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Review

Hsp90 structure and function studied by NMR spectroscopy ☆

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ABSTRACT

The molecular chaperone Hsp90 plays a crucial role in folding and maturation of regulatory proteins. Key aspects of Hsp90's molecular mechanism and its adenosine-5'-triphosphate (ATP)-controlled active cycle remain elusive. In particular the role of conformational changes during the ATPase cycle and the molecular basis of the interactions with substrate proteins are poorly understood. The dynamic nature of the Hsp90 machine designates nuclear magnetic resonance (NMR) spectroscopy as an attractive method to unravel both the chaperoning mechanism and interaction with partner proteins. NMR is particularly suitable to provide a dynamic picture of protein–protein interactions at atomic resolution. Hsp90 is rather a challenging protein for NMR studies, due to its high molecular weight and its structural flexibility. The recent technologic advances allowed overcoming many of the traditional obstacles. Here, we describe the different approaches that allowed the investigation of Hsp90 using state-of-the-art NMR methods and the results that were obtained. NMR spectroscopy contributed to understanding Hsp90's interaction with the co-chaperones p23, Aha1 and Cdc37. A particular exciting prospect of NMR, however, is the analysis of Hsp90 interaction with substrate proteins. Here, the ability of this method to contribute to the structural characterization of not fully folded proteins becomes crucial. Especially the interaction of Hsp90 with one of its natural clients, the tumour suppressor p53, has been intensively studied by NMR spectroscopy. This article is part of a Special Issue entitled: Heat Shock Protein 90 (HSP90).

1. Introduction

Protein folding in the cell is organised by molecular chaperones [1,2]. The major chaperone systems are ATP regulated machines conserved from bacteria to man: Hsp60, Hsp70, Hsp90 and Hsp100 [3]. The chaperone classes differ dramatically in sequence and structure, which relates to different function. The Hsp90 family is of particular interest because some of its members are especially dedicated to signal transduction [4].

The number of Hsp90 homologues per cell correlates with complexity of the organism [5]. Bacteria such as *Escherichia coli* have only one homologue, HtpG, while yeast cells have one or two homologues in the cytosol and one in mitochondria, but none in the endoplasmic reticulum (ER). In human cells there are four Hsp90 isoforms: the heat shock inducible Hsp90α and the constitutively expressed Hsp90β both in the cytosol, mitochondrial Trap1 and Grp94 located in the ER. Conservation within the family is high; the sequence homology between *E. coli* HtpG and human Hsp90s is around 50%.

Hsp90β is one of the most abundant proteins in the human cytosol, it makes up 1–2% of cytosolic protein [6]. Hsp90 can suppress protein aggregation in vitro, independent of ATP [7,8]. In vivo, however, ATPase activity is essential for the Hsp90 working cycle hence cell viability. Mutant Hsp90s with either faster or slower ATPase rate result in temperature-sensitive growth phenotypes and they are unable to fully activate their client proteins [9,10].

Hsp90 is a rather dynamic molecule and ATP hydrolysis is associated with conformational changes (Fig. 1). Those conformational changes are conserved from bacteria to man [11] and most likely linked to substrate interaction [12]. The functional role of Hsp90's conformational dynamics, however, remains elusive [3].

A zoo of co-chaperones controls Hsp90's ATPase activity and conformational changes. The co-chaperones Cdc37 and p23 prevent ATP hydrolysis while Aha1 stimulates it [13]. Several of Hsp90's substrate classes require specific co-chaperones for recruitment of Hsp90. For the progesterone and glucocorticoid receptors and the kinase Chk1 the minimal set of co-chaperones had been determined [14,15]. For reconstitution of steroid binding activity in vitro, both steroid receptors required an Hsp70 and its co-chaperone Hsp40 for initial complex formation, Hop, which links Hsp70 and Hsp90, then Hsp90 itself and its co-chaperone p23. Chk1 needs the same set of co-chaperones to become active but it requires the kinase-specific co-chaperone Cdc37 instead of p23 [16]. The contribution of each co-chaperone to folding and maturation of substrate proteins, however, is poorly understood.

NMR spectroscopy plays a key role in elucidating the mechanism of the Hsp90 machine and its interactions with co-chaperones and substrates. In this review, we will give a short overview of the key questions that can be targeted by NMR, and the progress that was already made using this technique.

☆ This article is part of a Special Issue entitled: Heat Shock Protein 90 (HSP90).

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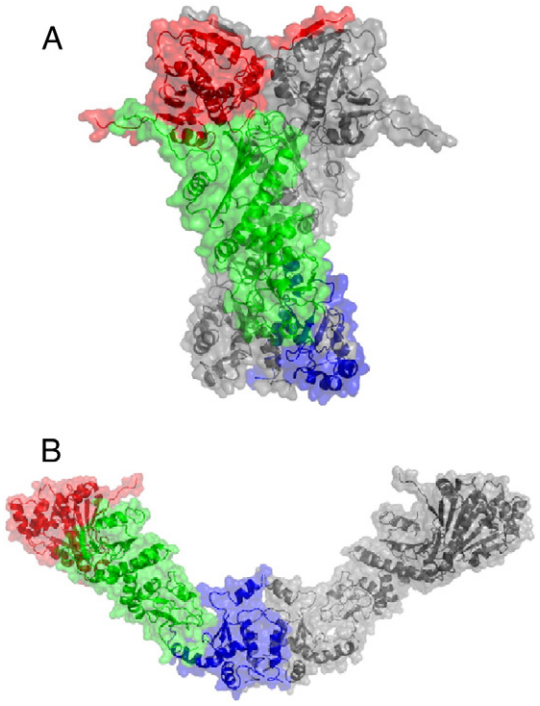

Fig. 1. Hsp90 dimer exhibits different conformations. A, Structure of the closed conformation of the yeast Hsp90 in complex with p23 and AMP-PNP [22]. B, Structure of the extended Hsp90 homologue HtpG from bacteria [43].

2. Hsp90 has a limited but diverse range of substrate proteins

Hsp90 plays an important regulatory role for a limited but rather diverse set of substrate client proteins, such as activation of kinases, maturation of steroid receptors, and assisting in folding of e.g. the tumour suppressor p53 or the cystic fibrosis transmembrane conductance regulator (CFTR) [4]. The oncogenic nature of a significant fraction of its substrate pool made Hsp90 a target for anti-cancer drugs [17]. Interestingly, Hsp90 also interacts with model proteins such as luciferase and citrate synthase [7,18].

Hsp90 is believed to act at late folding stages [7], which may suggest a similar structural motif present in all substrates. Such a motif, however, never was found. This might be related to the fact that the substrate proteins identified so far differ dramatically in sequence and structure (an updated list of Hsp90 interactors is maintained at http://www.picard.ch/downloads/Hsp90interactors.pdf). Interestingly, while functional studies discovered mainly substrate proteins with regulatory function, large-scale proteomics studies revealed more diversity. A study of the interactome of the yeast homologue described 627 putative Hsp90 substrates and co-factors, which represent ~10% of the yeast proteome [19]. Such diversity in the substrate pool suggests a rather flexible substrate selection, possibly regulated by co-chaperones. It should be pointed out, though, that the molecular understanding of Hsp90's substrate recognition principles is still limited, and the paradigms may change when more data become available. A crucial bottleneck is the lack of detailed structural information on Hsp90–substrate complexes.

3. Structural organisation of Hsp90

Hsp90 chaperones are homodimers consisting of three conserved domains (Fig. 1). The N-terminal domain (Hsp90-N) contains the ATP binding site [20]. The basal ATPase rate is rather low ($k_{cat} = 1.7 \times 10^{-3}\ s^{-1}$, for human Hsp90β) and requires both Arg391 of the catalytic loop of the middle domain and the transient dimerization of the N-terminal domain [21,22]. Therefore, isolated Hsp90-N does not have significant ATPase activity [23]. It is involved in binding to several co-chaperones, including p23, Cdc37, Aha1 and Sgt-1 [13,24,25]. Substrate binding properties have also been allocated to Hsp90-N [26–30].

The middle domain (Hsp90-M) is required for activating the ATP hydrolysis in Hsp90-N and contributes to binding of p23 and Aha1 [13,22,31]. Hsp90-M was implied to be involved in binding of substrates [29,30,32,33].

