Molecular Biology

Entry Clone Accession:
Entry Clone Source: Site directed mutagenesis
SGC Construct ID: PG1424PGA-c081
Protein Region: N49-A484
Vector: pNIC28-Bsa4
Tag: N-6HIS;N-TEV
Host: BL21(DE3)-R3-pRARE2

Sequence (with tag(s)):
MHHHHHHSSGVDLGTENLYFQSMNPPAGPVRAIAEYERSAAVLVRYPFGIPMELIKELAK
NDKVITIVASESQKNTVITQYTQSGVNLSNCDFIIAKTDSYWTRDYTGWFAMYDTNKVG
LVDFIYNRPRPNDDEFPKYEAQYLGIEMFGMKLKQTGGNYMTDGYGSAVQSHIAYTENS
SLSQAQVNQKMKDYLGITHHDVVQDPNGEYINHVDCWGKYLAPNKILIRKVPDNHPQH
QALEDMAAYFAAQTCAWGTKYEVYRALATNEQPYTNSLILNNRVFVPVNGPASVDNDA
LNVYKTAMPGYEIIGVKGASGTPWLGTDALHARTHEVADKGYLYIKHYPILGEQAGPDY
KIEADVVSCANATISPVQCYYRINGSGSFKAADMTMESTGHYTYSFTGLNKNDKVEYYIS
AADNSGRKETYPFIGEPDPFKFTCMNETNTCTVTGAAKALRAWFNA
Sequence after tag cleavage:
SMNPPAGPVRAIAEYERSAAVLVRYPFGIPMELIKELAKNDKVITIVASESQKNTVITQYTQ
SGVNLSNCDFIIAKTDSYWTRDYTGWFAMYDTNKVGLVDFIYNRPRPNDDEFPKYEAQY
LGIEMFGMKLKQTGGNYMTDGYGSAVQSHIAYTENSSLSQAQVNQKMKDYLGITHHDV
VQDPNGEYINHVDCWGKYLAPNKILIRKVPDNHPQHQALEDMAAYFAAQTCAWGTKY
EVYRALATNEQPYTNSLILNNRVFVPVNGPASVDNDALNVYKTAMPGYEIIGVKGASGTP
WLGTDALHARTHEVADKGYLYIKHYPILGEQAGPDYKIEADVVSCANATISPVQCYYRIN
GSGSFKAADMTMESTGHYTYSFTGLNKNDKVEYYISAADNSGRKETYPFIGEPDPFKFT
CMNETNTCTVTGAAKALRAWFNA

