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Structural Characterisation of Tpx from Yersinia pseudotuberculosis Reveals Insights into the Binding of Salicylidene Acylhydrazide Compounds

Mads Gabrielsen1, Katherine S. H. Beckham1, Victoria A. Feher2, Caroline E. Zetterström3, Dai Wang1*, Sylke Müller4, Mikael Elofsson3, Rommie E. Amaro2, Olwyn Byron5*, Andrew J. Roe1*

1 Institute of Infection, Immunity and Immunology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom, 2 Departments of Pharmaceutical Sciences, Computer Science, and Chemistry, University of California Irvine, Irvine, California, United States of America, 3 Department of Chemistry and Umeå Centre for Microbial Research, Umeå University, Umeå, Sweden, 4 Wellcome Centre for Molecular Parasitology, Institute of Infection, Immunity and Immunology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom, 5 School of Life Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom

Abstract

Thiol peroxidase, Tpx, has been shown to be a target protein of the salicylidene acylhydrazide class of antivirulence compounds. In this study, we present the crystal structures of Tpx from Y. pseudotuberculosis (ypTPx) in the oxidised and reduced states, together with the structure of the C61S mutant. The structures solved are consistent with previously solved atypical 2-Cys thiol peroxidases, including that for “forced” reduced states using the C61S mutant. In addition, by investigating the solution structure of ypTPx using small angle X-ray scattering (SAXS), we have confirmed that reduced state ypTPx in solution is a homodimer. The solution structure also reveals flexibility around the dimer interface. Notably, the conformational changes observed between the reduced states at the catalytic triad and at the dimer interface have implications for substrate and inhibitor binding. The structural data were used to model the binding of two salicylidene acylhydrazide compounds to the oxidised structure of ypTPx. Overall, the study provides insights into the binding of the salicylidene acylhydrazides to ypTPx, aiding our long-term strategy to understand the mode of action of this class of compounds.

Introduction

Thiol peroxidase (Tpx, p20, scavengase) is an atypical 2-Cys peroxiredoxin present throughout the eubacteria, including pathogenic strains, such as Escherichia coli O157:H7 [1], Yersinia spp., Haemophilus influenzae, Streptococcus pneumoniae and Helicobacter pylori [2]. Tpx constitutes part of the bacterial defence system against reactive oxygen species (ROS) and, correspondingly, is upregulated when E. coli is exposed to oxidative stress [1]. Tpx functionality specifically relies on the reducing equivalents from thioredoxin (Trx1) and thioredoxin reductase (TrxR) [3]. The catalytic cycle of peroxiredoxin activity consists of three steps: 1) peroxidation, 2) resolution and 3) recycling [4]. Atypical 2-Cys peroxiredoxins are functionally monomeric, in contrast to the typical peroxiredoxins, i.e. the resolving (Cr) and the peroxidasic (Cp) cysteines (C61 and C95, respectively in the case of Tpx) are situated on the same subunit. Structurally, this involves the reduced Tpx encountering a ROS, such as hydrogen peroxide or an alkyl hydroperoxide, and the covalent binding of O2− to Cp. The ROS is released as H2O resulting in the formation of a disulphide bridge between Cp and Cr. The cycle is completed by a transient interaction with Trx1, ending with two separate cysteine side-chains on Tpx (Figure 1).

Tpx contains three cysteine residues, two of which (C61 and C95) form the redox active disulphide bond. The third cysteine (C82) is not involved in the redox activities of Tpx [3], and is not involved in any covalent interactions. Until now, twelve structures of Tpx have been elucidated, from E. coli [4,5], Bacillus subtilis [6], Aquifex aeolicus, Mycobacterium tuberculosis [7,8], H. influenzae, and S. pneumoniae. Most of these structures have been solved in the oxidised state, or in the “forced” reduced state of the C61S (or equivalent) mutant. Two wild-type reduced structures have been solved, one by NMR [6] and one by X-ray crystallography (Structural Genomics Consortium).

Initially presumed to be localised in the periplasm [1], recent work using cross-linking and fractionation studies [9] has shown...
that Tpx is one of several peroxiredoxins in the cytosol of *E. coli*. Tpx has been shown to be important for the survival of *S. typhimurium* in macrophages, where the oxidative burst can be particularly acute [10].

