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Stefan H. Millson[†], Cara K. Vaughan[‡], Chao Zhai[§], Maruf M. U. Ali[‡], Barry Panaretou[§], Peter W. Piper[†], Laurence H. Pearl[‡] and Chrisostomos Prodromou[‡, #]

[†]Department of Molecular Biology and Biotechnology, The University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK; [‡]Section of Structural Biology, The Institute of Cancer Research, Chester Beatty Laboratories, 237 Fulham Road, London SW3 6JB, UK; [§]Pharmaceutical Science Research Division, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, UK

[#] To whom correspondence should be addressed: Tel: (44) (0) 207 153-5449, Fax: (44) (0) 207 153-5457, Email: chris.prodromou@icr.ac.uk
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Abbreviations: tetratricopeptide, TPR; ITC, isothermal titration calorimetry

Tah1 has been identified as a tetratricopeptide (TPR)-domain protein. TPR-domain proteins are involved in protein-protein interactions and a number have been characterized that interact either with Hsp70 or Hsp90, but a few can bind both chaperones. Independent studies suggest that Tah1 interacts with Hsp90, but whether it can also interact with Hsp70/Ssa1 has not been investigated. Amino acid sequence alignments suggest that Tah1 is most similar to the TPR2b-domain of Hop which when mutated reduces binding to both Hsp90 and Hsp70. Our alignments suggest that there are three TPR-domain motifs in Tah1 which is consistent with the architecture of the TPR2b-domain. We find that Tah1 is specific for Hsp90, able to bind tightly the yeast Hsp90, and the human Hsp90α and Hsp90β proteins, but not the yeast Hsp70, Ssa1 isoform. Tah1 acheives ligand discrimination by favourably binding the methionine residue in the conserved MEEVD-motif (Hsp90) and positively discriminating against the first valine residue in the VEEVD-motif (Ssa1). We also show that Tah1 can affect the ATPase activity of Hsp90, in common with some other TPR-domain proteins.

INTRODUCTION

Heat shock protein 90 (Hsp90) molecular chaperone plays a central role in the maturation and activation of key signalling proteins (reviewed in [1, 2]) that include steroid hormone receptors [3, 4] and protein kinases [5-9] amongst others [1, 2, 10, 11]. An up-to-date comprehensive list can be found at http://www.picard.ch/downloads/downloads.htm The activation of such proteins (client proteins) is regulated by the association of specific co-chaperones that complex with Hsp90, at varying stages of the chaperone cycle [12, 13]. A number of these co-chaperones play an apparent role in recruiting client proteins to the Hsp90 chaperone complex. In the case for steroid-hormone receptors it appears that the receptor is complexed with the teratricopeptide (TPR)-domain containing co-chaperone

Hop/Sti1 and Hsp70. Binding of Hop/Sti1 to the conserved MEEVD-motif at the extreme C-terminal end of Hsp90 delivers the steroid hormone receptor to Hsp90 (reviewed in [4, 14]). Protein kinases on the other hand are complexed with Cdc37/p50 (reviewed in [15]) that binds the N-terminal domain of Hsp90 [16]. In *Sacharomyces cerevisiae*, Tah1 (YCR060W), consisting of 111 amino acid residues, was identified as an Hsp90 interacting co-chaperone in three independent studies [17-19]. Tah1 is thought to associate with Pih1 and together complex with the essential DNA helicases, Rvb1 and Rvb2 that are key components of the chromatin remodelling complexes Ino80 and SWR-C [19]. Tah1 contains a single TPR-domain with at least two TPR-motifs [19].

Numerous crystallographic structures of TPR-domain containing proteins have been solved [20-25]. These reveal a common motif that is composed of a degenerate 34 amino acid sequence that forms an antiparallel alpha-helical hairpin, which in turn cluster to form a domain with a grooved surface that acts as a peptide-binding site. TPR-domains mediate protein-protein interactions in numerous protein complexes [26-28]. The question of ligand discrimination by TPR-domains has been addressed [29-31]. TPR-domain proteins such as Hip have been implicated in the regulation of the ATPase activity of Hsp70, while Hop/Sti1 has been shown to inhibit the ATPase activity of Hsp90, to activate Ssa1, but to have no affect on the vertebrate Hsp70 [32-36]. The TPR-domains of Hsp70 and Hsp90 co-chaperones interact with the extreme C-terminal amino acid sequences of their respective chaperone [34, 37-41], which share a common motif (EEVD). Residues immediately upstream of the conserved EEVD-motif of Hsp70 and Hsp90 achieve selective binding to different TPR-domains [20-23, 25]. In contrast, the co-chaperone CHIP, involved in the ubiquitylation of chaperone-bound client proteins [42, 43], binds to both Hsp70 and Hsp90 by associating with the MEEVD- (Hsp90) or the IEEVD-motif (human Hsp70) and avoiding further interaction with upstream amino acid residues. This is achieved by a hydrophobic pocket that accommodates the methionine residue (M in MEEVD) from Hsp90 and the isoleucine residue (I in IEEVD) of human Hsp70 and in doing so twists the upstream peptide, which differs between the two chaperones, and hoists it clear of the TPR-domain groove [24]. In contrast, structural information showed that for Hop/Sti1 the N-terminal TPR-domain (TPR1) interacts with Hsp70, whereas the central TPR-domain (TPR2a) binds to Hsp90 [21]. However, the situation might be more complex in that mutations in the third TPR-domain (TPR2b) disrupt association with both Hsp70 and Hsp90 [39]. A high affinity ligand for the TPR2b-domain has not been found [21, 29].

In this paper we show that the TPR-domain of Tah1 most closely resembles the TPR2b-domain of Hop/Sti1 and address whether Tah1 is an Hsp90 specific co-chaperone or whether it can also associate specifically with Ssa1. We show that Tah1 is specific for Hsp90 and that ligand discrimination is achieved by favourable binding of the methionine residue of the conserved MEEVD-motif of Hsp90, and by positively discriminating against the first valine of the VEEVD-motif of Ssa1. We also characterise the oligomeric state of Tah1 and investigate its ability to regulate the ATPase activity of Hsp90.

MATERIALS AND METHODS

Sequence alignments

The amino acid sequences for Tah1, Sti1 and Hop were obtained from Genbank. The accession numbers were, Human Hop, NM_006819; *Saccharomyces cerevisiae* Sti1, Z74935 and *S. cerevisiae* Tah1, NC_001135. The sequence alignment was generated using Clustal W

[44] 'on-line' at the European Bioinformatic Institute, which is part of the European Molecular Biology Laboratory (EMBL-EBI).