Note: this sequence encodes the C351A variant of PPAD
DNA Sequence:
CATATGCACCATCATCATCATCATTCTTCTGGTGTAGATCTGGGTACCGAGAACCTGTA
CTTCCAATCCATGAATCCCCCTGCAGGTCCTGTGCGTGCTATCGCTGAGTACGAACGC
TCTGCAGCCGTTTTGGTACGCTACCCGTTCGGTATCCCGATGGAATTGATCAAAGAGC
TGGCCAAGAACGACAAGGTGATTACCATTGTGGCGAGTGAAAGCCAAAAAAACACC
GTTATAACCCAGTACACCCAAAGCGGTGTGAATCTCTCTAATTGCGATTTCATCATTGC
GAAAACTGACTCTTACTGGACACGCGACTATACCGGTTGGTTCGCAATGTACGATACG
AACAAAGTAGGTCTCGTGGACTTTATTTATAACCGCCCTCGTCCTAACGATGATGAATT
CCCCAAATACGAAGCACAATATCTGGGCATCGAGATGTTCGGGATGAAGCTCAAGCAG
ACCGGTGGCAACTACATGACGGACGGATATGGATCCGCTGTGCAGTCACATATCGCAT
ATACGGAGAACTCCTCTCTGTCTCAAGCTCAAGTAAATCAAAAGATGAAAGACTATCT
CGGCATCACACATCATGATGTGGTACAAGATCCGAACGGCGAATATATCAACCATGTG
GACTGTTGGGGCAAGTATTTGGCACCGAACAAAATCCTCATCAGGAAAGTGCCTGAC
AATCACCCTCAGCACCAAGCCCTGGAAGATATGGCAGCCTACTTCGCAGCACAGACC
TGCGCATGGGGAACGAAGTACGAGGTATATCGCGCTTTGGCCACCAATGAACAACCG
TACACGAACTCTCTGATTCTGAACAACAGGGTATTTGTTCCTGTCAATGGCCCCGCCT
CCGTGGACAACGATGCTCTGAACGTCTATAAGACGGCAATGCCCGGTTACGAAATTAT
AGGTGTCAAAGGGGCTTCAGGAACACCTTGGTTAGGAACAGATGCCCTGCATGCCCG
TACTCACGAGGTAGCGGATAAGGGCTATCTCTATATCAAGCACTACCCGATACTGGGCG
AACAGGCAGGCCCTGATTATAAGATCGAAGCAGATGTCGTCTCATGCGCCAATGCTAC
TATCTCGCCGGTACAATGTTACTATCGTATCAATGGTTCCGGTAGCTTTAAGGCTGCTG
ATATGACGATGGAATCAACAGGTCACTATACTTATAGCTTTACAGGTCTTAACAAGAAT
GATAAGGTAGAATACTATATCTCTGCCGCTGACAATAGTGGTCGCAAAGAGACTTATCC

CTTTATCGGCGAACCTGATCCTTTCAAGTTTACGTGTATGAACGAAACCAATACATGTA
CTGTGACCGGAGCTGCCAAAGCTCTTCGTGCATGGTTCAACGCCTGACAGTAAAGGT
GGATACGGATCCGAA

Protein Expression

Medium: TB
Antibiotics: Kanamycin
Procedure: An overnight culture was made by inoculated 2ml/250ml terrific broth (TB) (with 1:1000 Kan & Chloramphenicol (Chlor)). The overnight culture inoculated 1:100 in TB (with 1:1000 Kan/Chlor), which was incubated at 37°C at 180rpm until OD600 of 0.6-0.8 was reached (2-3hours), at which point temperature was reduced. When cultures reached 18°C protein expression was induced with a final conc. of 0.1mM IPTG and incubated O/N at 18°C. Cells were harvested by centrifugation at 3000rpm for 15mins.

Protein Purification

Procedure: Pellets were resuspended in 2:1 (v:v) binding buffer with 1:1000 (v:v) Ethylenediaminetetraacetic acid (EDTA) free protease inhibitors (Merck-Millipore) and lysed by sonication. Soluble cell extract was isolated by centrifugation at 35,000g for 60mins. Supernatant containing target protein was purified by affinity chromatograhy (Talon Co^{2+} resin, Clontech), followed by size exclusion chromatography using Superdex S200. His-fusion tags were removed by *Tobacco Etch Virus (TEV)* cleavage using 1 mg/20 mg protein and incubated o/n before Co^{2+}-NTA affinity chromatography was repeated to remove free fusion tag.
Concentration: 15.0 mg/ml
Mass-spec Verification: Yes

Structure Determination

Crystallization: Crystals of PPAD-C351A variant were obtained by mixing 150 nL of protein solution and 300 nL of crystallization solution containing 22% PEG3350, 100 mM lithium sulfate and 100 mM bis-tris pH 5.5. All crystals were cryoprotected with 25% ethylene glycol solution mixed with reservoir buffer and cryocooled in liquid nitrogen.
Data Collection: *Beamline:* Dmnd I03; *Resolution:* 1.6 Å
Data Processing: The diffraction data were collected at Diamond Light Source (Didcot, UK). The diffraction data were processed using XIA2 and scaled with Aimless. The structure was solved and initial model was built using phenix.mr_rosetta. Coot and refmac5 were used for further building and refinement, respectively.