



Protein Design Based on a PHD Scaffold

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A thesis submitted in fulfillment
of the requirements for the degree
of Doctor of Philosophy

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June, 2004

Declaration

The work described in this thesis was performed between February 2001 and June 2004 in the School of Molecular and Microbial Biosciences (formerly The Department of Biochemistry) at the University of Sydney. The experiments described were performed by the author unless otherwise stated. This work has not been submitted, in part or in full, for the purpose of obtaining any other degree.

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Abstract

The plant homeodomain (PHD) is a protein domain of ~45–100 residues characterised by a Cys₄-His-Cys₃ zinc-binding motif. When we commenced our study of the PHD in 2000, it was clear that the domain was commonly found in proteins involved in transcription. Sequence alignments indicate that while the cysteines, histidine and a few other key residues are strictly conserved, the rest of the domain varies greatly in terms of both amino acid composition and length. However, no structural information was available on the PHD and little was known about its function. We were therefore interested in determining the structure of a PHD in the hope that this might shed some light on its function and molecular mechanism of action.

Our work began with the structure determination of a representative PHD, Mi2 β -P2, and this work is presented in Chapter 3. Through comparison of this structure with the two other PHD structures that were determined during the course of our work, it became clear that PHDs adopt a well-defined globular fold with a superimposable core region. In addition, PHDs contain two loop regions (termed L1 and L3) that display increased flexibility and overlay less well between the three PHD structures available. These L1 and L3 regions correspond to variable regions identified earlier in PHD sequence alignments, indicating that L1 and L3 are probably not crucial for the PHD fold, but are instead likely to be responsible for imparting function(s) to the PHD. Indeed, numerous recent functional studies of PHDs from different proteins have since demonstrated their ability in binding a range of other proteins.

In order to ascertain whether or not L1 and L3 were in fact dispensable for folding, we made extensive mutations (including both insertions and substitutions) in the loop regions of Mi2 β -P2 and showed that the structure was maintained. We then went on to illustrate that a new function could be imparted to Mi2 β -P2 by inserting a five-residue CtBP-binding motif into the L1 region and showed this chimera could fold and bind CtBP.

Having established that the PHD could adopt a new binding function, we next sought to use combinatorial methods to introduce other novel functions into the PHD scaffold. Phage display was selected for this purpose, because it is a well-established technique

and has been used successfully to engineer zinc-binding domains by other researchers. However, in order to establish this technique in our laboratory, we first chose a control system in which two partner proteins were already known to interact *in vitro*.

We chose the protein complex formed between the transcriptional regulators LMO2 and ldb1 as a test case. We have examined this interaction in detail in our laboratory, and determined its three-dimensional structure. Furthermore, inappropriate formation of this complex is implicated in the onset of T-cell acute lymphoblastic leukemia. We therefore sought to use phage display to engineer ldb1 mimics that could potentially compete against wild-type ldb1 for LMO2, and this work is described in Chapter 4. Using a phage library containing $\sim 3 \times 10^7$ variants of the LMO2-binding region of ldb1, we isolated mutants that were able to interact with LMO2 with higher affinity and specificity than wild-type ldb1. These ldb1 mutants represent a first step towards finding potential therapeutics for treating LMO-associated diseases.

Having established phage display in our laboratory, we went on to search for PHD mutants that could bind selected target proteins. This work is described in Chapter 5. We created three PHD libraries with eight randomized residues in each of L1, L3 or in both loops of the PHD. These PHD libraries were then screened against four target proteins. After four rounds of selection, we were able to isolate a PHD mutant (dubbed L13-FH6) that could bind our test protein Fli-ets. This result demonstrates that a novel function can be imparted to the PHD using combinatorial methods and opens the way for further work in applying the PHD scaffold to other protein design work.

In summary, the work detailed in Chapters 3 and 5 demonstrates that the PHD possesses many of the properties that are desirable for a protein scaffold for molecular recognition, including small size, stability, and a well-characterised structure. Moreover, the PHD motif possesses two loops (L1 and L3) of substantial size that can be remodeled for target binding. This may lead to an enhancement of binding affinities and specificities over other small scaffolds that have only one variable loop. In light of the fact that PHDs are mainly found in nuclear proteins, it is reasonable to expect that engineered PHDs could be expressed and function in an intracellular environment, unlike many other scaffolds that can only function in an oxidizing environment. Therefore, our results together with

other currently available genomic and functional information indicate PHD is an excellent candidate for a scaffold that could be used to modify cellular processes.

Appendices 1 and 2 describe completed bodies of work on unrelated projects that I have carried out during the course of my PhD candidature. The first comprises the invention and application of DNA sequences that contain all N-base sequences in the minimum possible length. This work is presented as a reprint of our recently published paper in *Nucleic Acids Research*. The second Appendix describes our structural analysis of an antifreeze protein from the shorthorn sculpin, a fish that lives in the Arctic and Antarctic oceans. This work is presented as a manuscript that is currently under review at the *Journal of the American Chemical Society*.

