

Crystallization and initial crystallographic analysis
of the Kelch domain from human Keap1Xuchu Li,^a Donna Zhang,^b Mark
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The human Keap1 protein is a substrate adaptor for an E3 ubiquitin ligase complex that specifically targets the transcription factor Nrf2 for degradation. Keap1 functions as a sensor of oxidative stress, such that the inhibition of Keap1-dependent degradation of Nrf2 activates a genetic program that protects cells from reactive chemicals and maintains cellular redox homeostasis. Keap1 interacts with Nrf2 through its C-terminal Kelch-repeat domain. Kelch-repeat domains are found in a large number of proteins and are predicted to assemble into a β -propeller structure. Only a single Kelch-repeat domain, that from the fungal enzyme galactose oxidase, has had its structure determined. Here, the crystallization of the Kelch domain of human Keap1 protein by hanging-drop vapor diffusion is reported in space group *P*6₅22. Crystals diffract to 1.85 Å resolution under cryocooling conditions. A selenomethionine-substituted version of the Kelch domain has also been purified and crystallizes isomorphously with the native protein. Structure determination by MAD phasing is under way. The role of Keap1 in oxidative stress and cytoprotection suggests that the Kelch domain will be an attractive target for therapeutic drug design.

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1. Introduction

A major mechanism for protection against oxidative damage in eukaryotic cells involves the coordinated induction of a group of cytoprotective genes including the glutathione-S-transferases, NAD(P)H oxidoreductase and γ -glutamylcysteine synthetase (Dalton *et al.*, 1999; Nguyen *et al.*, 2003). Induction of these cytoprotective genes during conditions of oxidative stress enables the neutralization of chemically reactive molecules and the restoration of cellular redox homeostasis. These genes share common *cis*-acting DNA sequences termed antioxidant-response elements (AREs) that mediate transcriptional induction following exposure to oxidative stress (Dalton *et al.*, 1999; Nguyen *et al.*, 2003; Wasserman & Fahl, 1997).

The transcription factor Nrf2 has emerged as the critical regulator of ARE-dependent transcription (Nguyen *et al.*, 2003; Jaiswal, 2004). Nrf2 is a member of a small family of transcription factors that share a conserved bZIP-dimerization/DNA-binding domain and the ability to bind ARE-like DNA-sequence motifs (Nguyen *et al.*, 2003; Motohashi *et al.*, 2002). The major regulator of Nrf2 is a BTB-Kelch protein termed Keap1, which regulates both the subcellular localization and steady-state levels of Nrf2 (Wakabayashi *et al.*, 2003; Itoh *et al.*, 1999; Zhang & Hannink, 2003).

Recent work from our laboratory has demonstrated that Keap1 targets Nrf2 for ubiquitination in the context of a Cul3-dependent E3 ubiquitin ligase complex (Zhang *et al.*, 2004). Exposure of cells to reactive chemicals or oxidative stress enables Nrf2 to escape Keap1-mediated repression, leading to increased transcription of Nrf2-dependent genes (Zhang & Hannink, 2003).

Keap1 is one of more than 50 human proteins that share an N-terminal BTB domain, a central linker domain and a C-terminal Kelch domain. BTB-Kelch proteins are generally regarded as actin-binding proteins with diverse biological roles in the regulation of the cytoskeleton (Adams *et al.*, 2000). For example, a number of single point mutations within the GAN1 gene have been described that result in giant axonal neuropathy, an autosomal recessive disease characterized by defects in intermediate filament organization in sensorimotor neurons (Bomont *et al.*, 2000). The crystal structures of several mammalian BTB domains have been reported, including the human PLZF transcription factor and the Skp1 protein, which bridges the Cul1 protein and numerous F-box substrate-adaptor proteins in the SCF1 E3 ubiquitin ligase complexes (Ahmad *et al.*, 1998; Wu *et al.*, 2003). The high degree of homology between different BTB domains has allowed the construction of homology-based models that

accurately predict the amino acids in BTB-containing proteins, such as MEL-26 and Keap1, that are important for association with Cul3 (Zhang *et al.*, 2004; Xu *et al.*, 2003).

The structure of only a single Kelch domain has been reported, that of the fungal enzyme galactose oxidase (Ito *et al.*, 1991). The Kelch domain of galactose oxidase contains seven Kelch-motif repeats that assemble into a β -propeller structure. In contrast, the mammalian BTB-Kelch proteins are predicted to contain six Kelch-motif repeats. The low degree of sequence identity between Keap1 and galactose oxidase (<16%) has precluded the development of homology-based models that provide an accurate representation of the structure of the Kelch domain of Keap1. In this report, we describe the initial crystallization of the Kelch domain of Keap1. Structural determination of the Kelch domain of Keap1 will provide significant insight into understanding how Keap1 binds to Nrf2 as well as providing a general model for understanding the structure of mammalian BTB-Kelch proteins.