Protein production and Hsp90 ATPase activity assays

Expression and purification of wild type and mutant forms of His-tagged yeast Hsp90, His-tagged Hsp90β, mouse His-prescission tagged CHIP and His-Tagged C-terminal domain of Sti1 (cSti1) was previously described [24, 35, 45]. The yeast Tah1, the human Hsp90α and the C-terminal domain of yeast Hsp90 were cloned into pRSETA as *Nhe*I-*Eco*RI, *Nhe*I-*Pst*I and *Nhe*I-*Hin*dIII DNA fragments, respectively, and expressed as His-Prescission tagged proteins. The yeast Ssa1 gene was cloned into pRSETA as an *Nhe*I-*Hin*dIII DNA fragment and expressed as a His-tagged fusion. The same protein purification procedure as used for yeast Hsp90 was employed to purify the C-terminal domain of Hsp90, Hsp90α, Ssa1 and Tah1. PreScission cleavage of the His-tags was carried out overnight at 4°C, as described by the manufacturer (GE Healthcare). The cleaved sample was then passed through a 1 ml GST-Hi Trap column, equilibrated in 20 mM Tris pH 7.5, 140 mM NaCl and 2.7 mM KCl, to remove GST-tagged prescission. The flow through was collected, desalted and then subjected to Talon-affinity chromatography to remove uncleaved His-Prescission Tah1 protein. The Talon-resin flow through was then concentrated and subjected to Superdx 75- or 200-HR gel-filtration chromatography, as appropriate. All purified proteins were dialysed against 20 mM Tris-HCl pH 7.5 containing 1 mM EDTA and 1 mM DTT and then concentrated using Vivaspin concentrators (Sartorius) with an appropriate molecular weight cut-off. The ATPase assays were performed in triplicate as previously described [45, 46], using 2 μM yeast Hsp90 and either 20 μM Tah1 or 30 uM geldanamycin. Protein concentration was determined by using the extinction coefficient of the protein (wild type and mutant yeast Hsp90, 54,050; cleaved C-terminal domain of yeast Hsp90, 18,260; cleaved Hsp90α, 59,625; Hsp90β 58,135; Ssa1, 20,045; cleaved CHIP, 29,380; cSti1, 34,480 and cleaved Tah1 10,295).

Isothermal titration calorimetry: stoichiometry and K_d determination.

Heat of interaction was measured on a MSC system (Microcal) with a cell volume of 1.458 ml. For Tah1 and CHIP interactions, 9-11 aliquots of 27 μl of 300 μM Tah1 or CHIP were injected into 30 μM of yeast Hsp90, yeast Hsp90 M705V, human Hsp90α, human Hsp90β or yeast Hsp90 C-terminal domain at 4°C (Tah1 experiments) or 30°C (CHIP experiments) in 20 mM Tris-HCl pH 8.0 containing 1 mM EDTA and 5 mM NaCl. For peptide interactions, either 9-11 aliquots of 27 μl of 300 μM peptide or 14-15 aliquots of 400 μM peptide were injected into 30 μM Tah1 at 4°C in 20 mM Tris-HCl pH 8.0 containing 1 mM EDTA and 5 mM NaCl. For cSti1 interactions 11 aliquots of 27 μl of 300 μM cSti1 were injected into 30 μM of yeast Hsp90 or yeast Hsp90 M705V at 30°C in 20 mM Tris-HCl pH 8.0 containing 1 mM EDTA and 5 mM NaCl. Heat of dilution was determined in a separate experiment by diluting protein or peptide into buffer, and the corrected data fitted using a nonlinear least square curve-fitting algorithm (Microcal Origin) with three floating variables: stoichiometry, binding constant and change in enthalpy of interaction. For the Ssa1-Tah1 and Tah1-VEEVD peptide experiment the stoichiometry was fixed at N = 1. All peptides were accurately weighed on an analytical balance and dissolved in dialysis buffer.

Relative molecular weight determination by Gel-filtration

0.5 ml aliquots of PreScission cleaved Tah1 (1.4 mg ml^{-1}) were loaded onto a Superdex 75 HR 16/60 column equilibrated in 20 mM Tris–HCl pH 7.4, 1 mM EDTA and 250 mM NaCl. The column was run at 0.5 ml min^{-1} and was calibrated using gel-filtration standards from Bio-Rad (bovine thyroglobulin, *M*r 670,000; bovine γ-globulin, *M*r 158,000; chicken ovalbumin, *M*r 44,000; horse myoglobin, *M*r 17,000 and vitamin B12, *M*r 1,350) and GE Healthcare (blue dextran 2,000; bovine albumin, *M*r 67,500; chicken ovalbumin, *M*r 43,000; bovine chymotrypsinogen A, *M*r 25,700 and bovine ribonuclease A, *M*r 13,700). The relative molecular weight of Tah1 was determined by logarithmic interpolation.

Analytical ultracentrifugation

PreScission cleaved Tah1 was dialyzed against 20 mM Tris pH 7.5 containing 1 mM EDTA and 5 mM NaCl. Sedimentation equilibrium analysis was performed at 20ºC in a Beckman XL-I Analytical Ultracentrifuge, following a Standard Operating Procedure SE_IO_07.1 at sample concentrations of 7.0 and 0.5 mg ml^{-1} (547 and 39, μM, respectively). After initial scans at 3000 rpm to check for the presence of any large aggregates, a final rotor speed of 22,000 rpm was selected. A 12 mm optical path length centerpiece was used in the cell. Scans were taken every 60 minutes and data logged to disk using the Beckman software. After 24 hours, no further change could be detected in the scans, and the run was terminated. The data were analysed by three methods using the INVEQ-, the recently derived Mfit- and a local (OS-X-profit) version of the widely used NONLIN-algorithm. *K*a values were converted to *K*d values by simple reciprocation.

RESULTS

Tah1 selectively binds Hsp90

Tah1 has been identified as a TPR-domain protein that interacts with Hsp90. Our sequence alignments suggest that Tah1 is most similar to the TPR2b-domain of Hop/Sti1 (Supplementary Figure 1), which influences the binding of Hop to both Hsp70 and Hsp90 [39]. In order to investigate the ability of Tah1 to bind both Ssa1 and Hsp90 we used isothermal titration calorimetry (ITC) to determine the extent of any interaction. Figure 1a shows that Tah1 binds to the full-length yeast Hsp90 (K_d = 0.78 ± 0.06 μM) with a stoichiometry of 1:1, while Figure 1b shows that the binding site is solely located in the C-terminal domain of Hsp90 (K_d = 0.32 ± 0.03 μM; Table 1 summarises all ITC data presented in this paper). Tah1 was also shown to be able to interact with the human Hsp90α (K_d = 0.33 ± 0.03 μM) and Hsp90β (K_d = 0.34 ± 0.07 μM) paralogs with a similar affinity (Figure 1c, d). However, its interaction with the yeast Ssa1 was significantly weaker (K_d = 16 ± 2.5 μM; Figure 1e), where the strength of the interaction is reflected in the shape of the binding curve for which a steeper gradient in the observed curve indicates tighter binding (compare Figure 1a and 2e), and suggests that Tah1 is an Hsp90 specific co-chaperone.