The C-terminal domain (Hsp90-C) is the dimerization domain. It was suggested to contribute to substrate binding [27,28,34,35]. An alternative ATP binding site in Hsp90-C is discussed [36,37], but structural studies failed to identify a specific nucleotide-binding pocket [22,34]. The C-terminus of the cytosolic eukaryotic homologues has a MEEVD motif that is recognised by TPR domains of several co-chaperones, some of which link the Hsp90 system to the Hsp70 system (via the adaptor protein Hop) and the proteasome (via the E2 ubiquitin ligase CHIP) [38–41].

While the three domains have a high degree of identity, the N- and C-terminal extensions and the linkers connecting the domains differ between homologues. Most notable is the charged, disordered linker between Hsp90-N and Hsp90-M, which is around 80 residues long in human Hsp90β which is almost missing in bacterial and mitochondrial homologues [42].

4. Hsp90 is a plastic molecule

In recent years several structural studies of Hsp90 provided considerable progress to the mechanistic understanding of this chaperone machine. High-resolution X-ray structures of full-length Hsp90 were obtained for several homologues and several nucleotide states. The structure of the yeast Hsp90 bound to the ATP analogue AMP-PNP and to the co-chaperone p23 revealed a picture of the ATP-bound state, while crystallographic studies of the HtpG revealed pictures of the nucleotide-free and the ADP-bound state [22,43]. Those structures demonstrate that Hsp90 can adopt different conformations triggered by nucleotides. Interestingly, structures of the ER homologue Grp94 in various nucleotide states failed to show similar nucleotide-dependent changes as they were observed for the cytosolic counterparts [44]. At present it is an open question to which extent the nucleotide state alone is able to define specific conformational states in the absence of co-chaperones, which are still to be discovered for Hsp90s in the ER and the bacterial cytosol.

Structural studies employing X-ray crystallography, electron microscopy (EM) and small-angle X-ray scattering (SAXS) revealed significant structural flexibility of Hsp90 and a species-dependent equilibrium between conformations [11,13,45]. Surprisingly, recent single molecule studies indicated that Hsp90's conformational space contains dimers that are connected via their N-terminal domains but open at the C-terminus [46]. At present the connection between conformational plasticity and function is only poorly understood. The most promising way to proceed would be a comprehensive study of the same homologue in the different nucleotide states in solution, as it is possible with NMR spectroscopy.

5. Hsp90 is a challenging molecule for NMR spectroscopy

The current state of the Hsp90's structural biology now allows focussing on the dynamic nature of Hsp90's functional cycle. NMR spectroscopy is particularly suitable to reveal a dynamic picture of protein complexes in solution at atomic resolution [47]. One of the advantages of NMR is the visualisation of the labelled component only, e.g. Hsp90, a co-chaperone or a client protein. High-resolution NMR spectra allow mapping of both interaction surfaces and conformational changes in a protein structure upon binding of partner proteins. This dual ability makes NMR spectroscopy a powerful research tool to address biochemical research questions. At times it can be a challenge,

though, to distinguish between both possibilities. Complementary NMR experiments such as cross-saturation transfer and hydrogen-deuterium exchange studies or additional biochemical work such as introducing specific mutants dissolve such ambiguities.

The high molecular weight (around 170 kDa for human cytosolic Hsp90 dimer) and its dynamic nature make the full length Hsp90 a rather challenging subject for NMR spectroscopy. Protein NMR studies usually require uniform ^{15}N labelling and well-resolved 2D ^{1}H–^{15}N correlation spectra. The traditional protein size limit for high-resolution ^{1}H–^{15}N NMR spectroscopy, however, is around 30 kDa for typical globular proteins [47]. This makes the isolated domains of Hsp90 accessible for such methods.

6. High resolution studies of Hsp90 domains

The shape of the individual domains in isolation and within the context of the full-length protein is rather similar [22, 43]. Structural studies of the separate domains can, therefore, be meaningful, even though they do not allow conclusions on the interdomain dynamics. Detailed structural data on domains are useful for protein–protein/ligand interaction studies to identify local perturbations.

The N-terminal domain is the part of Hsp90 that is best characterised by NMR spectroscopy [48–50]. It is a stable and well-folded 25 kDa protein that has excellent spectroscopic properties (Fig. 2). The assignment of Hsp90-N is of particular interest since it is the ATP-binding domain and the target for inhibitors blocking its nucleotide-binding site.

The first ^{1}H–^{15}N spectra of the middle domain of Hsp90 became available for the chicken homologue of Hsp90α, along with Hsp90-NM and -MC constructs (Table 1) [51]. The spread of signals in the ^{1}H–^{15}N spectra indicates that the protein is well folded and has only a few unstructured regions. The spectra of Hsp90-M of the human homologue Hsp90β are also well resolved (Fig. 3). ^{1}H–^{15}N correlation spectra of the middle domain for both Hsp90 homologues are rather similar, with small differences probably derived from minor distinctions in conformations, e.g. of loops between helices, and variability of experimental conditions. The protein behaves well in solution, although it tends to aggregate at concentrations higher than 500 μM (Rüdiger group, unpublished).

Hsp90-C is more difficult to assess by NMR spectroscopy. The size of the Hsp90-C dimer, 37 kDa for human Hsp90β, is suitable for NMR experiments. However, flexible regions and aggregation at NMR concentrations render high-resolution analysis of human Hsp90-C more complex than for the other Hsp90 domains (Rüdiger group, unpublished). A dimeric construct of yeast Hsp90-C (529–689), however, shows good spectroscopic properties and is partially assigned (Table 1) [35].

Table 1
Assignments of Hsp90 and Hsp90 domains.

Species	Protein	Assignments	Reference
Yeast	Hsp90-N	95% of backbone ^{1}H, ^{15}N and ^{13}C	BMRB: 5355 (Salek et al.) [50]
Human	Hsp90-N	82% of backbone ^{1}H, ^{15}N and ^{13}C	BMRB: 7003 (Jakobs et al., 2006) [103]
Yeast	Hsp90-M	85% of backbone ^{1}H, ^{15}N and ^{13}C	BMRB: 16279 (Hagn et al.) [35]
Human	Hsp90-M	backbone ^{1}H, ^{15}N and ^{13}C	(Park et al.) [71]
Yeast	Hsp90-C	85% of backbone ^{1}H, ^{15}N and ^{13}C	(Hagn et al.) [35]
Human	full length Hsp90α	Isoleucine δ-methyl groups partially assigned	(Park et al.) [71]
Human	full length Hsp90β	18 out of 48 isoleucines δ-methyl groups	BMRB: 17933 (Karagöz et al.) [63]

The most attractive application of NMR spectroscopy to isolated domains of Hsp90 is the analysis of anti-cancer drug binding. Hsp90 is an attractive target for drug design since it assists in the proper folding of signal transduction proteins [17,52]. Such Hsp90 substrates as p53, Cdk4, c-Src/v-Src, and HER-2/HER-3 are oncogenic. There are several approaches for inhibitors design: strong binding to ATP-binding pocket at the N-terminal domain (geldanamycin, radicicol), targeting a putative ATP-binding site in the C-terminal domain (novobiocin) and blocking interaction with co-chaperones (celastrol) [17,53,54]. Inhibitors currently undergoing clinical trials all target the N-terminal domain. The putative C-terminal nucleotide-binding site was neither found in any of the crystal structures of various Hsp90 homologues nor in NMR experiments of full length Hsp90 [22,34,44,55]. Nucleotides and such inhibitors as geldanamycin and radicicol bind to the same binding pocket in the N-terminal domain. An NMR analysis showed that ATP and ADP interact with Hsp90-N in the same manner with only small differences [49]. The drugs geldanamycin and radicicol induce additional ligand-specific perturbations therefore may affect the overall conformation of the Hsp90-N [49]. In general, effective drug design profits from the combination of the high-resolution crystal structures and NMR experiments, which give insights into the dynamics of the Hsp90–ligand complexes.