2. Materials and methods

Efforts in our laboratory to express Keap1 from *Homo sapiens* (624 amino acids in total) or domains thereof in a recombinant system at levels sufficient for structural studies were most successful for the Kelch domain (residues 321–609). This domain was cloned into a pET15b vector (Novagen) between an *NdeI* site and a *BamHI* site using standard techniques (Sambrook & Russell, 2001). The resulting construct was used to transform *Escherichia coli* BL21(DE3)pLysS cells. Freshly transformed cells were grown to an optical density at

595 nm of ~ 0.6 and expression of the Kelch-domain protein was induced by the addition of IPTG to a final concentration of 1 mM. Cells were harvested 3–4 h after induction and stored at 200 K. The Kelch domain was purified to homogeneity on an ÄKTA FPLC System (Pharmacia) using an Ni-NTA column (Novagen) followed by a Mono Q column (Pharmacia); approximately 6 mg of pure protein was obtained per litre of bacterial culture. MALDI-TOF mass-spectrometric analysis, performed by the Proteomics Core, University of Missouri, Columbia, MO, USA, confirmed the correct molecular weight of the expressed protein.

Initial crystallization screens employed conditions found in Hampton Research Crystal Screen kits 1 and 2. The Kelch-domain solution was at 10 mg ml⁻¹ in 20 mM Tris buffer pH 7.5, 5 mM DTT. Crystals were grown by hanging-drop vapor diffusion at 277 K from 4% PEG 4000 and 100 mM Na HEPES pH 7.5 from drops containing 2 μ l protein solution and 2 μ l well buffer; the *de novo* growth time was 7–10 d. A solution of well buffer containing 40% saturated sucrose was used to cryoprotect the crystals. Crystals were flash-frozen in a stream of liquid nitrogen for data collection.

To facilitate phase determination, a Kelch domain containing selenomethionine (SeMet) substituted for seven methionine residues was purified. Incorporation of selenomethionine was verified by MALDI-TOF mass spectrometry. The SeMet protein crystallizes isomorphously with the native enzyme under the same conditions and the cryoprotectant used for the native crystals is also suitable for the SeMet crystals. The SeMet crystals diffract to a nominal resolution of 1.8 Å on a home X-ray source.

3. Results and discussion

A native data set to 1.85 Å was collected from a single Kelch-domain crystal using a rotating-anode X-ray source and an R-AXIS IV detector (Table 1 and Fig. 1). Data were collected at approximately 100 K. The X-ray wavelength was 1.54 Å, the oscillation angle was 0.5° and the crystal-to-detector distance 140 mm. Crystals belong to space group *P*6₅22, with unit-cell parameters $a = b = 85.75$, $c = 147.81$ Å, $\alpha = \beta = 90$, $\gamma = 120$ °. Data were processed using *DENZO* and *SCALE-PAK* (Otwinowski & Minor, 1997). Packing-density calculations for the Kelch domain indicate that $V_M = 2.6$ Å³ Da⁻¹ assuming one molecule of protein per asymmetric unit. This corresponds to a solvent fraction of about 52.6%, a typical value for protein crystals (Matthews, 1968).

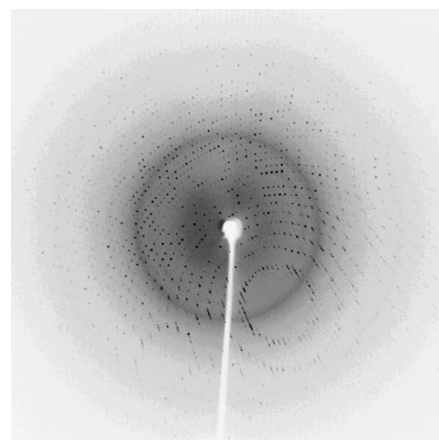


Figure 1
Diffraction pattern of native Kelch-domain crystals on a rotating-anode X-ray generator.

Table 1
Data-collection statistics for native crystals of the Kelch domain.

Values in parentheses refer to statistics for the outer resolution shell (1.92–1.85 Å).

Resolution (Å)	100–1.85
R_{sym} (%)	8.1 (52.0)
$I/\sigma(I)$	33.9 (4.2)
Completeness (%)	99.9 (100.0)
Reflections $I > 3\sigma(I)$ (%)	82.7 (48.1)
Multiplicity	14.4 (10.0)
No. unique reflections	28169

Several models derived from the Kelch domain of galactose oxidase (PDB code 1gof) were used for attempts at molecular replacement. However, no solution was apparent, presumably owing to the low sequence identity between the two proteins. Structure-determination efforts will proceed *via* the SeMet Kelch crystals, which will be used in MAD experiments at a synchrotron beamline. Refinement of the Kelch domain with the 1.85 Å resolution native data should provide an excellent starting point for functional studies.

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