A novel chaperone-activity-reducing mechanism of the 90-kDa molecular chaperone HSP90

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The 90-kDa heat shock protein (HSP90) acts as a specific molecular chaperone in the folding and regulates a wide range of associated proteins such as steroid hormone receptors. It is known that HSP90 possesses two different chaperone sites, both in the N- and C-domains, and that the chaperone activity of HSP90 is blocked by binding of geldanamycin (GA) to the N-domain, the same as the ATP-binding site. Here we show that Cisplatin [*cis*-diamminedichloroplatinum (II), CDDP], an anti-neoplastic agent, associates with HSP90 and reduces its chaperone activity. In order to analyse the binding proteins, bovine brain cytosols were applied to a CDDP-affinity column and binding proteins were eluted by CDDP. In the elutants, only 90-kDa protein bands were detected on SDS/PAGE, and the protein was cross-reacted with the anti-HSP90 antibody on immunoblotting. No protein bands were detected in the elutants from the control column on SDS/PAGE. These results indicated that CDDP has a high affinity for HSP90. On CD spectrum analysis, the binding of CDDP to HSP90 resulted in a conformational change in the protein. Although HSP90 inhibited the aggregation of citrate synthase as a molecular chaperone *in vitro*, the activity was suppressed almost completely in the presence of CDDP. Mg/ATP has an influence on the chaperone activity to some extent. The CDDP binding region of HSP90 is near the C-terminal which is quite different from the GA-binding site. Our results suggest that the chaperone activity of HSP90 may be inhibited by the binding of CDDP or GA by different mechanisms.

Key words: heat shock protein, stress protein, Cisplatin.

INTRODUCTION

The 90-kDa heat shock protein (HSP90) is a highly conserved molecular chaperone, a constitutively expressed stress protein in eukaryotes and prokaryotes with protein folding [1–3]. HSP90 can prevent the aggregation of unfolded proteins and cooperate with the HSP70/HSP40 chaperone system in the ATP-dependent refolding of unfolded model proteins [2,4]. In eukaryotes, the cytoplasmic HSP90s act as specific chaperones for a wide range of client proteins such as the cytoplasmic receptor steroid hormones [5]. HSP90 alone can act to prevent protein aggregation and promote refolding *in vitro* [6], but *in vivo* it is functionally associated in multiprotein complexes with a range of associated proteins such as p23 [7], HSP70/HSP40 [8], FKBP52 [9], Hop (p60/Sti1) [10], Cdc37/p50 [11] and Cyp40 (cyclophilin 40) [12].

Several HSP90-associated proteins contain multiple copies of the tetratricopeptide repeat (TPR) motif [10], a degenerate consensus sequence that mediates protein–protein interactions in diverse cellular pathways [13]. The TPR-containing domain of FKBP52, Cyp40, and protein phosphatase pp5 mediate the binding of these proteins to HSP90 [14–16]. Only a specific subset of TPR-containing proteins, with closely related TPR motifs, can bind HSP90. Although Hip (p48) contains four TPR motifs, the HSP70 cofactor protein Hip does not bind directly to HSP90. It is generally thought that two models of interactions with HSP90 and a polypeptide are as follows [7,17]. Unfolded polypeptide substrates associate first with the HSP70 system and are then targeted to HSP90 via Hop, TPR that interacts with both HSP70 and HSP90 (intermediate complex of Hip, Hop, HSP70 and HSP90). Further remodelling results in the mature complex consisting of HSP90, p23, an immunophilin, or Cyp40.

It has been reported that HSP90 has been dissected by proteolysis into three domains, an N-terminal, a middle and a C-terminal domain [18]. HSP90 is the cellular target protein of geldanamycin (GA), a novel anti-tumour drug and a specific inhibitor of HSP90-mediated protein folding [18]. The crystal structure of the N-terminal fragment of HSP90 (about 25 kDa) has recently been reported [18,19]. This domain contains a unique site for the high affinity binding of GA, and this site is the same domain for the binding of ATP [19], suggesting that GA is an inhibitor of ATP binding to HSP90. GA also prevents p23 binding to HSP90 and inhibits the proper folding of the steroid hormone receptor [7,17].