We have recently shown that Tpx is one of several proteins bound by a class of “anti-virulence” compounds, the salicylidene acylhydrazides [11]. These compounds are broadly effective in reducing the expression of the type three secretion system (T3SS) of a range of Gram-negative pathogens including *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, *Yersinia pseudotuberculosis*, and *E. coli* O157 [12]. The T3SS is a critical determinant used by pathogens to modulate host cell processes and facilitate processes such as binding and invasion [13] so compounds that interfere with its expression or function have the potential to become novel anti-infective agents [12,14].

The precise molecular mechanism of action of the salicylidene acylhydrazides is not fully understood, although our identification of multiple binding proteins suggests a synergistic effect arising from a modulation of the activity of several proteins, including Tpx. The binding affinity of the salicylidene acylhydrazide compound ME0052 [N’-(3,5-dibromo-2-hydroxy-benzylidene)-nicotinic acid hydrazide] to Tpx from *Y. pseudotuberculosis* (*yp* Tpx) was measured using analytical ultracentrifugation (AUC) and showed that a catalytically inactive mutant of Tpx displayed a two-fold reduction in binding [11]. In this mutant, cysteine 61 was specifically mutated to a serine residue (C61S). The C61S mutant Tpx is present only in its reduced form and cannot undergo the intramolecular disulfide bond formation critical for the catalytic cycle of the protein.

In the current study, we present the crystal structures of *yp* Tpx in the oxidised and reduced states together with the structure of the C61S mutant. These structural data, combined with our previous NMR chemical shift analysis, allow us to perform detailed molecular modelling of how the salicylidene acylhydrazides bind to target proteins. This work helps our understanding of the mode-of-action of this class of anti-virulence compounds.

**Results and Discussion**

Purified Tpx is active and bound by the salicylidene acylhydrazides

Tpx can be readily purified using nickel affinity chromatography, thereby facilitating structural and subsequent biochemical studies. The activity of purified *yp* Tpx was tested using a glutamine synthetase (GS) assay, where an active peroxiredoxin protects against ROS [15]. Two micrograms of *yp* Tpx rescued 50% of initial GS activity, with 15 μg of *yp* Tpx raising GS activity to 90% of initial activity, clearly demonstrating that *yp* Tpx was indeed active and reduced H2O2 (Figure 2). Previous characterisation of Tpx from *E. coli* (eTpx) has shown a substrate specificity for alkyl hydroperoxides over H2O2, with a *Km* of 9 μM for cumene hydroperoxide compared with a *Km* > 1.7 mM for H2O2 [3]. However, despite this lower substrate specificity, analysis of mutants in *Salmonella* revealed that a Tpx mutant was highly susceptible to exogenous H2O2 [10].

Crystal structures

To determine the high-resolution structure of Tpx, recombinant *yp* Tpx in reduced and oxidised state, and the mutants *yp* TpxC61S were crystallised. *yp* Tpx crystallised in three crystal forms, 1, 2 and 3, in three different space groups, *P*2₁, *P*6₄ and *P*2₁2₁₂₁ respectively, as described elsewhere [16]. Crystal forms 1 and 2 grew in conditions containing DTT, and the solved structures were in the reduced state. Crystal form 1 diffracted to 2.00 Å, and the structure comprised three dimers in the asymmetric unit. The six chains superpose well, with root-mean-square-deviations (r.m.s.d.) of less than 0.5 Å. Crystal form 2, diffracting to 2.35 Å, comprises a single subunit in the asymmetric unit, the complete dimer being made up by symmetry operators.

As there are only minor differences between the two reduced structures, with an r.m.s.d. of 0.4 Å over 160 Cα, only the highest resolution structure (space group *P*2₁) will be discussed here. Most residues are accounted for in the electron density, apart from the hexa-histidine tag. The reduced structure refined to *R*<sub>free</sub> of 22.2% and 26.8%, respectively. Refinement statistics for all structures are presented in Table 1.

*yp* Tpx has a regular thioredoxin-like fold: a seven-stranded β-sheet, with β2 and β6 running anti-parallel to the rest, although with an inserted N-terminal β-hairpin (βN1-βN2) (Figure 3) absent

**Figure 1. Redox cycle of Tpx.** The reduction of ROS by oxidation (step 1) of Tpx producing H₂O (step 2). The oxidised form (disulphide bond) of Tpx is recovered by thioredoxin (Trx), and returned to the reduced form (step 3).

