 10
RP-HPLC	<b>Reversed phase - high performance liquid chromatography</b>
rpm	<b>Revolutions per minute</b>
rrPar-4	<b>recombinant rat Prostate apoptosis response factor-4</b>
$R_s$	<b>Stokes radius</b>
rTEV	<b>recombinant tobacco etch virus protease</b>
SAC	<b>Selective apoptosis induction in cancer cells</b>
SAXS	<b>Small-angle X-ray scattering</b>
SEC	<b>Size exclusion chromatography</b>
SDS	<b>Sodium dodecyl sulphate</b>
SMAC	<b>Second mitochondria-derived activator of caspase</b>
SOC	<b>Super optimal broth with catabolite repression</b>
SP	<b>Sulfopropyl</b>
SSB1, 2, 4	<b>SPRY-domain containing SOCS box proteins 1, 2, 4</b>
TCEP	<b>Tris(2-carboxyethyl)phosphine</b>
TFA	<b>Trifluoroacetic acid</b>
TFE	<b>Trifluoroethanol</b>
$T_m$	<b>Melting temperature</b>
TNF $\alpha$	<b>Tumour necrosis factor <math>\alpha</math></b>
TNFR1	<b>Tumour necrosis factor receptor 1</b>
TOP1	<b>Topoisomerase 1</b>
TRAIL	<b>TNF-related apoptosis inducing ligand</b>
WT	<b>Wild type</b>
WT1	<b>Wilm's tumour protein 1</b>
XIAP	<b>X-linked inhibitor of apoptosis</b>



# Contents

Glossary of Abbreviations.....	i
Contents.....	v
List of Figures.....	xi
List of Tables.....	xiii
<b>1. Introduction</b> .....	<b>1</b>
<b>1.1. The pro-apoptotic protein Par-4 - a tumour-suppressor with biological significance.....</b>	<b>2</b>
1.1.1. Apoptosis and human disease.....	2
1.1.2. Discovery of Par-4.....	2
1.1.3. Par-4 as tumour-suppressor.....	3
1.1.4. Par-4 in neurodegenerative disease.....	4
1.1.5. Other cellular roles of Par-4.....	5
<b>1.2. Structure and function of Par-4.....</b>	<b>5</b>
1.2.1. Structure of Par-4.....	5
1.2.2. The pro-apoptotic function of Par-4.....	8
1.2.3. Two cellular targets are essential for the apoptosis-inducing function of Par-4.....	10
1.2.4. The importance of the SAC domain.....	11
<b>1.3. Par-4 interaction partners and their involvement in apoptosis.....</b>	<b>12</b>
1.3.1. Programmed cell death.....	12
1.3.2. Caspases.....	14
1.3.3. The extrinsic and intrinsic pathways of apoptosis.....	15
1.3.4. Interaction partners of Par-4.....	17
a) Atypical protein kinase C $\zeta$ and $\lambda$ 1 (aPKC $\zeta$ and $\lambda$ 1).....	18
b) p62.....	20
c) Protein kinase B.....	20
d) DAP like kinase.....	21
e) Actin filaments.....	22
f) Amida.....	23
g) THAP1.....	23
h) Wilm's tumour protein 1.....	26
i) Androgen receptor.....	27
j) E2F1.....	27
k) Topoisomerase 1.....	28
l) $\beta$ -site APP cleaving enzyme-1.....	28
m) Apoptosis-antagonising transcription factor.....	29
n) Glucose-regulated protein-78.....	29
o) Choline transporter CHT1.....	30