7. Full-length Hsp90 studied by NMR

The synergistic interaction of the domains within the Hsp90 dimer requires analysis of the full-length molecule to understand the conformational changes throughout its active cycle. This is particularly highlighted by the fact that the isolated N-terminal domain does not show any dimeric interaction in solution both in the presence and absence of ATP [20,26]. The Hsp90 full-length protein, however, requires more sophisticated NMR experiments than those described

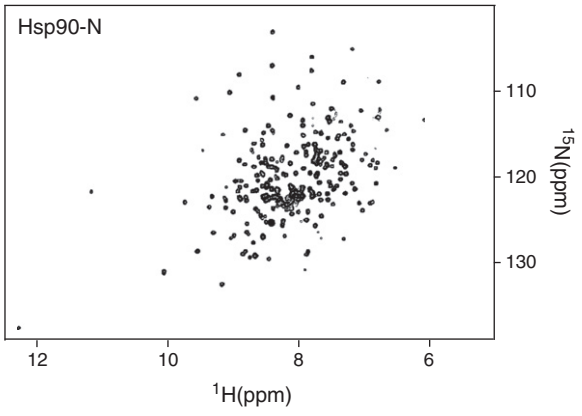

Fig. 2. ^{1}H–^{15}N HSQC NMR spectroscopy spectra reveal excellent spectroscopic properties of the N-terminal domain of human Hsp90β.

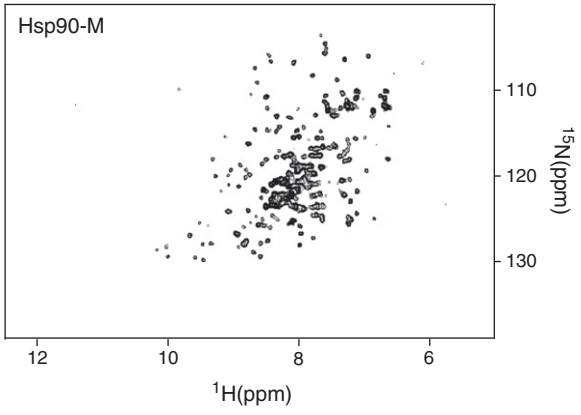

Fig. 3. ^{1}H–^{15}N HSQC NMR spectroscopy spectra show that the middle domain of human Hsp90β is well folded.

for the isolated domains. The major complications are the high molecular weight and the presence of disordered regions.

7.1. NMR of backbone amides of full length Hsp90

The molecular weight of 170 kDa of the Hsp90 dimer leads to slow tumbling in solution. This results in a loss of spectral sensitivity of large proteins due to an effect called transverse relaxation [56]. In a two-dimensional heteronuclear NMR experiment proton magnetization is created as a first step. Then it is transferred to either ^{15}N or ^{13}C nuclei via polarisation transfer step. The magnetisation evolves there for a time period called "evolution" and acquires a frequency label specific for the nuclei it was transferred to. After the frequency labelling step the magnetisation is transferred back to proton via reverse polarisation step and there it is detected during the acquisition period. Transverse relaxation occurs both during polarisation transfer steps and frequency labelling periods leading to signal loss and line broadening of spectra.

The development of novel NMR techniques by the Wüthrich laboratory allowed overcoming size obstacles [57]. In particular the combination of TROSY (transverse relaxation optimised spectroscopy) and CRINEPT (cross-relaxation enhanced polarisation transfer) techniques made full-length Hsp90 accessible for NMR spectroscopy. TROSY extends NMR spectroscopy to up to 100 kDa by suppressing transverse relaxation during evolution and acquisition periods, therefore reducing signal loss and line broadening [57,58]. A successful application of TROSY techniques in the chaperone field is the work or the Zuiderweg group on allostery of Hsp70 [59,60]. CRINEPT extends the effect of TROSY to heteronuclear polarisation transfer periods. It provides a significant gain in sensitivity for big protein complexes of 150 kDa and larger in comparison to the commonly used polarisation transfer techniques like INEPT [57]. The combination of TROSY with CRINEPT makes even the 900 kDa GroEL-GroES complex accessible for NMR experiments [61].

Another cause of sensitivity loss that is especially pronounced for high molecular weight proteins is dipole-dipole relaxation. Dipole-dipole relaxation of spin magnetization is not affected by TROSY and CRINEPT. It occurs due to magnetic properties of protons: ^{1}H as the nucleus with highest magnetic dipole moment is the most effective for causing this type of relaxation. In practice, dipolar interaction occurs between observed ^{1}H with remote protons and leads to signal loss. The exchange of those protons to deuterium overcomes this problem and decreases relaxation. Deuterium has a considerably lower magnetic dipole moment than protons. It is possible to keep the protons of backbone amides or methyl groups protonated and therefore NMR visible while deuterating the rest of the protein [56].

The application of CRINEPT–TROSY to perdeuterated ^{15}N-labelled full length Hsp90 results in a complex spectra of the backbone amides (Fig. 4) [62]. The CRINEPT–TROSY spectra of Hsp90 show the typical distribution of signals in the ^{1}H dimension that is typical for folded proteins, including signals in the region typical for β-sheet secondary structure between 8.5 and 10 ppm in the ^{1}H dimension. Most of those signals are not visible when TROSY is used without CRINEPT [62]. The most intensive signals in the spectra are, however, localised to the random coil region. Those signals appear with doublets separated by 90 Hz in both the ^{1}H and the ^{15}N dimension. It is a potential disadvantage of CRINEPT–TROSY pulse sequences that for highly dynamic regions an additional set of signals is retained. The presence of those signals next to signals corresponding to rigid parts of the molecule indicates a significant content of flexible regions in Hsp90. A comparison with spectra of the isolated domains revealed that most of those signals represent the C-terminal domain (Rüdiger group, unpublished).

While the CRINEPT–TROSY technique allows studying the backbone of full-length Hsp90, the complexity of the spectra makes peak assignment difficult. This limits the resolution at which interactions

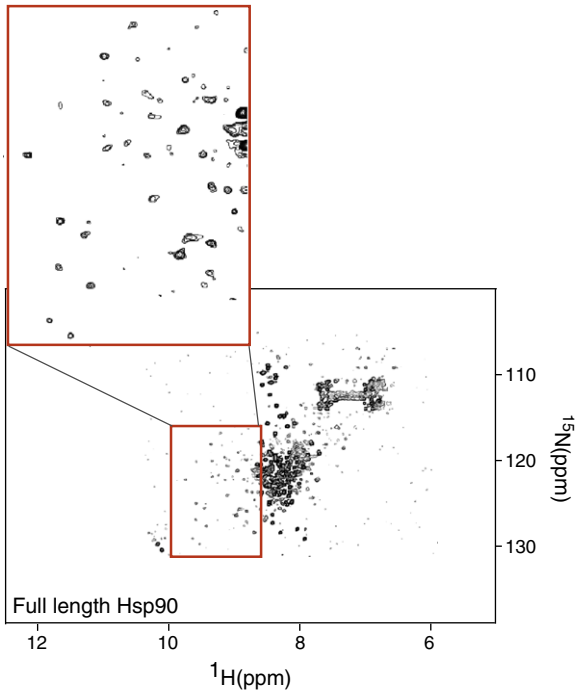

Fig. 4. CRINEPT–TROSY NMR spectroscopy allows studying of the backbone of full-length Hsp90. The perdeuterated 170 kDa Hsp90 dimer was measured using ^{1}H–^{15}N CRINEPT–TROSY pulse sequences [62]. The inset is a magnification of signals in the β-sheet region.

with partners such as drugs, co-chaperones and substrate proteins can be studied. The combination of CRINEPT–TROSY with protein engineering techniques such as segmental isotope labelling might be used in the future to reduce the spectral complexity of full length Hsp90 [63].

Independent from the relaxation problems, large proteins such as Hsp90 also face challenges that result from the shear number of residues. The interpretation of NMR experiments of large proteins becomes increasingly complex when they contain a large number of disordered and highly dynamic parts. Hsp90 owns long disordered stretches in the N- and C-termini and in the inter-domain linkers, which together account for more than 120 amino acids for human Hsp90β. In principle, those stretches lead to comparable sharp and intensive peaks. Their signal is enhanced due to the fast intrinsic dynamics, while for the rigid parts signal strength is determined by the tumbling of the molecule. However, the lack of a stable fold in the linkers and terminal stretches limits differentiation of chemical shifts for those stretches on the proton scale. This results in significant signal overlap in the random coil region of Hsp90's spectra (8.0–8.5 ppm in the proton dimension; Fig. 4). Hsp90 is, therefore, a particularly attractive target for techniques that limit complexity in NMR spectra.