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**Figure 2. Glutamine synthetase assay.** The activity of *yp* Tpx is confirmed by its ability to rescue the activity of glutamine synthetase (to 90% of original activity at 15 μg *yp* Tpx).

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from other peroxiredoxins. The central sheet is flanked by four β-helices following β3, β4, β5 and β7, and one short 3_10 helix following β2. The numbering of the β-strands is based upon that of α-Tpx [5] to make direct comparisons between all Tpx molecules easier. βN1-βN2 (Figure 3) forms an L-shaped hydrophobic cleft, and it has been speculated that this cleft allows Tpx to accommodate the long fatty acid hydroperoxides [17].

Crystal form 3 captured yTPx in the oxidised state with an intact intramolecular disulphide bond between Cys61 and Cys95. The crystal belonged to space group P2_1 and diffracted to 1.74 Å. The overall oxidised structure, diffracting to a resolution of 1.74 Å and presenting space group P2_1,2_1, is similar to that of the reduced structure, except for some differences that are mostly confined to the region around the active site (see below).

As part of this study, the structure of yTPxC61S was solved to a resolution of 2.55 Å in space group P6_4. This structure represents the “forced” reduced form of the protein, as the resolving cysteine has been mutated to a serine, rendering it catalytically inactive [3]. All of our solution data indicate that the mutant structure and the reduced wild type structure are identical, and that the oligomeric states are the same. When superimposed onto the reduced structure, the r.m.s.d. was 0.52 Å over 163 Cα (Figure S1). This fits well with the structural analysis of Hall et al. [4] who used the TpxC61S mutant from E. coli to describe the structure of reduced Tpx.

yTPx crystallised either as a dimer in the asymmetric unit, where two subunits superposed with an r.m.s.d. of less than 0.2 Å, or the dimer could be created by crystallographic symmetry operators. The dimer interface comprises about 20 residues from each subunit, corresponding to 12% of total surface residues, according to the PISA server [18]. The interfaces are formed mostly by hydrophobic interactions, with a few hydrogen bonds, mainly between R110NH1 and three main-chain carboxyl groups on the opposing subunit (G125, P126, A128). There are no salt bridges or covalent bonds between the two dimers in the reduced structure. The dimer interfaces are identical in the structures of reduced Tpx and C61S.

Table 1. Refinement statistics for reported structures.

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doi:10.1371/journal.pone.0032217.t001

Active site

The redox active site of Tpx is made up by CP and CR (C61 and C95 in yTPx, respectively). There is a conformational change between the two states, involving the partial unfolding of helices α1 and α2, and a shift of 0.4 Å for C61 and 5.1 Å for C95, respectively, as presented in Figure 4. The two structures superpose well, in particular the core parts, with an r.m.s.d. of 0.7 Å over 135 Cα. Inclusion of the unfolding helices increases the r.m.s.d. to 1.02 Å.

The partial unfolding of α1 opens a cleft in Tpx formed between the loops connecting β1 and α1, α3 and β6, and α7 and β5 on subunit Α and connecting β1 and β2, and β4 and α2 on subunit B. When in the reduced state, C61 is orientated into the pocket where it is available for oxidation by H2O2 or alkyl peroxides. This cleft makes up the active site of Tpx, and has been described in detail by Hall et al. [4]. In this manuscript they present the fully intact peroxide binding site (a reduced C61S mutant), the locally unfolded binding site (oxidised), and a partially unfolded transitional state (seen only for the double C82, 95S mutant) for Tpx from E. coli that shares an identical active site.

Oligomeric state

Peroxiredoxins exhibit a wide variety of oligomeric states, ranging from monomeric (YPrx, [19]), to large decameric or dodecameric assemblies like TryP [20], AhpC [21] and other typical 2-Cys peroxiredoxins, including PrxIII from bovine mitochondria, which forms two concatenated dodecamers [22]. These assemblies are often dependent on redox state, dissociating into homodimers upon oxidation [23]. Previous studies of α-Tpx showed that the protein is a homodimer, regardless of the redox state, and despite the lack of any inter-subunit disulphide bond [3]. When superimposed onto the reduced structure, the r.m.s.d. was 0.52 Å over 163 Cα (Figure S1). This fits well with the structural analysis of Hall et al. [4] who used the TpxC61S mutant from E. coli to describe the structure of reduced Tpx.