p) Dopamine D2 receptor.....	30
q) SPRY-domain containing SOCS box proteins 1, 2 and 4.....	31
1.3.5. The oligomeric state of homo- and heteromeric Par-4 complexes.....	32
<b>1.4. Coiled Coils and Leucine Zippers.....</b>	<b>32</b>
<b>1.5. Intrinsically disordered proteins.....</b>	<b>35</b>
<b>1.6. Assessing intrinsic disorder.....</b>	<b>38</b>
1.6.1. Computational methods.....	38
1.6.2. Biochemical methods.....	38
1.6.3. Methods to assess the protein dimension and shape.....	38
1.6.4. Methods for the assessment of secondary structure.....	39
1.6.5. Methods to assess tertiary structure.....	41
<b>1.7. Research Aims.....</b>	<b>42</b>
<b>2. Expression and Purification of Par-4.....</b>	<b>45</b>
<b>2.1. Basic microbiologic techniques.....</b>	<b>46</b>
2.1.1. Preparation of ultra-competent cells.....	46
2.1.2. Transformation of E. coli cells.....	46
2.1.3. Preparation of glycerol stocks.....	47
<b>2.2. Expression and purification of recombinant proteases.....</b>	<b>47</b>
2.2.1. Expression and purification of HMBP-3Cpro.....	47
2.2.2. Expression and purification of rTEV protease.....	48
2.2.3. Summary.....	49
<b>2.3. Preparation of cell free expression vectors.....</b>	<b>49</b>
2.3.1. Materials and Methods.....	49
2.3.2. Results and Discussion.....	50
<b>2.4. Preparation of Par-4 expression vectors.....</b>	<b>53</b>
2.4.1. Materials and Methods.....	53
2.4.2. Results and Discussion.....	54
<b>2.5. Protein expression and labelling techniques.....</b>	<b>56</b>
2.5.1. Expression of unlabelled Par-4.....	56
2.5.2. Expression of <sup>13</sup> C/ <sup>15</sup> N-labelled Par-4.....	56
2.5.3. Deuteration of Par-4.....	57
2.5.4. REDPRO expression of Par-4.....	57
<b>2.6. Purification of Par-4(1-332).....</b>	<b>58</b>
2.6.1. Purification of Par-4(1-332)WT from HpMal-c2P.....	58
a) Materials and Methods.....	58
b) Results.....	59
2.6.2. Purification of Par-4(1-332)WT from pProEX-HTb.....	59
a) Materials and Methods.....	59
b) Results.....	59

2.6.3. Purification of Par-4(1-332)G40G from pET32a.....	60
a) Materials and Methods.....	60
b) Results.....	60
2.6.4. Native purification of Par-4(1-332)G40G from pET32TEV.....	60
a) Materials and Methods.....	60
b) Results.....	60
2.6.5. Denaturing purification of Par-4(1-332)G40G from pET32TEV – Purification of rrPar-4FL.....	61
a) Materials and Methods.....	61
b) Results.....	63
<b>2.7. Purification of Par-4(1-265).....</b>	<b>63</b>
2.7.1. Purification of Par-4(1-265)WT from HpMal-c2P.....	63
a) Materials and Methods.....	63
b) Results.....	63
<b>2.8. Purification of Par-4(1-290).....</b>	<b>64</b>
2.8.1. Purification of Par-4(1-290)WT from HpMal-c2P.....	64
a) Materials and Methods.....	64
b) Results.....	64
2.8.2. Purification of Par-4(1-290)G40G from pCFE-TrxH-TEV – Purification of rrPar-4ΔLZ.....	64
a) Materials and Methods.....	64
b) Results.....	65
<b>2.9. Purification of Par-4(137-195).....</b>	<b>65</b>
2.9.1. Purification of Par-4(137-195)WT from pET32TEV – Purification of rrPar-4SAC.....	65
a) Materials and Methods.....	65
b) Results.....	66
<b>2.10. Purification of Par-4(240-332).....</b>	<b>66</b>
2.10.1. Denaturing purification of Par-4(240-332)WT from pGex-6P-3 – Purification of rrPar-4CC.....	66
a) Materials and Methods.....	66
b) Results.....	67
<b>2.11. Purification of Par-4(286-332).....</b>	<b>67</b>
2.11.1. Purification of Par-4(286-332)WT from HpMal-c2P.....	67
a) Materials and Methods.....	67
b) Results.....	69
2.11.2. Purification of Par-4(286-332)WT from pCFE-GST-3C.....	70
a) Materials and Methods.....	70
b) Results.....	71
2.11.3. Denaturing purification of Par-4(286-332)WT from pGex-6P-3 – Purification of rrPar-4LZ.....	71
a) Materials and Methods.....	71