7.2. High resolution spectra of full-length Hsp90 using side chain labelling

Selective isotope labelling of a defined subset of side chains is an effective way to reduce spectral complexity [64]. It decreases the total number of signals and, therefore, results in spectra with better resolution. This makes it easier to assign signals and interpret results. Most importantly, certain side chains result in higher signal intensity compared to backbone amides. The hydrophobic amino acids Ile, Leu and Val contain methyl groups that are rotationally not restricted, resulting in intensive signals. Methyl-groups are the very attractive targets for selective labelling not only because of their good spectroscopic properties but also because of effective labelling techniques [65,66].

Kay and co-workers pioneered the use of specific α-keto acid precursors to selectively label the methyl groups Ile, Leu, and Val [67]. Highly deuterated samples that are $^{1}H–^{13}C$ labelled at selective methyl positions result in intense and well-dispersed $^{1}H–^{13}C$ correlation spectra even for large structures such as the 20S proteasome and the SecA translocation pore [68–70]. Recently the approach was successfully applied first to human Hsp90β and later also to Hsp90α [55, 71].

Human Hsp90β contains 48 Ile side chains, which are well dispersed throughout the Hsp90 structure and present in every crucial region (Fig. 5A). Twenty of them reside in the N-terminal domain. The N-terminal domain is of particular interest due to its binding to nucleotides and inhibitors and its transient dimerisation in the ATP cycle. This makes Ile side chains rather attractive targets for selective labelling strategies. All δ-methyl groups of Ile side-chains can be specifically ^{1}H and ^{13}C labelled in an otherwise deuterated human Hsp90β [55].

The Ile-labelled Hsp90 has excellent properties for methyl-TROSY experiments (Fig. 5B) [55]. The spectra indicate the presence of rigid next to dynamic segments, resulting in significant variation of the relative intensity and line width. This is consistent with similar findings for the amide backbone in CRINEPT–TROSY experiments [62]. Remarkably, 45 peaks appear in the spectra, which indicate that the approach is sufficiently sensitive to monitor most of 48 possible Ile side chains (Fig. 5B). Hsp90 behaves similar to the sum of its individual domains. The comparison with spectra of the isolated Hsp90-N, -M and -C constructs allowed annotation of the signals to one of the domains [55,72]. Assignments of Hsp90-N and Hsp90-M can be transferred to the full-length protein and allow high-resolution NMR studies of the entire protein (Fig. 5C). Despite some signal overlap 12 Iles are assigned in Hsp90-N and 5 Iles in Hsp90-M (Table 1). Those assignments provide a basis to study Hsp90's interaction with co-factors, ligands and substrates.

Recently, Ile-specific labelling succeeded for Hsp90α, too [71]. The $^{1}H–^{13}C$ spectra of Hsp90β and Hsp90α show a good correlation in pattern though with distinct differences that may be caused by slightly different structural organisation of the isoforms. In the Hsp90α study the flexible linker between Hsp90-N and Hsp90-M had been omitted to improve spectra. Good correlation between spectra of Hsp90β full length and Hsp90α with deleted linker indicates that the linker does not result in significant structural changes. Nevertheless, it cannot be excluded that removal of the linker might have caused the minor discrepancies in the spectra of both Hsp90 homologues. Future NMR experiments comparing Hsp90α and Hsp90β under identical conditions in solution may shed a light on possible differences between both homologues.

In addition to NMR methods that provide high-resolution structural information and therefore mostly deal with local perturbation it is also possible to monitor the overall hydrodynamic behaviour of Hsp90 by NMR. Diffusion Ordered NMR spectroscopy (DOSY) allows to determine a translation diffusion coefficient which is a function of protein size and shape [73]. For Hsp90 where conformational flexibility is crucial for function the diffusion studies provide important information on structural changes upon interaction with ligands. The translational diffusion coefficient is determined by monitoring the decrease in peaks intensity upon increasing the pulsed gradient strength or diffusion time. Therefore the combination of DOSY with selective $^{1}H–^{13}C$ labelling of methyl groups (3D DOSY–TROSY) is particularly suitable for high molecular weight proteins and results in precise determination of the translation diffusion coefficient

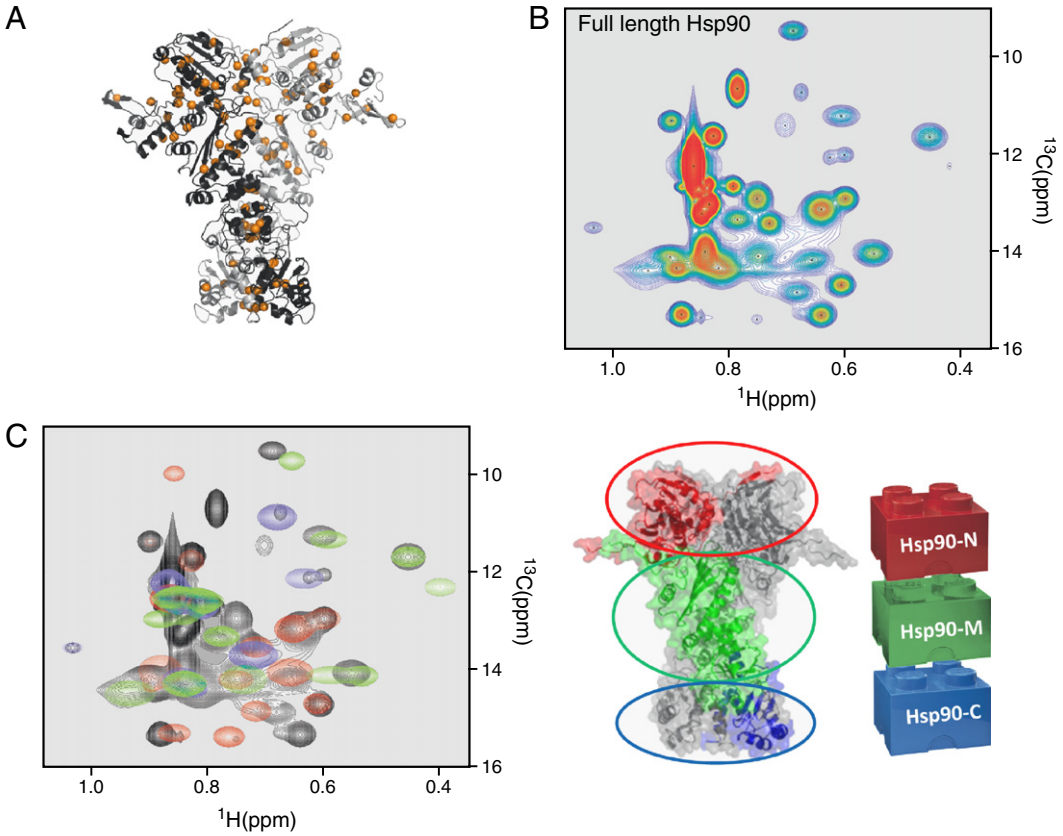

Fig. 5. Isoleucines can be used as NMR markers for full-length Hsp90β. A, Homology model of human Hsp90β with both monomers in dark and light grey. Isoleucine methyl groups depicted as orange spheres. B, ^{1}H-^{13}C-Ile methyl-TROSY spectrum of full-length Hsp90. C, Methyl groups of individual Ile residues were assigned via the ^{1}H-^{13}C-Ile methyl-TROSY spectra of individual domains. Overlay of full-length Hsp90β ^{1}H-^{13}C-Ile methyl-TROSY spectrum with spectra of the individual domains (full-length Hsp90β, black; Hsp90-N, red; Hsp90-M, green; Hsp90-C, blue), homology structure of Human Hsp90β (Hsp90-M, green; Hsp90-C, blue). The spectra of the individual domains can be put on top of each other such as Lego bricks to generate an assignment for the full-length protein [55].