Acknowledgements

First and foremost, I would like to give the most sincere thanks to my supervisor Joel, for his help, advice, support and never-ending encouragement throughout the years. Joel has been a superb supervisor, but he is also a great mentor and a very good friend. I am especially grateful for his willingness and patience in teaching me MANY things (from NMR to how to slither down steep hills during bushwalks, and everything else in between). I have had a brilliant time in the last few years and I thank him for making my PhD experience such a wonderful one.

I am also very grateful to Jacqui for her help and advice with the molecular biology aspects of my work and introducing me to the world of LMO proteins. I thank her for always giving me time whenever I needed it, whether it was for proofreading or other things. But more importantly, I am overwhelmed by her kindness — from sewing pants for Pokemonk to making birthday cakes on my birthdays.



I would also like to give special thanks to Manuel for his help with the phage display work. Not only has he kindly offered us his vectors and constructs, he has also freely given me many tips, help and advice, which have been absolutely invaluable.

Thanks must also go to Big Chu, Dave, Belle, Janet, Raina and Fionna for their help with experiments and a countless numbers of things throughout the years. During my PhD, I have been extremely fortunate to be able to take part in other people's projects or have other people involved in mine. Thanks to everyone whom have played a role in my project and have allowed me to be a part of theirs (this includes many members of the Mackay, Matthews, Crossley and Harding labs). I am grateful for their generosity and help. And of course, a big thank-you to my fellow lab members (both ex and present) for their help and support, laughs, jokes and their company throughout the years. Many thanks also for their tolerance towards Pokemonk's daily visits. I must also thank Pokemonk, my favorite stuffed monkey for always being there (literally)! Last, but not least, I would like to thank my family for their support throughout the years. I am also grateful for the financial support provided by an Australian Postgraduate Award.

Contents

Declaration	ii
Abstract	iii
Acknowledgements	vi
Contents	vii
List of Abbreviations	xii
Publications arising from my PhD work	xv
Chapter 1. Introduction	
1.1 Proteins as drugs	1
1.2 Protein engineering - designing for a specific binding activity	2
1.2.1 Scaffold-based engineering of small proteins	4
1.2.2 Natural vs. artificially designed scaffolds	4
1.2.3 Criteria for a good scaffold	6
1.2.4 From scaffolds to novel functional proteins – rational design strategies	7
1.2.5 From scaffolds to novel functional proteins – combinatorial methods	9
1.3 Natural scaffolds — antibodies	13
1.3.1 The complementarity-determining regions (CDRs)	15
1.3.2 Extracellular vs intracellular use	15
1.4 Other small protein scaffolds not based on antibodies	16
1.4.1 Example 1: Fibronectin III (FN3)	17

1.4.2	Example 2: Zinc fingers	18
1.5	Aims of my PhD project	21
 CHAPTER 2. MATERIALS AND METHODS		
2.1	Materials	23
2.1.1	Chemicals, reagents and consumables	23
2.1.2	Buffers and solutions	24
2.1.3	Plasmids, phagemids, helper phage and bacterial strains	25
2.1.4	Culture media and plates	26
2.2	Methods for Chapter 3	27
2.2.1	Subcloning and overexpression of Mi2 β -P2 and mutants	27
2.2.2	Pellet processing for large-scale overexpression	28
2.2.3	Sample preparation for SDS-PAGE	28
2.2.4	Purification by GSH-affinity chromatography	29
2.2.5	Ion exchange chromatography	29
2.2.6	Reverse phase high pressure liquid chromatography (rpHPLC)	30
2.2.7	Electrospray mass spectrometry (ESMS)	30
2.2.8	NMR spectroscopy	30
	2.2.8.1 Acquisition of NMR experiments	30
	2.2.8.2 NMR data processing	31
2.3	Methods for Chapter 4 & 5	32
2.3.1	Expression and purification of target proteins	32
2.3.2	Cloning of proteins of interest into phagemid vectors	32
2.3.3	Transformation by electroporation	33

2.3.4	Phage infection and propagation	34
2.3.5	Phage stock production	35
2.3.6	Phage titering by infection	36
2.3.7	ssDNA production for Kunkel mutagenesis	36
2.3.8	Kunkel mutagenesis	37
2.3.9	Panning of the phage library	38
2.3.10	Simple phage enzyme-linked immunosorbent assay (ELISA)	39
2.3.11	Competition phage ELISA	40
2.3.12	One-spot ELISA for screening of potential binders	41
2.3.13	Sequencing of binding clones	41
CHAPTER 3. PHD as a protein scaffold		
3.1	Introduction	42
3.1.1	The sequence and functional diversity of PHDs	42
3.1.2	The PHD as a protein scaffold?	44
3.2	Paper: Engineering a protein scaffold from a PHD finger	45
3.3	Discussion	57
CHAPTER 4. Finding the perfect LID for LMO proteins		
4.1	Introduction – LMO proteins	59
4.1.1	The interactions of the LMO proteins and ldb1	60
4.1.2	The potential for modulating LMO:ldb1 interactions	61
4.1.3	Molecular details of the interactions between LMO2/4 and ldb1-LID	62