b) Results.....	73
2.11.4. Purification of Par-4(286-332)WT from pCFE-GST-TEV.....	73
a) Materials and Methods.....	73
b) Results.....	73
2.11.5. Purification of Par-4(286-332)W285 from HpMal-c2P.....	73
a) Materials and Methods.....	73
b) Results.....	74
2.11.6. Purification of Par-4(286-332)D305K from HpMal-c2P – Purification of rrPar-4LZD305K.....	74
a) Materials and Methods.....	74
b) Results.....	74
2.11.7. Purification of Par-4(286-332)E310K from HpMal-c2P – Purification of rrPar-4LZE310K.....	75
a) Materials and Methods.....	75
b) Results.....	75
2.11.8. Denaturing purification of Par-4(286-332)E310K from HpMal-c2P.....	75
a) Materials and Methods.....	75
b) Results.....	76
2.11.9. Denaturing purification of Par-4(286-332)N313I from pGex-6P-3 – Purification of rrPar-4LZN313I.....	76
a) Materials and Methods.....	76
b) Results.....	76
<b>2.12. Summary.....</b>	<b>77</b>
2.12.1. General purification problems of Par-4.....	77
2.12.2. Purification problems of Par-4(286-332).....	81
 <b>3. Intrinsic Disorder in Par-4</b> .....	 <b>87</b>
<b>3.1. Bioinformatic analysis.....</b>	<b>88</b>
<b>3.2. Electrophoretic mobility, hydrodynamic and proteolytic analysis.....</b>	<b>92</b>
3.2.1. Materials and Methods.....	92
a) Dynamic Light Scattering.....	92
b) Limited Proteolysis.....	92
3.2.2. Results and Discussion.....	93
<b>3.3. Characterisation of Par-4 by size exclusion chromatography.....</b>	<b>97</b>
3.3.1. Materials and Methods.....	97
3.3.2. Results and Discussion.....	98
<b>3.4. Secondary structure assessment by CD and NMR spectroscopy.....</b>	<b>101</b>
3.4.1. Materials and Methods.....	101
a) Circular Dichroism.....	101
b) NMR spectroscopy.....	101
3.4.2. Results and Discussion.....	102

<b>3.5. Influence of pH and TFE on the conformation of the Par-4 SAC domain.....</b>	<b>105</b>
3.5.1. Materials and Methods.....	105
a) Circular Dichroism.....	105
b) NMR spectroscopy.....	105
3.5.2. Results and Discussion.....	106
<b>3.6. Evidence for self-association through coiled coil formation.....</b>	<b>110</b>
<b>3.7. The advantage of disorder in regulatory processes.....</b>	<b>113</b>
<b>4. Order-disorder Equilibria in the Coiled Coil region of Par-4</b>	<b>115</b>
<b>4.1. Sequence analysis of the Par-4 C-terminus.....</b>	<b>116</b>
4.1.1. Materials and Methods.....	117
4.1.2. Results and Discussion.....	117
<b>4.2. Coiled coil formation of the Par-4 LZ domain - Assessing the oligomeric state.....</b>	<b>122</b>
4.2.1. Materials and Methods.....	122
a) Combined sample preparation for CD and DLS.....	122
b) Circular Dichroism.....	123
c) Dynamic Light Scattering.....	123
d) Size Exclusion Chromatography.....	124
e) Size Exclusion Chromatography - Multi Angle Laser Light Scattering.....	124
f) Crosslinking.....	125
4.2.2. Results and Discussion.....	125
a) Circular Dichroism.....	125
b) SEC and MALLS.....	130
c) Dynamic Light Scattering.....	133
d) Par-4 LZ Crosslinking.....	137
e) Future directions.....	139
<b>4.3. Coiled coil formation of the Par-4 LZ domain analysed by NMR spectroscopy.....</b>	<b>140</b>
4.3.1. Materials and Methods.....	140
a) Chemical shift assignment and CSI calculation.....	140
b) $^1\text{H}$ , $^{15}\text{N}$ -SOFAS-T-HMQC spectra of Par-4LZ peptides.....	140
c) $^{15}\text{N}$ -relaxation measurements of rrPar-4LZ at pH 6.0.....	141
d) NMR spectroscopy of REDPRO rrPar-4LZE310K.....	141
e) NMR spectra processing and analysis.....	142
4.3.2. Results and Discussion.....	142
<b>4.4. The effect of TFE on coiled coil formation of rrPar-4LZ.....</b>	<b>156</b>
4.4.1. Materials and Methods.....	156
a) Sample preparation.....	156
b) Circular Dichroism.....	156
c) NMR spectroscopy.....	157
4.4.2. Results and Discussion.....	157
<b>4.5. Asparagine residue contributions to Par-4 LZ stability.....</b>	<b>163</b>