[74]. Strikingly, this approach allows detecting significant differences for the ATP and ADP states of Hsp90 (TD and SGDR, unpublished).

8. Hsp90 co-chaperones: binding sites and stoichiometry

The activity of eukaryotic cytosolic Hsp90s is controlled by co-chaperones regulating ATP hydrolysis, conformation, dynamics and substrate binding and activation [75]. Insights into Hsp90's complexes with co-chaperones allow conclusions on the individual steps of its working cycle. Genetic studies on ten distinct Hsp90 co-chaperones in different eukaryotic species showed that the presence of co-chaperones is species dependent [5]. For example, the most dispersed co-chaperone is Hop/Sti1, while the kinase-specific co-chaperone Cdc37 presents only in nine out of 19 species. This divergence is contrasting the conserved nature of Hsp90 itself. It suggests that regulation by co-chaperones could be adapted to specific substrate requirements.

Co-chaperone binding to Hsp90 regulates its conformation state. p23 stabilises the closed state of Hsp90, while in contrast Hop/Sti1 and Cdc37/p50 prevent the N-terminal closing [22,76–78]. Since Hsp90 is rather flexible even upon interaction with nucleotides, co-chaperones may stabilise the individual steps in the ATPase cycle. Solution NMR studies thus may shed a light on the mechanism of Hsp90's interaction with Aha1, Cdc37 and p23.

8.1. Mapping of the Aha1 binding site by NMR

Aha1 is the only one known ATPase activator among Hsp90 co-chaperones [31,79]. Aha1 is involved in a vast set of cellular processes. It is involved in the activation of v-Src, glucocorticoid receptors and regulates the quality control of CFTR [79,80]. The stability of mutant CFTR is specifically enhanced in the absence of Aha1 [81]. The crystal structure of Hsp90-M of the yeast homologue in complex with the N-terminal domain of Aha1 disclosed the interaction surface and indicates the mechanism of Hsp90 activation [31]. So far it was not possible to solve a crystal structure of a complex of Aha1 with full length Hsp90.

Insights into the mechanism of the ATPase activation of Hsp90 by Aha1 came from NMR studies of domain complexes of Hsp90 and Aha1 [82,83]. Monitoring of hydrogen to deuterium exchange of ^{15}N-labelled Aha1 in the presence and absence of Hsp90-M allowed mapping the binding surface in solution [82]. Backbone assignment of domains of Aha1 and Hsp90 allowed conclusions about the binding interface. The resulting interaction pattern is consistent with previous crystallographic data [31]. Remarkably, the data also indicate a possible secondary binding site on the opposite site of the Aha1 molecule.

A recent study combining NMR and FRET experiments elucidates the binding mode of Aha1 in full length Hsp90 [82]. Interestingly, not only the N-terminal but also the C-terminal domain of Aha1 turned out to be involved in interaction with Hsp90. The Buchner and Kessler groups probed ^{15}N-labelled Hsp90-N and Hsp90-M with various Aha1 constructs and analysed the chemical shift perturbations [82]. Backbone assignments of Hsp90-N and Hsp90-M allowed mapping of Hsp90 residues affected by Aha1 binding (Fig. 6). Amino acids involved in interaction with Aha1 form a large hydrophobic groove at Hsp90 surface that suggest a possible binding surface supported by crystallographic data [31]. The N-terminal domain of Aha1 interacts nucleotide-independent with Hsp90-M, consistent with previous crystallographic data. Strikingly, the C-terminal domain of Aha1 interacts with Hsp90-N only in the presence of ATP or an ATP analogue such as AMP-PNP, suggesting an important role of ATP-determined Hsp90 conformational rearrangement.

Hsp90 is a symmetric dimer. From a structural point of view, the Aha1 binding site on the N-terminal and middle domains of Hsp90 would be compatible with symmetric binding of two Aha1 molecules in a symmetric complex. Interestingly, ATPase assay indicated that one molecule of Aha1 could already induce maximal stimulation of ATP hydrolysis of the Hsp90 dimer. Aha1 activates the ATPase of one particular N-terminal domain in both cis- and trans-positions. The active asymmetric complex consists of one closed Hsp90 dimer and one Aha1 molecule [82]. This could be crucial for the functional cycle since binding of a second Aha1 on the other side of the dimer might interfere with substrate interaction.

8.2. The Hsp90–Cdc37 complex by NMR

Cdc37 is a kinase-specific co-chaperone. It consists of three domains: an N-terminal substrate binding domain, a middle Hsp90-binding domain and C-terminal domain. Cdc37 inhibits Hsp90 ATPase and traps Hsp90 in an open conformation [84,85]. A crystal structure of a complex of yeast Hsp90-N and a fragment consisting of the middle and C-terminal domains of Cdc37 explains that Cdc37 inhibits Hsp90's ATPase through interaction of the side chain of R167 in Cdc37's middle domain with the catalytic Glu in Hsp90-N [84].

A complex of Hsp90-N and the middle domain of Cdc37 was characterised both by NMR chemical perturbation analysis and cross-saturation transfer experiments that allowed direct mapping of binding surface between both proteins [86]. The analysis of ^{1}H–^{15}N correlation spectra of the complex reveals the interaction interface on both sides of the Hsp90 dimer (Fig. 7). The combination of residual dipolar couplings, NOE experiments and HADDOCK modelling provided further insights into the Hsp90–Cdc37 complex. As a result a detailed structure of the complex was built in general similar to the one obtained by X-ray for yeast Hsp90–Cdc37 [86,87] . The surface area of the NMR-based structure is distinctly larger (1600 A^2) than the one of the crystal structure (1100 A^2) therefore the NMR-based structure is less compact. The residue Leu205 in Cdc37 is crucial for interaction driving the complex formation by its hydrophobic nature.

8.3. Hsp90 interaction with p23 by NMR

Similar to Cdc37, the co-chaperone p23 inhibits Hsp90 ATPase. Some substrates including glucocorticoid and progesterone receptors are trapped by p23 in a complex with Hsp90 [14,15]. The co-crystal

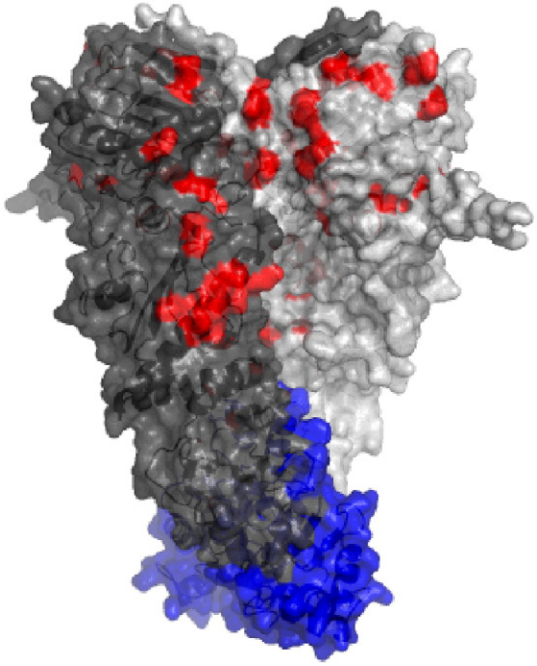

Fig. 6. The Aha1 binding site in Hsp90 maps to the N-terminal and middle domains. Residues that shift upon interaction with Aha1-N and Aha1-C are depicted in red (Hsp90-N and Hsp90-M, grey; Hsp90-C, blue) [82].

structure of a complex between p23, Hsp90 and an ATP analogue indicates that the interaction requires contacts of p23 to both of Hsp90-N of the dimer as well as both of Hsp90-M [22]. Interestingly, NMR experiments with constructs comprising N-terminal and/or the middle domain of Hsp90 revealed that only those fragments containing Hsp90-M bound to p23 [51]. In contrast, isolated Hsp90-N does not bind to p23, not even in the presence of nucleotide [51]. This emphasizes the importance of studying the full-length protein to understand the mechanism of the Hsp90 machine.