Comparison of MEEVD-peptide bind-ing to Tah1 and TPR2a

In the TPR1-VEEVD and TPR2a-MEEVD complex most of the direct hydrogen bonded interactions with bound-peptide involve the main-chain and are therefore sequence independent. Both complexes have a highly conserved two-carboxylate clamp. In the TPR2a complex the terminal main-chain carboxylate of MEEVD is hydrogen bonded to Lys229, Asn233 and Asn264. These residues are also conserved in Tah1 (Lys8, Asn12 and Asn43, respectively; see Supplementary Figure 1 and Figure 2). However, in contrast, residues that interact with the side-chain of the terminal aspartate, which is bound by Lys301 and Gln298 in TPR2a, are not conserved in Tah1 (Thr70 and His73, respectively). Consequently, if a two-carboxylate clamp exists in the peptide-bound Tah1 complex it is clearly different to that seen in TPR2a. Other electrostatic interactions include a tetrahedrally coordinated water molecule that interacts with the carboxylate side-chain of the terminal aspartate, and with the main-chain carbonyl of Thr263 (Ser42 in Tah1), the guanidinium group of Arg305 (Arg77 in Tah1) and the side-chain carbonyl of Asn264 (Asn43 in Tah1). The side-chain amide of Asn264 is also involved in main-chain interactions with the backbone amide of the terminal aspartate residue, while the guanidinium group of Arg305 also contacts the main-chain carbonyl and amide (via a water molecule) of Glu-2 (S^{-6}RMEEVD^{0}). Furthermore, the hydroxyl of Tyr236 (Phe15 in Tah1; Supplementary Figure 1 and Figure 2) contacts the main-chain carbonyl of Glu-3, an interaction not possible with Phe15 of Tah1, while the side-chain carbonyl group of Glu271 (Lys50 in Tah1) contacts the main-chain amide of Glu-3. The only interactions to side-chains (excluding the two carboxylate clamp) are between the guanidinium group of Arg305 and the side-chain amide of Asn308 (Lys 79 in Tah1) to the carboxylate group of Glu-3. Additionally, Val-1 is involved in a hydrophobic interaction with a pocket formed by Asn233, Tyr236 (Phe15 in Tah1), Asn264 and Ala267 (Met46 in Tah1), whereas the side-chain of Met-4 binds between Tyr236 (Phe15 in Tah1) and Glu271 (Lys50 in Tah1). Two further Van der Waals contacts involve the side chain of Glu-3 and Glu-0 with Phe270 (Ile49 in Tah1) and Thr260 (Val39 in Tah1), respectively. It should be noted that the Glu-2 side-chain is not involved in interactions. In summary, our alignments suggest that there are significant residue differences between the TPR2a- and the Tah1 TPR-domain that point to differences in the precise way they would bind the MEEVD peptide (Supplementary Figure 1 and Figure 2).

Ligand discrimination by Tah1 is similar to that of the TPR2a domain of Hop

For Hop the residue at position -4 of Hsp70 (I^{-4}EEVD^{0} or VEEVD for SsaI) and Hsp90 (MEEVD) primarily determines the specificity for the TPR1- and TPR2a-domains, respectively [29]. To determine whether Tah1 ligand discrimination was similar to that of TPR2a we investigated the ability of Tah1 to bind peptides representing the C-terminal ends of Hsp90 and Ssa1. The results in Figure 3 show that the binding affinity for the C-terminal peptides of yeast Hsp90 increases with increasing peptide length (MEEVD, K_d = 35.4 ± 4.4 μM; TEMEEVD, K_d = 0.95 ± 0.04 μM; PADTEMEEVD, K_d = 0.73 ± 0.04 μM; Figure 3a-c) and that the decapeptide most likely represents the intact binding site for Tah1 (PADTEMEEVD, K_d = 0.73 ± 0.04 μM and full-length yeast Hsp90, K_d = 0.78 ± 0.06 μM; Figure 3c and 2a). Indeed, the binding of Tah1 to the human Hsp90α- and Hsp90β-decapeptide was also similar to that of the corresponding full-length proteins (Hsp90α decapeptide, DDTSRMEEVD, K_d = 0.9 ± 0.06 μM; Hsp90β decapeptide, EDASRMEEVD, K_d = 1.0 ± 0.19 μM; full length Hsp90α, K_d = 0.33 ± 0.03 μM and full length Hsp90β, K_d =

0.34 ± 0.07 μM; Figure 1c, d and 3d, e). In contrast to the Hsp90 decapeptides, peptides representing the C-terminal end of Ssa1 showed a significantly weaker affinity for Tah1 (VEEVD, K_d = 48.8 ± 5.5 μM; PTVEEVD, K_d = 10.6 ± 1.4 μM and AEGPTVEEVD, K_d = 28.9 ± 3.1 μM; Figure 4a-c).

The methione residue of the conserved MEEVD-motif confers tight Tah1 binding

The results so far show that Tah1 preferentially binds Hsp90 over Ssa1. Consequently, we next investigated the mechanism behind this selectivity. A comparison of the decapeptide sequences from a variety of cytoplasmic Hsp90s suggests that there is very little sequence conservation upstream of the conserved MEEVD-motif, except at the -6 position (91% conservation) and -5 position (68% conservation) [29]. This suggests that position -6 (which is mainly serine and rarely a threonine residue) may be critical for the specificity in the interaction with Tah1. Using ITC the binding of the PAD*AA*MEEVD decapeptide (changes to wild type sequence are shown in italic; K_d = 0.75 ± 0.04 μM; Figure 5a) was found to be similar to the decapeptide with wild type sequence (PADTEMEEVD, K_d = 0.73 ± 0.04 μM; Figure 3c). Consequently, tight binding of Tah1 to Hsp90 is determined within the conserved MEEVD-motif, and upstream amino acids appear to be involved in mainly main-chain interactions. However, since the EEVD-motif is common to both Ssa1 and Hsp90 it follows that the conserved methionine of the MEEVD-motif is responsible for affecting tight binding to Tah1. Our results show that a decapeptide containing an alanine (PADTE*A*EEVD) or valine (PADTE*V*EEVD) in place of the conserved methionine in the MEEVD-motif significantly reduced the affinity for Tah1 (K_d = 3.3 ± 0.2 and 9.8 ± 0.7 μM, respectively; Figure 5b, c) relative to wild type decapeptide (K_d = 0.73 ± 0.04 μM; Figure 3c). Therefore, the results suggest that ligand discrimination for the MEEVD-peptide by Tah1 and the TPR2a domain of Hop [21] is similar in that both favour a methionine at position -4 of the MEEVD motif of Hsp90.

Tah1 positively discriminates against the valine at position -4 in the AEGPTVEEVD-motif of Ssa1

The results so far suggest that the first valine in the VEEVD-motif of Ssa1 prevents tight association with Tah1. Thus introducing an alanine or methionine residue at position -4 of the Ssa1 decapeptide should increase the binding affinity relative to a decapeptide containing a valine at this position. Our results show that this is indeed the case, where the decapeptides AEGPT*A*EEVD and AEGPT*M*EEVD, were found too bind with increasing affinity (K_d = 14.8 ± 0.7 and K_d = 4.2 ± 0.5 μM, respectively; Figure 5d, e) relative to the wild type sequence decapeptide (K_d = 28.9 ± 3.1 μM; Figure 4c).