Sedimentation velocity (SV) experiments revealed that all three forms of yTPx were completely monodisperse in solution, as evidenced by a single dominant peak in the c(s) distribution (Figure 5). Infinite dilution sedimentation coefficients (S0,20,w) were determined from the concentration dependence of c20,w (obtained from fitting the SV data with a non-interacting discrete species model in SEDFIT [24]) for the oxidised and reduced forms of yTPx (S0,20,w = 3.04
Figure 3. Crystal structures. (A) Cartoon representation of oxidised ypTpx. Strands and loops are purple, helices are green, and the disulphide bond is represented as sticks. The N-terminal hairpin is highlighted in pink. The secondary structure elements are labelled. (B) Sequence alignment of Tpx from a number of pathogens, with the secondary structure based on ypTpx. Black represents identical, and red highly similar residues (based on...
an ALSCRIPT algorithm level of 0.7 [53]). The unravelling regions of α1 and α2, caused by the change of redox state, are highlighted by the lighter colour in the secondary structure elements. The reactive C residues are highlighted in yellow, and C61S is marked by a triangle. (C) Close-up of the dimer interface with the residues involved interactions marked as sticks, and salt bridges and hydrogen bonding partners are labelled. Bonds are shown in dashes.

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and 2.62 S, respectively). For yfpTpx C61S S0,0 is 2.80 S, suggesting that this mutation does not induce structural instability (i.e. the value is comparable with those determined for reduced and oxidised yfpTpx).

Sedimentation equilibrium (SE) data were fitted with the species analysis model in SEDPHAT [25]. From the concentration dependence of the resdient apparent mass of the single species the infinite dilution mass (M0) was determined to be 41.5±3.4, 38.4±2.9, 39.4±0.4 kDa for oxidised, reduced and C61S yfpTpx respectively. The mass of yfpTpx dimer, including the tag, calculated from its amino acid sequence is 42,382 Da, which is consistent with the experimentally determined masses. This indicates that yfpTpx is present solely as a dimer in solution. It was not possible to fit the SE data with a monomer-dimer (or any other plausible) self-association model, which is further consistent with the complete dimerisation of the protein in the concentration range studied.

Solution structure

The solution structures of yfpTpx and the C61S mutant were investigated using SAXS, a powerful method to structurally analyse proteins in solution under more physiologically relevant conditions [26]. Figure 6A shows a SAXS curve for yfpTpxC61S, representative of the data obtained for yfpTpx in both oxidising and reducing conditions. yfpTpxC61S was obtained at a higher concentration than the other samples, and subsequently produced better scattering data. The Dmax and Rg of yfpTpx and yfpTpxC61S, obtained by indirect Fourier transform with GNOM [27], were the same (70.5 Å and 24.0 S, respectively) indicative that conformational changes induced by disulphide bond formation are too small to be detected by SAXS. Theoretical scattering curves of monomeric and dimeric atomic structures of Tpx were calculated, and again confirm that Tpx is a dimer in solution (Figure 6A). A low-resolution (11 Å) envelope of yfpTpxC61S (Figure 6B) in solution was generated using the ab initio modelling program DAMMIN [28]. The fit of the model to the data is shown in Figure 6A.

The high-resolution structure superimposes well onto the low-resolution envelope (Figure 6B). The Dmax of the space-fill model of the dimer crystal structure is approximately 68 Å, which agrees with the Dmax obtained from the SAXS data (70.5 Å), indicating that the low-resolution envelope describes the yfpTpx dimer. The differences in the Dmax values obtained from the two methods are small, and may be explained by the fact that in the crystal structure there is no electron density to account for the two N-terminal residues of Tpx plus the hexa-histidine tag, therefore it has not been included in calculations. However, as these residues were present in the yfpTpx studied by SAXS, we would expect the Dmax value observed in solution by SAXS to exceed that calculated for the incomplete crystal structure.

Rigid body modelling of the oxidised Tpx crystal structure against the SAXS data, using BUNCH [29], based on a single chain, and imposing P2 symmetry yielded a model similar to that for the dimeric crystal structure. Comparison of the crystallographic model with the one fitted to the solution data using DYNDOM [30] yielded a rotation angle of 21.4° and a 5 Å translation. This freedom of movement corresponds well with that observed for the structures of Tpx from other different species [4].