4.5.1. Materials and Methods.....	163
a) Sample preparation.....	163
b) Circular Dichroism.....	164
c) Dynamic Light Scattering.....	164
d) NMR spectroscopy.....	164
4.5.2. Results and Discussion.....	164
<b>4.6. Structural characterisation of the Par-4 Coiled Coil domain.....</b>	<b>172</b>
4.6.1. Materials and Methods.....	172
a) Sample preparation.....	172
b) Circular Dichroism.....	173
c) Dynamic Light Scattering.....	173
d) Crystallisation trials.....	173
4.6.2. Results and Discussion.....	173
<b>4.7. The advantage of transient coiled coil formation in Par-4.....</b>	<b>181</b>
<b>5. Conclusions and Future work</b>	<b>187</b>
5.1. Conclusions.....	188
5.2. Contributions to the current knowledge.....	189
5.3. Future work.....	190
<b>Appendices</b>	<b>193</b>
A List of Suppliers.....	194
B Plasmid maps of cell free expression vectors.....	196
C Initial NMR spectroscopic characterisation of Par-4(286-332)WT.....	202
D NMR spectroscopy of the Par-4 LZ domain as GST fusion protein.....	204
E Calibration curves for size exclusion chromatography.....	207
F CD spectropolarimetry acquisition parameters.....	208
G NMR spectroscopy acquisition parameters.....	209
H Chemical shifts for Par-4(286-332)WT.....	210
I Bibliography.....	212

## List of Figures

Figure 1.1	Sequence conservation in Par-4.....	7
Figure 1.2	Model for the cancer-selective apoptosis by Par-4.....	10
Figure 1.3	The extrinsic and intrinsic pathways of apoptosis.....	16
Figure 1.4	The pro-apoptotic function of Par-4.....	24
Figure 2.1	Denaturing purification of rrPar-4FL.....	62
Figure 2.2	Purification of Par-4(286-332)WT as MBP fusion protein.....	70
Figure 2.3	Purification of Par-4(286-332)WT as GST fusion protein.....	72
Figure 2.4	Non-specific binding and aggregation of Par-4 constructs.....	79
Figure 3.1	Computational analysis of Par-4.....	89
Figure 3.2	Charge/hydrophobicity plot and sequence complexity of Par-4.....	91
Figure 3.3	Electrophoretic mobility of Par-4.....	93
Figure 3.4	DLS analysis of rrPar-4FL, rrPar-4ΔLZ and rrPar-4SAC.....	96
Figure 3.5	Limited proteolysis of rrPar-4FL, rrPar-4ΔLZ and rrPar-4SAC.....	97
Figure 3.6	Size exclusion chromatography of Par-4.....	99
Figure 3.7	CD spectropolarimetry indicates ID in Par-4.....	103
Figure 3.8	NMR spectroscopy displays typical features of ID in Par-4.....	104
Figure 3.9	pH and TFE dependence of the CD spectra of rrPar-4SAC.....	107
Figure 3.10	Influence of ionic strength and TFE on the NMR spectra of rrPar-4SAC.....	109
Figure 3.11	Influence of increasing concentrations of urea on rrPar-4FL.....	112
Figure 4.1	Coiled coil formation and stability - sequence analysis of Par-4.....	118
Figure 4.2	CD spectropolarimetry of Par-4 LZ peptides.....	126
Figure 4.3	Thermal stability of Par-4 LZ peptides.....	129
Figure 4.4	SEC and DLS of Par-4 LZ peptides.....	131
Figure 4.5	DLS of Par-4 LZ peptides.....	136
Figure 4.6	Crosslinking mechanism of EDC.....	137
Figure 4.7	Crosslinking of Par-4 LZ peptides.....	138
Figure 4.8	CSI for the Par-4 LZ domain.....	143
Figure 4.9	Influence of pH on the <sup>1</sup> H, <sup>15</sup> N-SOFAST-HMQC spectra of rrPar-4LZ.....	144
Figure 4.10	<sup>15</sup> N-relaxation and J-coupling of rrPar-4LZ at pH 6.0 and 5 °C.....	146
Figure 4.11	NMR peak intensity of Par-4 LZ peptides.....	147
Figure 4.12	<sup>1</sup> H, <sup>15</sup> N-SOFAST-HMQC spectra of the Par-4 LZ point mutants.....	151
Figure 4.13	A second set of peaks exist in the NMR spectra of the Par-4 LZ peptides.....	154
Figure 4.14	CD spectropolarimetry of rrPar-4LZ under various TFE concentrations.....	158