The M705V mutation of Hsp90 disrupts Tah1 binding

Having established with decapeptides that the methionine in the conserved MEEVD-motif of Hsp90 confers specificity for Tah1 binding we investigated whether this was also true for the intact yeast Hsp90. We therefore made the yeast Hsp90 M705V mutation to see whether it would bind Tah1 significantly less tightly. Firstly we tested the M705V mutation with CHIP, known to bind both Hsp90 and Hsp70, and then with the C-terminal domain of Sti1 (cSti1),

which favours Hsp90 binding. As expected, the M705V mutation bound CHIP with an more or less equal affinity (K_d = 6.7 ± 0.62 μM) relative to wild type Hsp90 (K_d = 4.2 ± 0.63 μM; Figure 6a, b). Previous estimate for the CHIP and human Hsp90β interaction by ITC (K_d =4.9 μM) is consistent with the value obtained here with yeast protein [24]. For cSti1 the M705V mutation bound less tightly (K_d = 0.20 ± 0.03 μM) than the wild type protein (K_d = 0.04 ± 0.01 μM; Figure 6c, d). It is noted that previous estimates with full-length Sti1 appear to be slightly lower in affinity (K_d = 0.24 ± 0.07 and 0.33 ± 0.03 μM; [35, 47]) against the wild type Hsp90, indicating that TPR1 may have a negative affect on the binding of the TPR2a-domain. However, having established that the M705V mutation affected the binding of both CHIP and the cSti1 in a predictable manner we next tested the binding of Tah1 and, as expected, we found that the M705V mutation inhibited the binding of Tah1 (K_d = 13 ± 1.7 μM) relative to wild type protein (K_d = 0.78 ± 0.06 μM; Figure 1a and 6e).

Tah1 weakly stimulates the ATPase activity of Hsp90

TPR-domain containing proteins have been implicated in the regulation of the ATPase activity of Hsp90. The first report of such regulation was for Sti1, which was shown to be a potent inhibitor of the ATPase activity of Hsp90 [35]. In contrast, the TPR-domain containing co-chaperone, Cpr6, has been shown to have a weak stimulatory affect on the ATPase activity of Hsp90 [45]. Using a ten-fold excess of Tah1 we were able to show a very weak stimulation of the ATPase activity of Hsp90 (Figure 7a).

Tah1 appears to be primerally monomeric

TPR-domain containing proteins have been shown to be both monomeric (immunophillins such as, FKBP51 and Cpr6) and dimeric (Hop/Sti1 and CHIP) in nature [24, 35, 48]. We were interested in determining the oligomeric state of Tah1. We employed gel-filtration chromatography to determine the relative molecular weight of Tah1. Tah1 eluted, as a single peak, with a *M*r (relative molecular weight) of 18,500 (Figure 7b). As Tah1 is basically a TPR-domain it is reasonable to expect this protein to be slightly elongated, rather than globular, in common with other TPR-domain motifs. Consequently, on gel-filtration it would appear slightly larger than 12.8 Kd (cleaved Tah1). Our gel-filtration results therefore suggest that Tah1 is most likely monomeric. To support this we conducted an analytical sedimentation equilibrium analysis on Tah1. Good results with high quality fits were obtained from both 7 and 0.5 mg ml^{-1} samples, and these are shown in Figure 7c and 7d. The Tah 1 sample clearly cannot be simply monomeric, as the average molecular weight (See Table 2) by two independent methods confirms this. The excellent fit obtained with the INVEQ approach is essentially conclusive evidence that a dimeric species is present (estimated to be around 19%), but probably not higher oligomers, at least in significant quantity. The single experiment at 7 mg ml^{-1} is however not by itself capable of distinguishing between the presence of a dimer in reversible equilibrium with monomer, and an irreversible dimer being present. To make this distinction we must compare the two, in this case widely separated, solute concentrations. The predictions for the effect of dilution are that if a reversible dimer is present the K_d value estimated will be the same, but the average molecular weight will decrease markedly. However, if an irreversible dimer is present the K_d value estimated will increase markedly, but the average molecular weight will remain the same. Inspection of Table 2 shows immediately that it is the second hypothesis that is supported.

The results therefore show that Tah1 is primerall y monomeric under the conditions used, but that some dimeric species is also present. The fact that the monomeric form is not in equilibrium with the dimeric species suggests that the dimer may not be a biologically native species, but rather aggregated molecules of Tah1. TPR-domains are known to self-associate [20] and we have in this study observed irreversible aggregation and precipitation of Tah1 on prolonged storage and during handling of the protein. Even though our samples were spun to remove such aggregates small soluble species might not be removed by centrifugation. We therefore conclude that Tah1 is most probably monomeric as a biologically active species and that the dimeric species is most probably irreversibly aggregated dimer.

DISCUSSION

This study has shown that the TPR-domain of Tah1 is similar to that of the TPR2b-domain of Hop. Tah1 was shown to selectively favour the binding of methionine over a valine at position -4, and therefore selectively binds Hsp90 over Ssa1, in common with the TPR2a-domain of Hop. However, although tight binding of ligand by Tah1 and the TPR2a domain of Hop is dependant on a methionine at this position the overall interactions that these domains make with the MEEVD-motif are actually different since the sequence conservation between the two domains is significantly diverged. This is even more remarkable as many of the upstream contacts from the MEEVD-motif and contacts made by the TPR2a-domain with MEEVD appear to be man chain contacts to their respective peptides.

The orientation of peptide binding in different TPR-domains has been shown to vary. For example the peptide in the TPR2a-MEEVD complex is bound in an antiparallel orientation to α-helix 1, 3, 5 and 7 of the domain. However, in the Cyp40- and FKBP51-complexes the EEVD is bound in the opposite orientation [21-23, 25]. Our amino acid sequence alignment showed that for residues involved in contacting the MEEVD-motif by the TPR2a-domain seven residues are identical and eight residues are different at equivalent positions in the Tah1-domain. Consequently, the orientation in which the MEEVD-peptide binds to Tah1 cannot be assumed to be the same as seen in the TPR2a-MEEVD complex, and must await structural determination to be resolved.

A high affinity-binding motif for the TPR2b domain of Hop has not been identified [21, 29], but surprisingly Tah1 was shown to be most similar to The TPR2b domain of Hop. This suggests that differences between these two TPR-domains direct their selectivity towards different binding sites. It is interesting to note that the TPR2b-domain has been shown to be critical for the interaction of the TPR2a-domain of yeast Sti1 with Hsp90[49].

It has also been reported that binding of TPR-domain containing co-chaperones to Hsp70 and Hsp90 can influence the ATPase activity of the chaperone [32-36]. Under the assay conditions that we applied we were able to detect a weak stimulation of the ATPase activity of Hsp90 with a ten-fold excess of Tah1. While this activation is very weak the ATPase activity of Hsp90 is critical to its proper functioning [46]. However, how TPR domain proteins bind the extreme C-terminus of Hsp90 and influence its ATPase activity is still not understood and may have to wait for structural studies to shed light on the precise mechanism.

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Table 1. Summary of the binding affinities of Tah1, Chip and cSti1.