Modelling of salicylidene acylhydrazide compounds to yfpTpx

We have previously used NMR chemical shift mapping to identify yfpTpx amide groups that were shifted upon addition of 200 μM ME0052. The study mapped these shifting residues onto the published TpxC61S structure from E. coli (PDB code 3HVV) to show they clustered to a defined region of the protein. Now we have obtained the high-resolution structure of yfpTpx itself, allowing us to model the binding of ME0052 and ME0055 to both the oxidised and reduced forms of the protein and examine how this correlates with the NMR data. These two compounds were docked into the receptor structures using MOE Dock, and the 25 best poses determined for each compound were ranked after energy minimisation and docking scoring. Figure 7 shows the lowest energy binding modes for ME0052 (Figure 7A) bound to oxidised yfpTpx. Docking using ME0055 gave equivalent binding poses (data not shown). The binding pocket is mostly hydrophobic (Figure 7A) with one hydrogen bond proposed between the ME0052 p-hydroxyl to the yfpTpx I153 carbonyl, which fits with the chemical shift change for the neighbouring T154 amide (Figure 7B). The chemical shift data indicated significant shifts in the amides of residues from both subunits, highlighting the importance of the dimer interface for the generation of the binding pocket and compound binding, as illustrated in Figure S2A. The binding site is also consistent with previously published Tpx-substrate models [32].

Figure 4. Comparison of the oxidised and reduced active site. (A) Close-up of the active cysteines in the reduced structure. C61 is shown to occlude the active site cleft. (B) Close-up of the reduced structure. The formation of the disulphide bond shifts the helix and opens a cleft, which allows substrate access.

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During transition to the reduced form, the loop containing G59 to C61 folds into the binding pocket predicted to accommodate the compound. Similarly, the side chain of R133 undergoes a significant conformational change. Collectively, these conformational changes reduce the overall volume of the binding pocket (Figure 7C) and are predicted to affect the binding of ME0052 to the reduced state of ypTpx by inducing steric clashes. In fact, MOE Dock as not able to find a suitable docking pose for the reduced state of ypTpx, presumably because the binding site is occluded by the folded extension of the α1 helix in the reduced state.

Accurately measuring the binding of the salicylidene acylhydrazides to any protein has proved problematic due to the low solubility of the compounds in physiologically relevant solvents. This has prohibited the application of techniques including isothermal calorimetry and surface plasmon resonance that would be default methods to measure binding of ligands to proteins. Previously we have used an AUC-based method to estimate \( K_d \), giving values of 51 and 71 \( \mu M \) for the binding of ME0052 to oxidised and reduced ypTpx, respectively \[11\]. These data suggest only minor differences in compound binding to ypTpx in the two oxidation states, although we would exercise caution in the interpretation of these data due to the inherent inaccuracy of measuring \( K_d \) by this method.

An alternative binding pose, that would accommodate binding of ME0052 or ME0055, to both the oxidised and reduced forms of ypTpx is presented as Figure S2B. Although this model would require some conformational accommodation of the binding site, the compounds are predicted to be less buried in the pocket and therefore binding would be largely equivalent irrespective of the oxidation state of Tpx. Such conformational accommodation is plausible given the large backbone fluctuations observed in each state.

**Far-western analysis**

To test the binding of ypTpx to salicylidene acylhydrazide compounds, far-western blotting was used in which the protein was resolved by SDS-PAGE, transferred to a nitrocellulose membrane and probed using biotinylated ME0052 (ME0052-bio) \[33,34\]. Interactions were then detected using a StreptAvidin-HRP conjugate in a process similar to that used for a routine western blot. Following SDS-PAGE and staining by Coomassie blue, Tpx could be seen as a monomer of 21 kDa as well as a dimer of 42 kDa (Figure 8). In comparison, the purified C61S mutant was present only as a monomer of 21 kDa. This difference can be attributed to the changes in the strength of the dimer interface, arising from the loss of two salt bridges in the ‘forced’ reduced C61S mutant, which in turn is more susceptible to the heat and detergent experienced during the far-western blotting procedure. Far-western analysis indicated that ME0052-bio binds with a far higher affinity to ypTpx dimer, as over four times the amount of signal was seen corresponding to the dimer compared with the monomer (see Material and Methods). This finding is particularly stark when the relative proportions of dimer and monomer indicated on the gel are considered: quantification of the monomer-dimer on the SDS-PAGE gel indicated the ratio of these two species was 15:1. This finding confirmed our previous data \[11\] showing binding of ME0052-bio to ypTpx dimer, as over four times the amount of signal was seen corresponding to the dimer compared with the monomer (see Material and Methods). This finding is particularly stark when the relative proportions of dimer and monomer indicated on the gel are considered: quantification of the monomer-dimer on the SDS-PAGE gel indicated the ratio of these two species was 15:1. This finding confirmed our previous data \[11\] showing binding of ME0052-bio to ypTpx dimer, as over four times the amount of signal was seen corresponding to the dimer compared with the monomer (see Material and Methods). This finding is particularly stark when the relative proportions of dimer and monomer indicated on the gel are considered: quantification of the monomer-dimer on the SDS-PAGE gel indicated the ratio of these two species was 15:1. This finding confirmed our previous data \[11\] showing binding of ME0052-bio to ypTpx dimer, as over four times the amount of signal was seen corresponding to the dimer compared with the monomer (see Material and Methods).
Therefore, the dimer refolds better than the monomer and gives more signal. However, despite this reservation, the preferential binding of the compound to the $\text{ypTpx}$ dimer is consistent with our modelling data and previous NMR studies that indicated both subunits contribute to the binding pocket [11].