Figure 4.15	$^1\text{H}, ^{15}\text{N}$ -SOFAST-HMQC spectra of rrPar-4LZ at various TFE concentrations.....	162
Figure 4.16	CD spectropolarimetry of rrPar-4LZN313I.....	168
Figure 4.17	NMR spectroscopy of rrPar-4LZN313I.....	169
Figure 4.18	CD spectropolarimetry of rrPar-4CC.....	176
Figure 4.19	Thermal stability of various Par-4 constructs.....	178
Figure 4.20	Crystallisation trials of rrPar-4CC.....	179
Figure 4.21	Potential order-disorder equilibria may be necessary for the function of Par-4...	182
Figure A.1	$^1\text{H}, ^{15}\text{N}$ -TROSY-HSQC spectra of Par-4(286-332)WT.....	202
Figure A.2	NMR spectroscopy of the Par-4 LZ domain as a GST-fusion protein.....	204

## List of Tables

Table 1.1	Par-4 interaction partners.....	18
Table 2.1	Preparation of cell free expression vectors.....	50
Table 2.2	Preparation of Par-4 expression vectors.....	54
Table 3.1	Hydrodynamic properties of rrPar-4 constructs using various biophysical techniques.....	94
Table 3.2	Comparison of experimental and theoretical Stoke's radii ( $R_s$ ).....	95
Table 3.3	Sample conditions of rrPar-4SAC at various pH values.....	106
Table 4.1	Potential electrostatic interactions within the Par-4 CC domain.....	121
Table 4.2	Sample conditions of Par-4 LZ peptides used for DLS and CD spectropolarimetry.....	123
Table 4.3	Hydrodynamic properties of various oligomers of a 6 kDa peptide.....	132
Table 4.4	DLS-determined hydrodynamic properties of Par-4 LZ peptides.....	135
Table 4.5	Sample conditions of rrPar-4LZ with increasing concentrations of TFE.....	157
Table 4.6	Influence of increasing TFE concentrations on the $\alpha$ -helicity of rrPar-4LZ.....	160
Table 4.7	Thermal stability and supercoiling of rrPar-4LZ under various TFE concentrations.....	161
Table 4.8	Sample conditions of rrPar-4LZN313I at various pH values.....	164
Table 4.9	DLS-determined hydrodynamic properties of rrPar-4LZN313I.....	166
Table 4.10	Sample conditions of rrPar-4CC used for DLS and CD spectropolarimetry.....	172
Table 4.11	DLS-determined hydrodynamic properties of rrPar-4CC.....	177