Tah1 interactions with	K_d (μM)	Stoichiometry (N)
Yeast Hsp90	0.78±0.06	1.1
C-terminal domain of Hsp90	0.32±0.03	0.93
Hsp90α	0.33±0.03	1.1
Hsp90β	0.34±0.07	0.84
Ssa1	16±2.5	1.0 (fixed)
YeastHsp90 peptides		
MEEVD	35.4±4.4	1.1
TEMEEVD	0.95±0.04	1.2
PADTEMEEVD	0.73±0.04	1.0
PADAAMEEVD	0.75±0.04	0.81
PADTEAEEVD	3.3±0.2	0.89
PADTEVEEVD	9.8±0.7	1.1
Human Hsp90 peptides		
DDTSRMEEVD	0.9±0.06	1.1
EDASRMEEVD	1.0±0.19	0.81
Ssa1 peptides		
VEEVD	48.8±5.5	1.0 (fixed)
PTVEEVD	10.6±1.4	1.0
AEGPTVEEVD	28.9±3.1	1.2
AEGPTAEEVD	14.8±0.7	1.1
AEGPTMEEVD	4.2±0.5	0.98
Hsp90 and M705V interactions		
CHIP-Hsp90	4.2±0.63	1.2
CHIP-Hsp90 M705V	6.7±0.62	1.1
cSti1-Hsp90	0.04±0.01	0.88
cSti1-Hsp90 M705V	0.2±0.03	0.98
Tah1-Hsp90 M705V	13±1.7	0.83

Table 2. Summary of values for parameters estimated via sedimentation equilibrium analysis for Tah1 at two cell loading concentrations:

Protein Concentration (mg ml^{-1})	K_d by INVEQ (mM)	M(z) by Mfit	M(z) by NONLIN' (kDa)	$K_{d'}$ by NONLIN' (mM)
7.0	3.9	15.968	16.062	1.80
0.5	0.51	16.106	15.845	0.15

FIGURE LEGENDS

Figure 1 Isothermal titration calorimetry of Tah1 with Hsp90 and Ssa1

ITC by injecting Tah1 into (a), yeast Hsp90 (yHsp90); (b), the C-terminal domain of yeast Hsp90 (C-yHsp90); (c), Hsp90α; (d), Hsp90β and (e), Ssa1. The results show that the Tah1 interaction with full length yeast Hsp90, Hsp90α, Hsp90β and C-yHsp is significantly tighter than against the yeast Hsp70, Ssa1.

Figure 2 Pymol diagram showing the interaction of the MEEVD peptide with the TPR2a domain of Hop.

Residues are labeled and those in brackets are the equivalent residues of Tah1. Single letter code is used for the MEEVD peptide sequence. Water molecules are shown as cyan coloured balls and polar interactions as dashed lines.

Figure 3 Isothermal titration calorimetry of Tah1 with Hsp90 peptides carrying the MEEVD-motif

ITC by injecting into Tah1 (a), the pentapeptide MEEVD; (b), the heptapeptide TEMEEVD and (c), the decapeptide PADTEMEEVD all representing the C-terminal end of yeast Hsp90 and (d), the decapeptides DDTSRMEEVD and (e), EDASRMEEVD, representing the human Hsp90α and Hsp90β C-terminal ends. The results show that the Tah1 interaction increases in affinity with increasing length of the peptide and that decapeptides representing the extreme C-terminus of Hsp90α and Hsp90β bind with a similar affinity to the equivalent decapeptide of yeast Hsp90.

Figure 4 Isothermal titration calorimetry of Tah1 and Ssa1 peptides containing the VEEVD-motif.

ITC by injecting into Tah1 (a), the pentapeptide VEEVD; (b), the heptapeptide PTVEEVD and (c), the decapeptide AEGEPTVEED representing the extreme C-terminus of Ssa1. The results show that the Tah1 interaction with Ssa1 peptides, remains weak, and does not increase in affinity with increasing peptide length, in contrast to equivalent peptides based on Hsp90.

Figure 5 Isothermal titration calorimetry with Tah1 and peptides with altered Hsp90 and Ssa1 sequence

ITC by injecting into Tah1 the modified decapeptides (a), PAD*AA*MEEVD; (b) PADTE*A*EEVD; (c), PADAA*V*EEVD; (d), AEGPT*A*EEVD and (e), AEGPT*M*EEVD. The results show that the upstream conserved TE motif (in PADTEMEEVD) of Hsp90 when replaced by Ala-Ala does not disrupt binding to Tah1, and that at position -4 a methionine residue is favored over an alanine or valine. The results also show that a methionine, and to a lesser extent an alanine, at position -4 of the Ssa1 decapeptide increases the binding affinity of the peptide over that containing a valine at this position.

Figure 6 Isothermal titration calorimetry of TPR-domain containing proteins and the Hsp90 M705V mutation

ITC by injecting CHIP into (a), yeast Hsp90 or (b), M705V mutant or by injecting (c), yeast Hsp90 or (d), M705V mutant into cSti1 or by injecting (e), Tah1 into the M705V mutant. The results show that the M705V mutation of Hsp90 does not affect the binding of CHIP, but in contrast the binding of the cSti1 and Tah1 is significantly weaker.

Figure 7 Tah1 activation of Hsp90 and determination of the relative molecular weight of Tah1

(a), ATPase activation of yeast Hsp90 by prescission cleaved Tah1; (b), gel-filtration chromatography of prescission cleaved Tah1; (c), analytical equilibrium sedimentation ultracentrifugation analyses (INVEQ, Mfit and NONLIN fits, top to bottom) of prescission cleaved Tah1 at 7.0 mg ml^{-1} and (d), 0.5 mg ml^{-1}. The dotted line represents the fit to the data point. The results show that prescission cleaved Tah1 can weakly activate the ATPase activity of Hsp90, elutes with a *Mr* of 18,500 on gel-filtration, which is close to the values estimated by analytical centrifugation (M(z) by Mfit = 15,968 and 16,106). The results indicate that Tah1 is mostly a monomer under the conditions used.