**Conclusion**

In summary, we have solved the high-resolution structure of $\text{ypTpx}$ in three forms enabling us to model the binding of salicylidene acylhydrazide compounds. Binding of the compound, ME0052, was found (by far-western blotting) to be markedly stronger to the Tpx dimer compared with the monomer. This is consistent with the surface area of the modelled binding site predominantly comprising one subunit yet also including the dimer interface. The solution structure confirms the oligomeric state of the protein for both redox states.

Overall, the study provides insights into the binding of the salicylidene acylhydrazide compounds to $\text{ypTpx}$, aiding our long-term strategy aiming to understand the mode of action of these compounds. Further studies characterising the role of the conformational flexibility observed around the catalytic triad and dimeric interface in ligand binding may yield additional insights into the binding mechanisms of these compounds and guide efforts to design even more effective inhibitors.

**Materials and Methods**

**Protein expression and purification**

$\text{ypTpx}$ and $\text{ypTpxC61S}$ were expressed and purified as described previously [16], and the N-terminal hexa-histidine tag formed part of the expressed protein.

**Glutamine synthetase assay**

The enzymatic activity of $\text{ypTpx}$ was demonstrated by a glutamine synthetase (GS) protection assay [15]. Briefly, 4 µl (6.7 U) of commercially purchased GS (Sigma) was mixed with increasing amounts of $\text{ypTpx}$ (1 µg, 2 µg, 4 µg, 6 µg, 8 µg, 10 µg, 15 µg, 20 µg and 50 µg) and 10 µl inactivation solution (50 mM DTT, 25 µM FeCl$_3$), in a final volume of 100 µl (made up in 100 mM HEPES pH 7.4). The mix was incubated for 30 min at room temperature. 2 µl of assay mix (100 mM HEPES, 10 mM KH$_2$AsO$_4$, 20 mM Na$_2$H$_2$O$_4$, 0.4 mM ADP, 0.5 mM MnCl$_2$, 100 mM glutamine, pH 7.0–7.2) was added to each solution and the incubation continued at 37°C. After 30 min, 1 µl of stop solution (5.5% (w/v) FeCl$_3$, 2% (w/v) TCA, 2.1% (v/v) concentrated HCl) terminated the reaction. Absorbance of the samples was measured at 540 nm.

**Protein crystallisation**

Purified proteins were dialysed overnight against 20 mM Tris pH 7.5, 50 mM NaCl and kept at a concentration of approximately 8 mg ml$^{-1}$ (based on the absorbance at 280 nm, and a calculated extinction coefficient of 4595 M$^{-1}$ cm$^{-1}$), for crystallisation studies using crystallisation conditions described previously [16].

**Diffraction data collection and structure solution**

All diffraction data were collected at Diamond Light Source (Oxfordshire UK), processed with MOSFLM [35] and scaled in SCALA [36], both parts of the CCP4 suite of programs [37,38], or d*TREK [39]. The relevant statistics are published elsewhere.
from the crystal structure coordinates using CRYSOL [50].

imposed, namely a maximum distance of 7 Å between D57 and
distance restraints between interacting interface residues were
maintenance of the correct dimer interface in the reconstruction,
data by rigid body fitting, using BUNCH [29]. In order to ensure

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