With help of Ile-specific labelling the sensitivity is sufficiently high to monitor binding of co-chaperones such as p23 to Hsp90 (Fig. 8A). The determination of the translational diffusion coefficient by 3D DOSY-TROSY experiments with Ile labelled p23 and unlabelled Hsp90 demonstrated complex formation of both proteins [74]. Analysis of chemical shift perturbations of $^{1}H–^{13}C$ spectra of Ile-labelled full-length Hsp90 in the presence of unlabelled p23 and ATP revealed an interaction site covering the ATP lid and adjacent regions in both Hsp90-N and Hsp90-M (Fig. 8B) [55]. The interaction pattern detected in NMR is in agreement with previous crystallographic data where binding of p23 to Hsp90-N was demonstrated [22]. Notably, the NMR spectra of the Hsp90–p23 complex also indicate chemical shifts corresponding to residues spreading over large parts of Hsp90-M [55]. Most of those are distant from the crystallographically identified p23-binding site. In fact, the disordered C-terminus of p23, which is lacking in the crystal structure, could potentially be responsible for additional contacts to Hsp90-M. Deletion of this stretch still does not significantly modify the chemical shift pattern. Together, those data indicate that p23 induces long-range conformational changes in Hsp90-M.

The chemical shift perturbation analysis can also provide some information on stoichiometry of interaction of Hsp90 upon interaction with co-factors. A subset of Hsp90 peaks shows p23-dependent doubling of signals (Fig. 8A) [55]. This could be caused by formation of an asymmetric $Hsp90_2p23_1$ complex, similar to the asymmetric $Hsp90_2Aha1_1$ [82]. Complementary experiments with native mass spectrometry demonstrated that human Hsp90 and p23 form a complex with 2:2 stoichiometries. The two p23 may bind to Hsp90 in slightly different mode, which could be responsible for the peak doubling in the NMR spectra. In this context it is notable that binding of p23 and Aha1 is mutually exclusive due to a partial overlap on the interaction interface [22,82]. It will be interesting to find out about conditions that favoured the formation of $Hsp90_2p23_1Aha1_1$ complexes and whether such a species may play a role in the ATPase cycle.

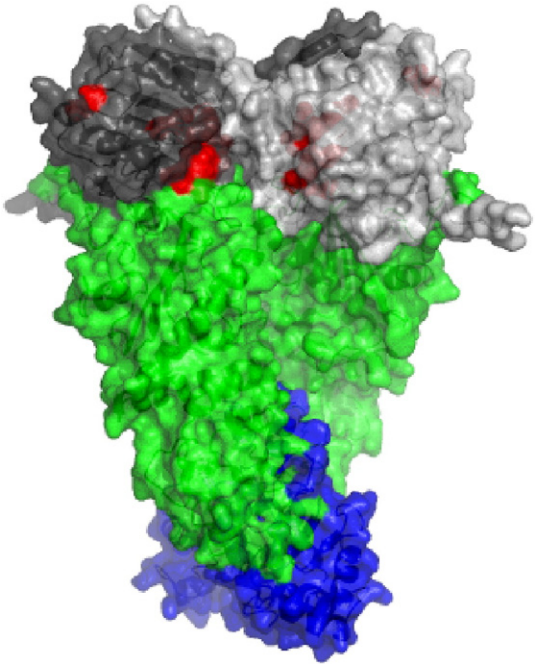

Fig. 7. The Cdc37 binding site in Hsp90 maps close to the contact site of both N-terminal domains in the closed dimer. Residues that shift upon interaction with Cdc37-M are depicted in red, C-terminal and middle domains of Hsp90 are shown in blue and green respectively [86].

It is evident from the structure of the yeast Hsp90–$Sba1^{p23}$ complex that the co-chaperone stabilises the ATP-dependent dimerisation of N-terminal domains [22]. This is consistent with the chemical shift pattern of the human Hsp90–p23 complex in solution and the NMR and MS data indicating that the interaction is ATP-dependent [55]. It is not consistent, however, with previous NMR experiments reporting that p23 binds to Hsp90 in the absence of ATP [51].

This apparent contradiction is most likely related to the different experimental conditions. The nucleotide independent interaction was monitored at very low ionic strength (50 mM KCl and 10 mM Tris pH 6.9). Those conditions favour protein–protein interaction, particularly at high protein concentrations typically used in NMR. ATP increases the affinity of p23 for Hsp90 by one order of magnitude, even at low ionic strength [88]. In turn, high ionic strength conditions (300 mM NaCl/KCl and 25 mM phosphate pH 7.2) may potentially suppress the physiologically less relevant ATP-independent interaction even at high protein concentrations in the NMR tube [55]. For protein concentrations used in most NMR experiments, even physiological ionic strength may support non-physiological associations, asking for independent verification. In case of the Hsp90–p23 interaction NMR conditions at high ionic strength were in pleasing congruence with crystallographic and biochemical data [22,88]. In conclusion, the role of experimental conditions in investigations of protein complexes can be crucial to avoid unspecific interactions at the non-physiologically high protein concentrations of most NMR experiments.

9. Hsp90 interaction with substrates studied by NMR

The key question in understanding the mechanism of the Hsp90 machine is to comprehend its interaction with substrate proteins. The nature of interaction of Hsp90 with substrates, however, remains largely elusive. There is only limited knowledge about the folding state of substrates that interact with the chaperone, the specificity of the interaction and the fate of the substrate during the Hsp90 working cycle. Some insights come from an electron microscopic reconstruction of an Hsp90/Cdc37/Cdk4 complex [29]. The EM pictures indicate an asymmetric complex in which only one substrate molecule is bound on the outside, possibly making contacts to the N-terminal and middle domains of Hsp90. So far, however, there is no high-resolution structure of Hsp90 complexes with bound substrate protein.

The dynamic nature of Hsp90–substrate interactions assigns a prime role to NMR spectroscopy to study those protein–protein interactions. In addition to the possibility to specifically monitor a labelled protein in complex mixtures it provides detailed information on the conformational state and on protein dynamics. Also, a key advantage of NMR spectroscopy is the possibility to monitor transient interactions between proteins.

9.1. The structure of the Hsp90-bound substrate protein

Hsp90 suppresses aggregation of proteins in vitro without being too particular about their whereabouts, whether it is a natural partner such as p53 or a model protein such as porcine mitochondrial citrate synthase [7,8,62]. It shows preference for destabilised proteins, such as for the destabilised viral v-Src kinase versus the constitutive c-Src or for structural mutants of p53 versus the wild type [62,89].