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Figure 1

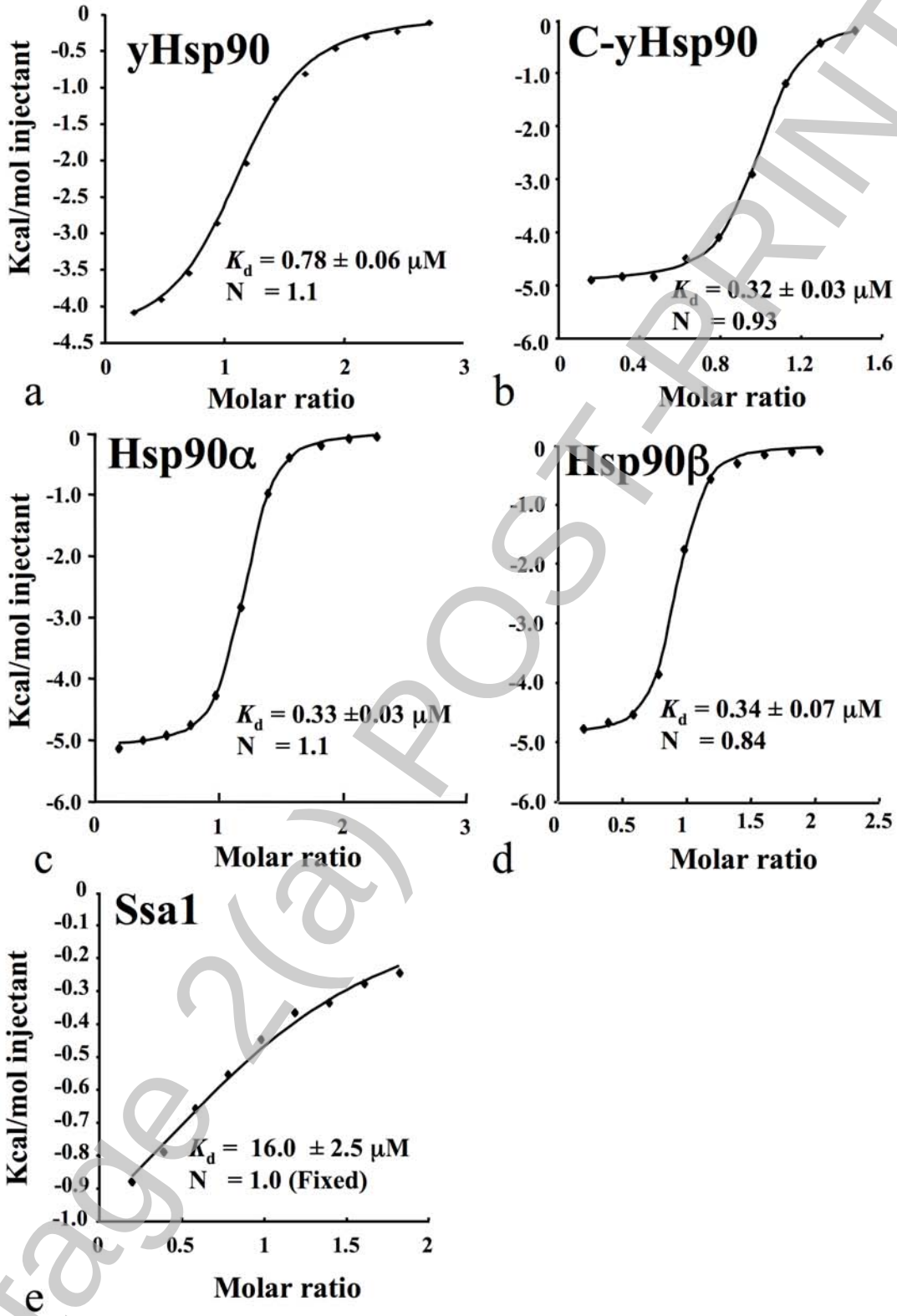

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Figure 2

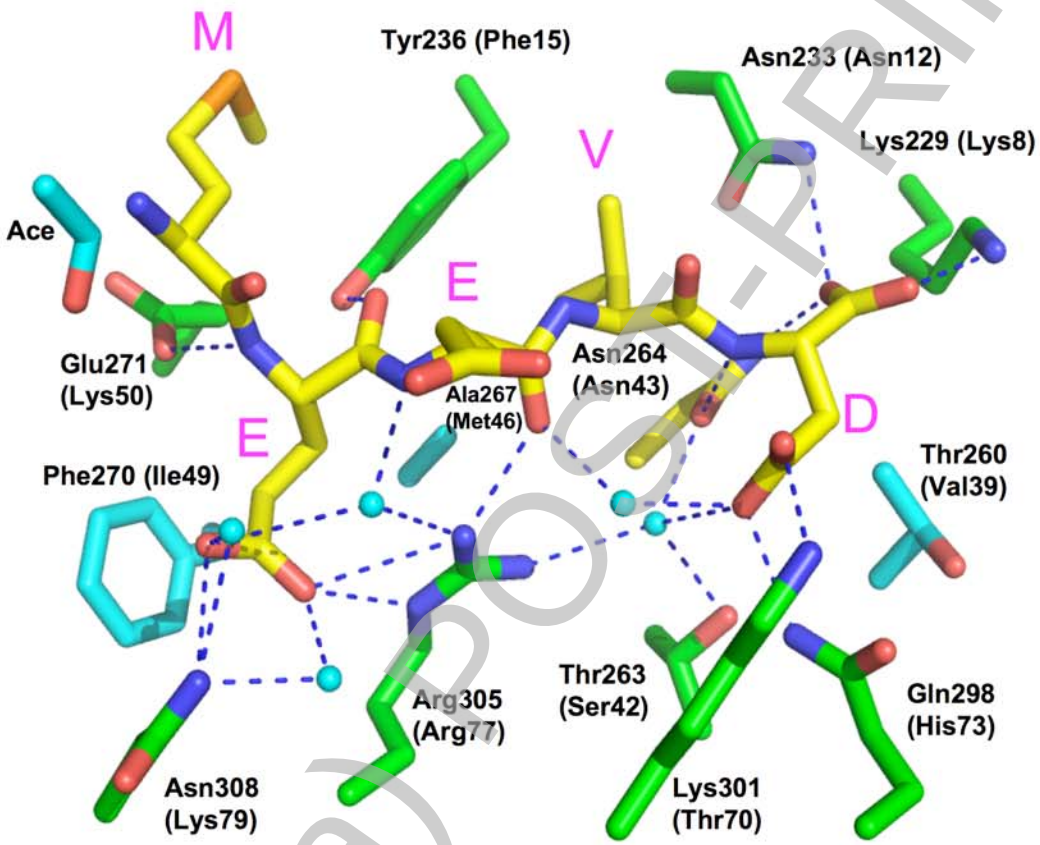