4.2	A phage display system for examining the LMO2:ldb1-LID interaction	68
4.2.1	Phage display	69
4.2.2	Choice of phagemid vector: geneIII or geneVIII	72
4.3	Confirmation of the LMO2:ldb1-LID interaction using phage display	74
4.4	Design of a ldb1-LID mutant library	77
4.5	Panning of ldb1-LID mutants with LMO2	80
4.6	Characterization of selected ldb1-LID mutants	81
4.7	Rationalizing the ldb1-LID mutations obtained from phage display	85
4.8	Panning of ldb1-LID mutants with LMO4	88
4.9	Discussion	89
 CHAPTER 5. PHD engineering using phage display		
5.1	Introduction	91
5.1.1	Phage display of a naive library	92
5.1.2	The phage display system chosen for PHD engineering	94
5.2	Confirming the expression of PHD on phage	94
5.3	Selection of target proteins	97
5.4	Library design of PHD mutants	99
5.5	Panning of PHD libraries with target proteins	102
5.5.1	Sequencing of clones from Fli-ets experiment	105
5.5.2	Sequencing of clones for BKLF-F3	105

5.5.3	Sequencing results for ldb1-LID and TACC77	106
5.6	Preliminary characterisation of selected clones	106
5.7	Phage ELISAs with L13-FH6	109
5.8	Discussion	111

CHAPTER 6. General summary and discussion

6.1	PHD as a protein scaffold	116
6.2	Some considerations about the engineering of binding loops	117
6.3	From a novel binder <i>in vitro</i> to useful therapeutics	119
	References	122

Appendix

List of Abbreviations

1D	one-dimensional NMR spectrum
BKLF-F3	basic Krüppel-like factor
BSA	Bovine serum albumin
CD	circular dichroism
CDR	complementarity-determining region of an antibody
cfu	colony forming unit
CV	column volume
DMSO	dimethyl sulfoxide
DQF-COSY	double-quantum-filtered <i>J</i> -correlated spectroscopy
dsDNA	double-stranded DNA
DSS	2,2-Dimethylsilapentane-5-sulfonic acid
DTT	dithiothreitol
EDTA	ethylene-diamine-tetraacetic acid (disodium salt)
ELISA	enzyme-linked immunosorbent assay
ESMS	electrospray mass spectrometry
Fli-ets	Ets domain of Fli-1
FN3	fibronectin type III domain
GSH	glutathione (reduced form)
GST	glutathione <i>S</i> -transferase
HSQC	heteronuclear single-quantum coherence
IC ₅₀	median inhibitory concentration
IPTG	isopropyl (β)-D-thiogalactopyranoside
K _d	dissociation constant

LB	Luria-Bertoni broth
LB-Amp	Luria-Bertoni broth with ampicillin
ldb1-LID	LID domain of ldb
L1	Loop 1 region of Mi2 β -P2
L1-lib	PHD library with randomized residues in L1
L13	Loop 3 region of Mi2 β -P2
L13-lib	PHD library with randomized residues in L1 and L3
L3-lib	PHD library with randomized residues in L3
LID	ldb1-interacting domain
LMO	LIM-only
LMO2-LIM1	N-terminal LIM domain of LMO2
LMO4-LIM1	N-terminal LIM domain of LMO4
Mi2 β -P2	second PHD from Mi2 β
MBP	maltose-binding protein
MQW	Milli-Q [®] water
mRNA	messenger RNA
MW	molecular weight
NMR	nuclear magnetic resonance
NOE	peak in NOESY spectrum resulting from dipolar connectivity
NOESY	nuclear Overhauser enhancement spectroscopy
OD	optical density
p3	coat protein 3 from the filamentous M13 bacteriophage
p8	coat protein 8 from the filamentous M13 bacteriophage
PBS	phosphate-buffered saline

PCR	polymerase chain reaction
PHD	plant-homeodomain
PMSF	phenylmethylsulfonylfluoride
ppm	parts per million
rpHPLC	reverse-phase high pressure liquid chromatography
scFv	single-chain variable fragment of an antibody
ssDNA	single-stranded DNA
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
T-ALL	T-cell acute lymphoblastic leukemia
TACC77	C-terminal coiled coil region of TACC
TCEP	tris(2-carboxyethyl)phosphine
TE	Tris-EDTA
TFA	trifluoroacetic acid
TOCSY	total correlation spectroscopy
Tris	Tris[hydroxymethyl]aminomethane

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