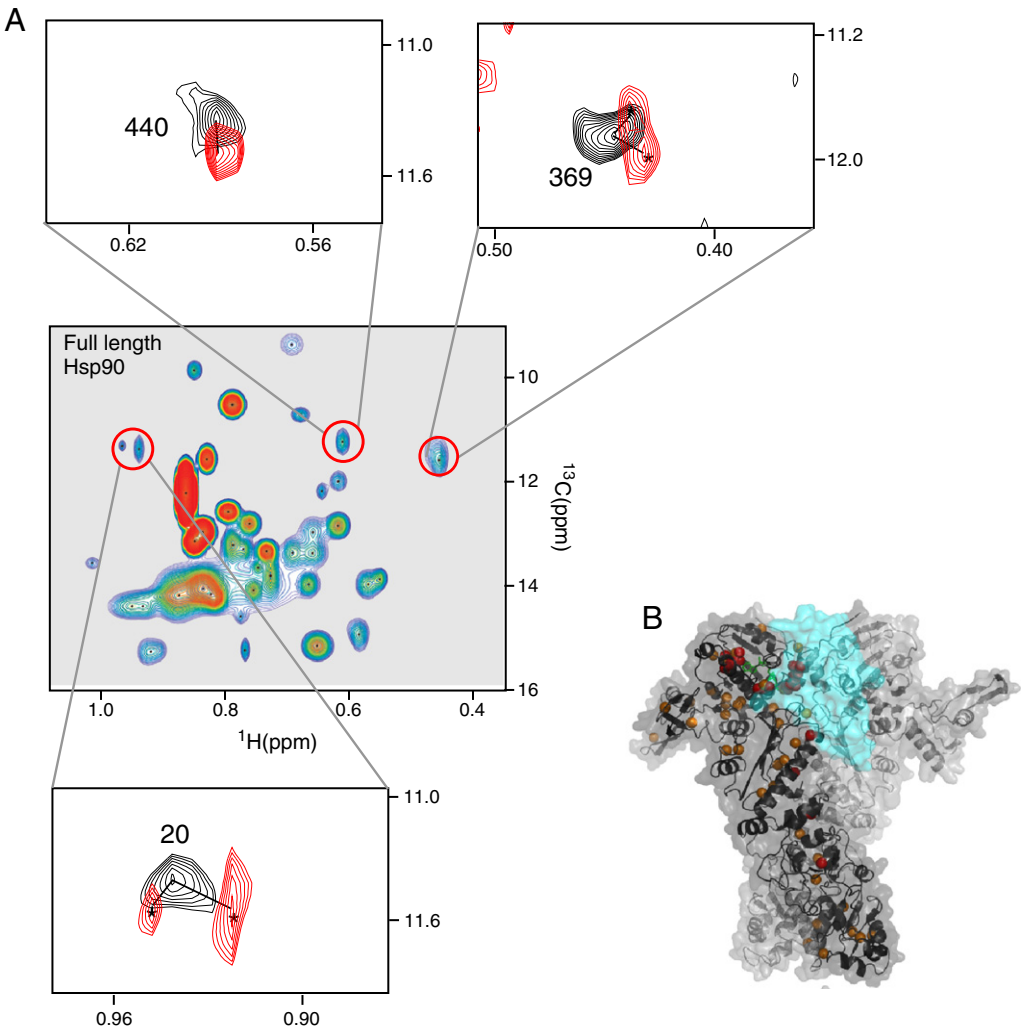

Fig. 8. ^{1}H-^{13}C-Ile methyl-TROSY allows the identification of the binding site of p23 in Hsp90β A, ^{1}H-^{13}C-Ile methyl-TROSY spectrum of full-length Hsp90, three insets are shown as examples of significant peak shifts that allow the identification of the binding region of p23 in full-length Hsp90β (Hsp90 + ATP, black; Hsp90 + ATP + p23, red). B, Ile residues that shift upon the interaction with p23, plotted on one monomer of the homology model of the Hsp90β (shifting residues, red; non-shifting residues, orange). The monomers of Hsp90β are depicted in grey and the location of p23 based on the X-ray structure from yeast Hsp90 in volume representation in light blue [22].

The combination of NMR and SAXS allowed investigating the interaction of *E. coli* Hsp90, HtpG, with a partially folded model substrate [32,90]. Specific structured regions of the 131 amino acid fragment of staphylococcal nuclease (Δ131Δ) are supposed to contribute to binding to HtpG. The interaction occurs via middle domain of HtpG triggering a structural rearrangement of the chaperone [90]. ^{1}H–^{15}N cross-peaks of Δ131Δ are non-uniformly affected upon addition of HtpG. In particular, the subset of signals representing the structured region decreases in intensity, while the disordered parts are less affected. The loss of peak intensities is thought to be due to the signal broadening caused by slow tumbling upon interaction with HtpG [32].

Such studies with model proteins may give general insights into the Hsp90–substrate interaction mechanism. Since Hsp90 is thought to interact with a limited subset of substrates, however, interaction studies with natural ligands are of particular importance. A particular attractive target is p53, for which wild type and mutant proteins have been assayed also with biochemical techniques for Hsp90 interaction [91–93]. Much of this work is beyond the focus of this review, here we would like to focus on the NMR data on this topic. The p53 protein is a homotetrameric transcription factor that is frequently hit by cancer mutations in its DNA binding core domain [94]. NMR studies on Hsp90 interaction with the monomeric 25 kDa p53 core domain provided the most detailed insights of Hsp90 interaction with natural substrates so far.

9.2. Natural substrates — the p53 case

The NMR analysis on a complex of ^{15}N-labelled DNA binding core domain of human p53 and non-labelled human full length Hsp90β revealed that p53 binds to Hsp90 when unfolded [62]. The complex of Hsp90 and p53 core is around 200 kDa and provides all the challenges for NMR experiments that are typical for molecules of this size. The most prominent effect is signal loss due to transverse relaxation. While the 25 kDa p53 core domain has very good NMR spectroscopic properties, all the constraints of a large protein complex apply when it binds to the 170 kDa Hsp90 dimer. The p53 core becomes part of a big complex that has tumbling properties of around 200 kDa particle, resulting in signal loss due to transverse relaxation [62].

This signal loss also has advantages, it is an indicator of p53 core binding to Hsp90, and it allows monitoring even by one dimensional ^{1}H NMR spectroscopy [62]. Interestingly, the signal of folded p53 core is not affected by the presence of Hsp90. The p53 core protein binds to Hsp90, however, when the sample is exposed to temperatures above 36 °C. In the absence of the chaperone, p53 aggregates visibly

under such conditions. Hsp90 prevents this aggregation. This is consistent with intrinsic fluorescence data that shows that Hsp90 forms complexes with p53 wild type and mutant protein upon temperature increase [62]. Strikingly, destabilised p53 mutants bind to Hsp90 already at lower temperature than wild type does.

The resulting complex can be analysed by CRINEPT–TROSY. In the absence of Hsp90, the $^{1}H–^{15}N$ correlation spectra of folded p53 core are characterised by the complex signal distribution typical for folded proteins [95]. Upon binding to Hsp90, the spectra are dominated by signals in the random coil region, including signals that were not present before in the absence of Hsp90, resulting in a pattern typical of an unfolded protein [62]. This might not be too surprising given that p53 was destabilised to force it into a complex with Hsp90. One could speculate that it might be possible to bind folded p53 to Hsp90 inducing an unfolded state. In that context it is interesting to note that under comparable experimental conditions Hsp90 did not facilitate the exchange of slow-exchanging hydrogens of p53 core over several days (SGDR, SMV Freund and AR Fersht, unpublished). Also, Hsp90 does not form a stable complex with the native, wild type protein in this environment despite the high protein concentration demanded by the NMR experiment [62].

The p53 protein is not the only example for which Hsp90 binding leads to destabilising of the protein. Significant progress in understanding Hsp90–substrate interactions derives from studies on steroid receptors. During assembly of a complex of Hsp90 and glucocorticoid receptor, the hydrophobic ligand-binding cleft gets exposed, which may destabilise the protein. Subsequent binding of steroid within the cleft triggers a transformation of the receptor and leads to release from the complex [96]. However, complexes of Hsp90 and steroid receptors so far escaped a successful analysis by NMR spectroscopy, which increases the importance of the Hsp90 interaction with p53 core domain for the deciphering of the substrate recognition mechanism.

The precise nature of p53 when bound to Hsp90 is currently a subject of a rather exciting mechanistic discussion. A recent NMR studies on the interaction of the p53 core domain and human Hsp90α described that this Hsp90 homologue associates with the native transcription factor [30]. The intensity of cross-peaks in $^{1}H–^{15}N$ spectra of p53 core decreases linearly upon addition of Hsp90. Interestingly, this did not only apply to the full length Hsp90 protein but extended to all fragments analysed, including Hsp90-N, -M, -NM and -MC. Also, the isolated middle domain of Hsp90 facilitates the HD exchange of native p53 core on a short time scale [30]. Nevertheless, significant peak shifts are not observed under these conditions for any of the domains. Given this complex conformational equilibrium and Hsp90's preference for a specific subset of substrates it is remarkable that various monomeric and dimeric Hsp90 domain constructs lacking a consensus site do not significantly differ in substrate binding properties. Extrinsic fluorescence experiments showed an increase of the signal of the dye ANS upon addition of a fragment lacking the flexible linker between Hsp90-N and Hsp90-M [30]. The authors concluded that p53 core interacts reversibly as a molten globule with Hsp90, without forming a stable complex and lacking a specific binding motif [30].

A recent study using Hsp90 domain constructs of the yeast homologue demonstrated interaction with folded p53 but came again to different conclusions [35]. Cross-peaks of p53 core also weaken and disappear upon titration with any of the Hsp90 domains. However this effect is specific to rigid parts and to the binding surface of p53 core and the magnitude of chemical shift differences is consisted only with minor structural changes. Chemical shift perturbations of p53 signals upon titration with Hsp90 domain constructs suggest a specific binding surface located at DNA-binding interface of p53 core.