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Figure 3

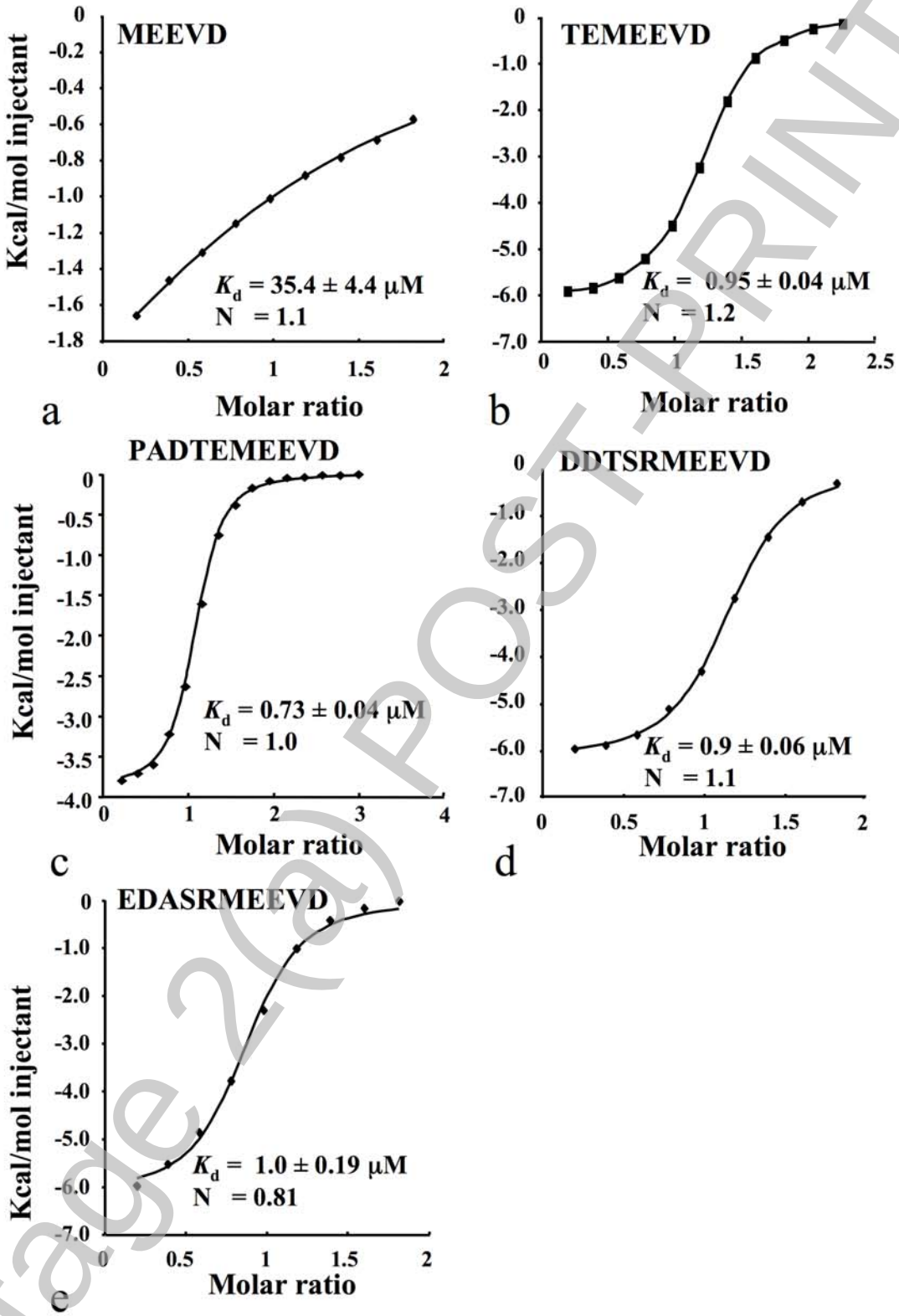

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Figure 4

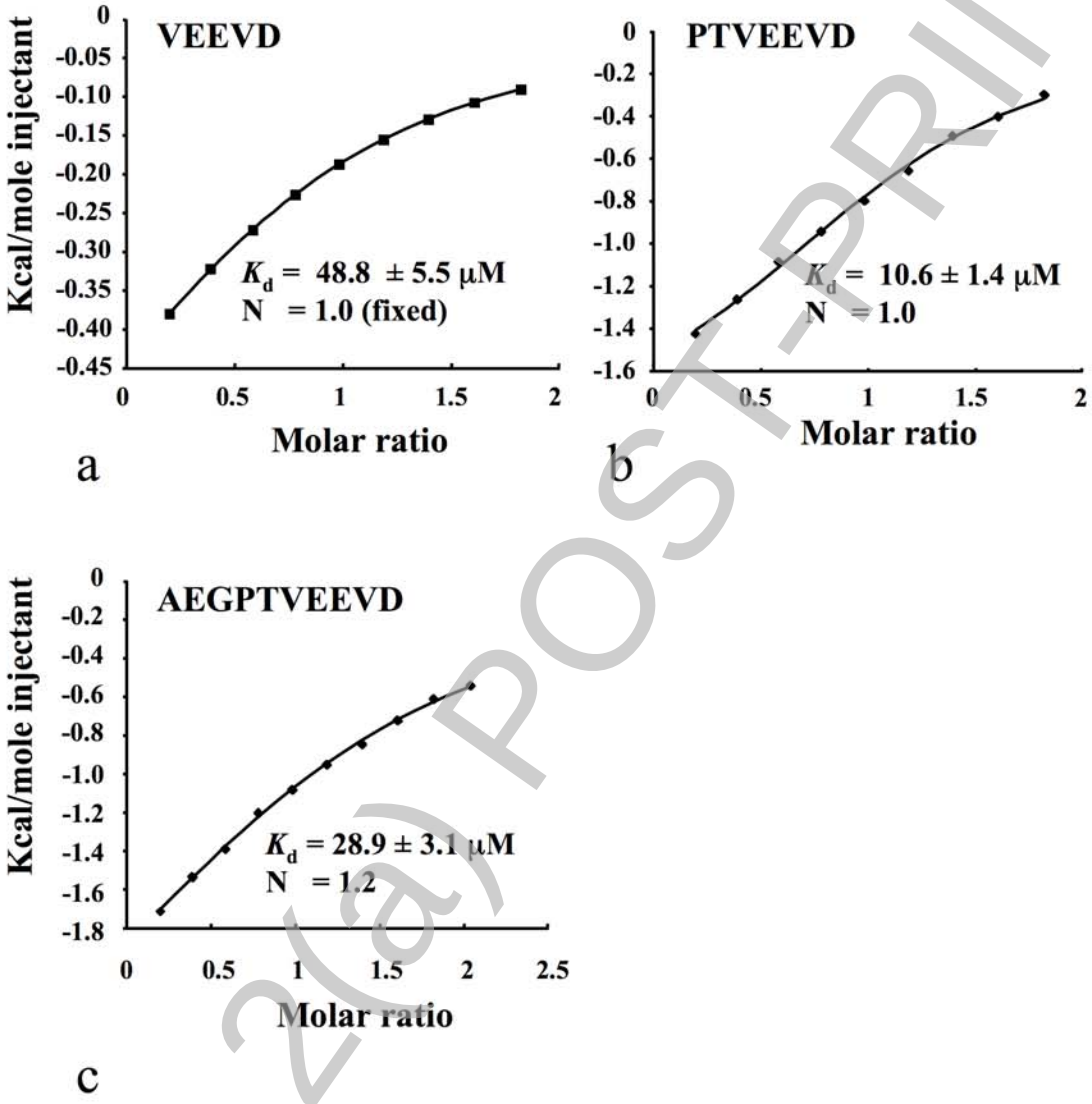

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Figure 5

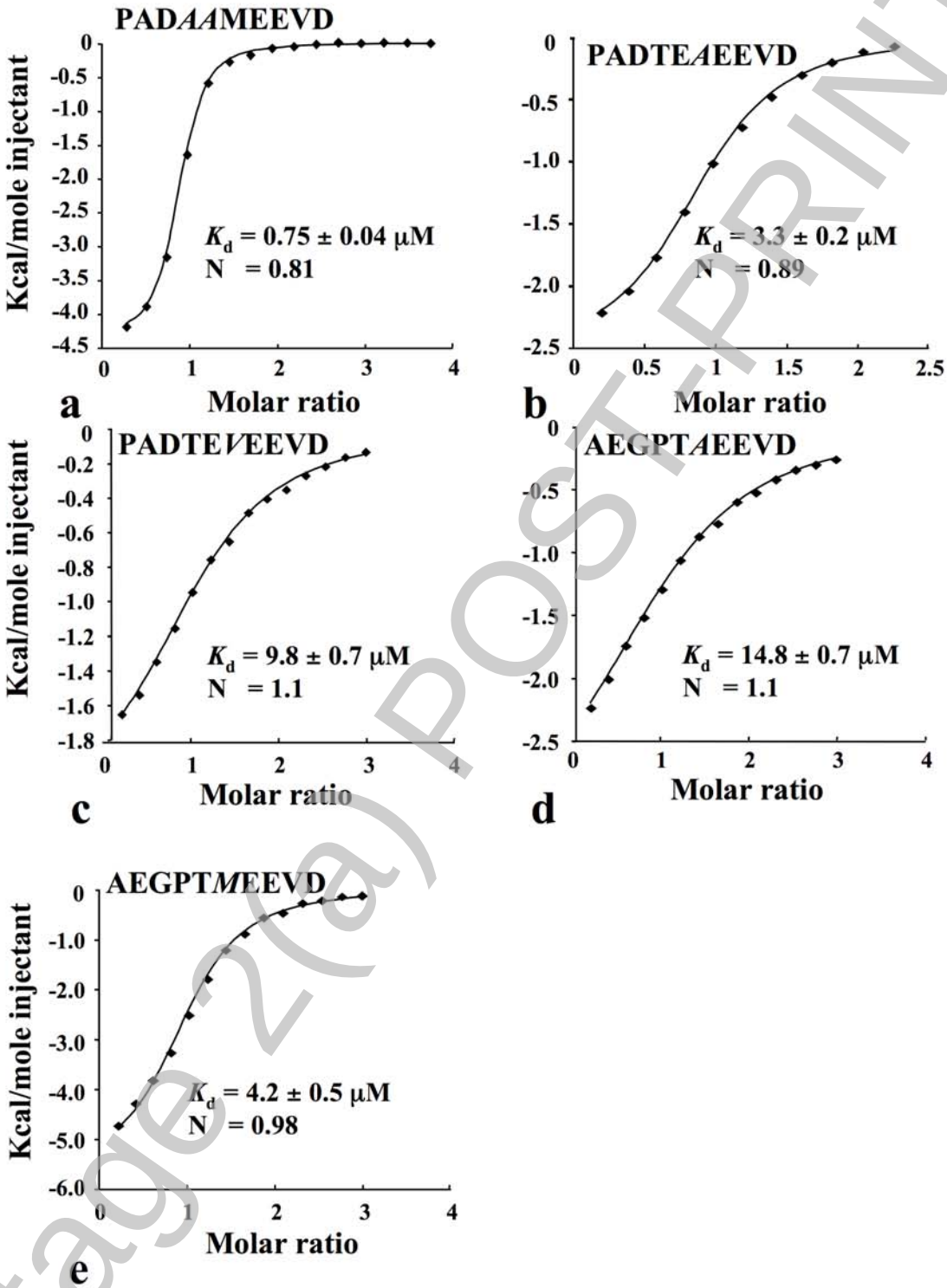

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FIGURE 6

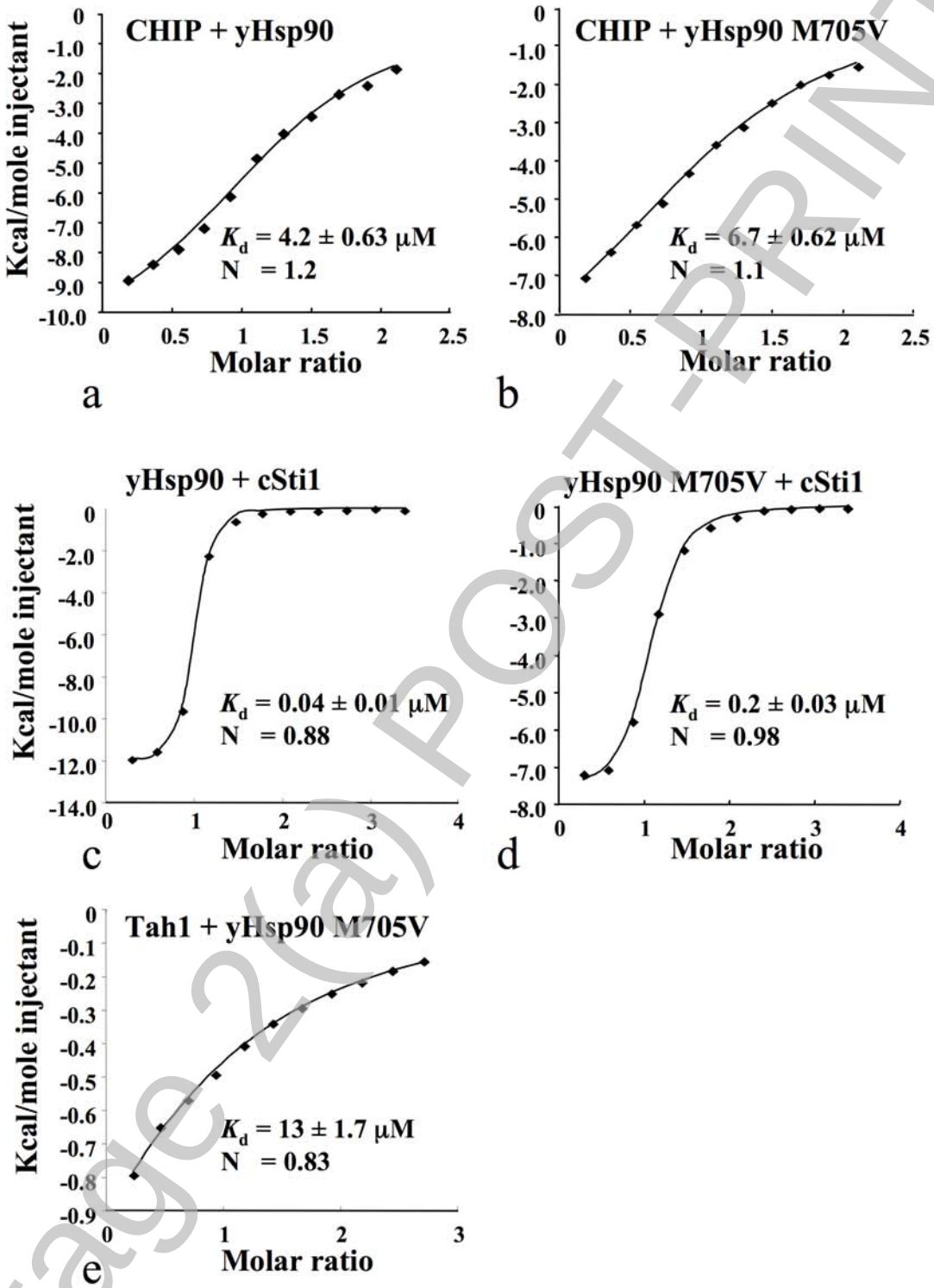

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FIGURE 7