9.3. Experimental conditions may influence p53 binding to Hsp90

At first glance, those three studies seem to be contradictory. Those apparent controversies might disappear when taken a look at the experimental conditions of those studies. One study uses human Hsp90β, the other human Hsp90α and the third — p53 titrations were performed with domain constructs of yeast Hsp90. It would be an attractive thought that the experimental differences might reflect the functional difference between the homologues. This seems to be unlikely, however, because such fundamental differences are not backed up by any functional data. We conclude that the differences are partly caused by experimental conditions, partly by differences in interpretation.

One important difference between those studies is ionic strength. The study involving Hsp90β used 300 mM salt, the studies analysing Hsp90α and yeast Hsp90 studied interactions at 50–100 mM salt [30,35,62]. Ionic strength affects protein stability and unspecific interactions, and both effects account for the reported differences. This is underlined by a recent study that identifies a charged segment near the Hsp90 C-terminus as salt-sensitive interaction site [35]. The major difference between those studies does not relate to the question whether unfolded p53 core interacts with Hsp90 but whether the native p53 core interacts with Hsp90. Wild type p53 core was previously shown to interact with Hsp90 under low salt conditions in biochemical binding assays [97]. The high protein concentration in the NMR experiment may force a substrate protein artificially onto the chaperone, which is a well-established drawback of this method. The co-chaperone p23, for instance, interacts with Hsp90 in low salt in nucleotide independent manner with Hsp90 in NMR experiments, which is most likely not physiologically relevant [11,51,55,88]. In high salt conditions, when unspecific protein–protein interactions are less favoured, ATP binding becomes essential to trigger association, even at NMR concentration [55,88]. We assume that similar effects may play a significant role for the Hsp90–p53 interaction.

Crucial for the NMR monitoring of complexes of full length Hsp90 protein with any substrate are the advances in technology. A significant difference of the Hsp90–p53 studies is the spectroscopic methodology. The use of CRINEPT–TROSY revealed additional p53 signals in the random coil region when bound to Hsp90 [62]. On the other hand, HSQC spectra did not allow detecting those signals, neither for Hsp90α nor Hsp90β [30,62]. Hsp90 is a complex and dimeric multi-domain protein in which interactions between both of its halves are crucial for its mechanism. Therefore, studies with full-length proteins are crucial to take the complex architectural constraints of the Hsp90 structure into account. While CRINEPT–TROSY allows detection of Hsp90–substrate complexes now for some time, future studies may make use of the recent developments using methyl-specific labelling techniques as they had been successfully used for Hsp90 interaction with the co-chaperone p23 [55,62,71,74].

A complication that was not so far taken into account for NMR studies on Hsp90–p53 is possible nucleotide-dependent effects. ATP-triggered conformational changes are supposed to be linked to Hsp90 interaction with substrate proteins [3]. A recent biochemical study analysed the role of ATP for Hsp90 chaperoning of p53 [98]. ATP binding but not hydrolysis was necessary and sufficient to support promoter binding of p53. It will be interesting to monitor the effect of ATP on Hsp90–substrate interactions in future NMR studies.

Another question is to which extent the putative difference between the molten globule and the unfolded state is in this context to some extent linguistic differences. In both studies signals in the β-sheet region disappeared, and both studies agree that they observe a rather dynamic species [30,62]. Neither of the studies using full-length Hsp90 and p53 core succeeded in observing signals of regular secondary structure in the NMR spectra. In turn the spectra involving fragments of yeast Hsp90 found that p53 core signals disappear due to increased molecular size upon interaction with Hsp90 domains, while eventual structural changes in p53 were minor, judged by the minor extent of the detected peak shifts [35].

9.4. The substrate-binding site of Hsp90

One of the central questions in the field is the identification of the substrate-binding site in Hsp90. It is remarkable that despite all the crystallographic information available for Hsp90 no obvious site had been identified [13]. The main reason for this is that the dynamic nature of the bound client makes co-crystallisation rather challenging. The EM reconstruction of a complex of the kinase Cdk4 bound to Hsp90 and Cdc37 revealed interaction of this substrate with both the N-terminal and middle domains. Similarly, NMR spectroscopy allowed mapping the binding of the protein Tau to those two domains in Hsp90 (Rüdiger group, unpublished).

In contrast to those observations, NMR studies of binding of Hsp90α to p53 core did not identify any specific binding site [71]. Resonances affected by addition of Hsp90 are spread through the entire molecule suggesting that the specificity of Hsp90's interaction with substrate is not stringently defined in those experiments.

Recent NMR studies on the interaction of domains of yeast Hsp90 with p53 core provide a more complex pattern [35]. Hsp90-C binds to p53 core with micromolar affinities in a salt-dependent manner. The responsible binding site is the strongly negatively charged pentapeptide motif DEDEE, close to Hsp90's C-terminus. Consequently, NMR mapping data at low salt identified a clustering of chemical shift changes around the disordered C-terminal extension of Hsp90 [35]. NMR mapping data also suggests that the negatively charged DEDEE motif binds to the positively charged DNA binding cleft of p53 core. DEDEE is a remarkably simple motif that can be found in several hundreds of proteins in the human genome. Nevertheless, in an in vitro aggregation assay, Hsp90 lacking the C-terminal disordered motif did prevent the precipitation of p53 core less effective than the wild-type chaperone [35].

Interestingly, the same study succeeded mapping p53 binding to negatively charged residues on middle domain, in contrast to the finding by similar experiments with the middle domain of human Hsp90α [35,71]. Substitution of those negatively charged amino acids E415 and E412 by alanine did not affect the ability of the chaperone to prevent p53 core precipitation in vitro, but the inversion of the charge had a significant effect for the E415K mutant [35].

A key aspect to take into account is related to the fact that one is studying interactions of a large molecular weight protein with a potentially dynamic and unstructured partner protein that can lead to extra experimental differences. The variety in results on Hsp90–substrate interactions might be caused in part by differences in Hsp90 homologs and constructs, in part by the experimental differences. The typically high protein concentrations in NMR experiments favour protein–protein interactions. This effect is amplified for samples that have subphysiological ionic strength, which favour charged interactions. High ionic strength conditions typically stabilise proteins. When interactions of Hsp90 and p53 core are detectable at low ionic strength but invisible at high ionic strength, this could be caused by one of two possible explanations: (i) Low salt may destabilise p53 core and generate a not fully folded species that binds to Hsp90, or (ii) p53 core at low ionic strength possibly in folded form to Hsp90 via ionic interactions.

10. Conclusions and outlook

The interactions observed by structural biology methods including NMR spectroscopy provide fundamental information regarding the protein's functional properties, however these have to be placed in the big picture of the cell were the chemical environment is different from the in vitro condition. Although traditionally an in vitro method, NMR has moved over the last decade inside living cells [99,100]. For a few example proteins NMR spectra in human cells reveal similar pattern as in the test tube, although the dynamics increase *in vivo* [101,102]. The complex nature of the Hsp90 machine has so far prevented similar in-cell NMR experiments. In particular the role of co-chaperones can be crucial for Hsp90 client recognition *in vivo*, as they may possibly trigger so far undetected interaction mechanisms.

Further structural studies of Hsp90–substrate complexes are required to elucidate the molecular mechanisms of the recognition of substrates. NMR spectroscopy will have a key role in this quest due to its ability to combine high-resolution data with the analysis of dynamics. Although Hsp90 is a highly challenging protein to study by NMR spectroscopy a significant progress has been made in the past 10 years. The particular advantage of NMR spectroscopy is the possibility to analyse dynamic complexes in solution. This is crucial for the analysis of chaperones with their substrate proteins, which may not be rigidly folded. Recent progress in selective labelling techniques and spectroscopic techniques allowed overcoming many of the typical restrictions of NMR spectroscopy. It is particularly important that it is now possible to apply this technology to the Hsp90 full-length protein. This is essential to study domain–domain interactions of this dynamic molecular machine. The combination of NMR experiments with crystallographic and SAXS data will reveal a comprehensive picture of Hsp90's molecular mechanism.

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