Recently, it has been reported that HSP90 possesses two chaperone sites located in the NH_2- and COOH-terminals [4,20]. The NH_2-terminal domain contains a peptide binding site that seems to bind preferentially unfolded proteins and the chaperone activity of the NH_2-domain is inhibited by GA in an ATP dependent manner. On the contrary, the COOH-terminal fragment binds to partially folded proteins in an ATP-independent manner. Recently, it has been reported that the *in vivo* function of HSP90 is dependent on ATP binding and ATP hydrolysis

Abbreviations used: HSP, heat shock protein; HSP90 and HSP70, heat shock proteins with subunit molecular masses of 90 and 70 kDa; CDDP, Cisplatin [*cis*-diamminedichloroplatinum (II)]; GA, geldanamycin; TPR, tetratricopeptide repeat; CD, circular dichroism; CS, citrate synthase; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TPCK-trypsin, treated with tosylphenylalanylchloromethane trypsin; HPLC, high performance liquid chromatography.

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[21,22]. Thus, HSP90 contains two independent chaperone sites; both NH_2- and COOH-domains suppress protein aggregation, with differential specificity.

We have previously reported that HSP90 is markedly induced in rat kidneys with CDDP-mediated acute renal failure [23]. The induction of HSP90 by CDDP was mediated in relation to renal injury and repair. The CDDP administration did not induce HSP70, another major molecular chaperone, in the affected tubular epithelial cells, in which HSP90 was induced. CDDP is an effective antineoplastic agent in the treatment of various solid tumours, whose full clinical utility is limited because of its renal toxicity [24]. It is therefore considered that the induction pattern of HSP90 in drug-mediated-renal injury may depend on the kind of nephrotoxic reagent. Based on the previous study, we observed the possibility that CDDP may have an interaction with HSP90 and studied its influence on the chaperone activity of HSP90 *in vitro*.

MATERIALS AND METHODS

CDDP-affinity column chromatography

CDDP-Sepharose was prepared using CDDP (Sigma, 3.33 mM in saline) and CH-activated Sepharose 4B (Pharmacia) according to the instruction manual. Bovine HSP90 was prepared from brain [25,26] and anti-HSP90 antibody was produced as described previously [25,26]. An anti-HSP70 antibody against bovine brain HSP70 was produced as described previously [27]. Bovine brain cytosols in 10 mM Tris/HCl (pH 7.4) were applied to the column equilibrated with the same buffer and washed with 20 column volumes of the buffer containing 0.15 M NaCl. After washing the column, binding proteins were eluted with a linear gradient of CDDP (0–3.33 mM in the buffer) in the same buffer. All these experiments were carried out under dark conditions. The elutants were analysed on SDS/PAGE (9 % gel) [28] stained with silver by the methods of Morrissey [29] or immunoblotting [30], using anti-HSP90 or anti-HSP70 antibody. In this study, we used CH-activated Sepharose 4B as a control. Bovine brain cytosols were applied to the control column, and binding proteins were eluted with 3.33 mM CDDP and analysed on SDS/PAGE or immunoblotting as described above.

Purified bovine HSP90 and/or trypsin digested HSP90 (see below) were also applied to the column, washed with the buffer containing 0.15 M NaCl and eluted with a linear gradient of CDDP as described above. The elutants were electrophoresed on SDS/PAGE (9 % gel) and stained with silver by the methods of Morrissey.

Far-UV circular dichroism

Circular dichroism (CD) measurements were performed on a J-720 spectropolarimeter (Jusco) as described previously [31]. Hepes buffer (50 mM at pH 7.4) in the presence or absence of CDDP (1.5 μM) was used as a blank. Purified HSP90 (1.5 μM) in 50 mM Hepes buffer (pH 7.4) in the presence or absence of CDDP (1.5 μM) was filtered through a 0.22-μm filter, and scans were carried out between wavelength 240 and 200 nm in a cuvette with a 0.5-mm path length at 25 °C. The observed specific ellipticity (difference between sample and blank) was converted to the mean residue ellipticity [θ] (degrees$\cdot$cm$^2\cdot$dmol^{-1}).

Measurement of protein aggregation

The influence of HSP90 and CDDP on the thermal aggregation of mitochondrial citrate synthase (CS; Boehringer–Mannheim) at 43 °C was monitored as described [32]. To monitor thermal unfolding/aggregation, the CS concentration was 0.075 μM in 40 mM Hepes buffer (pH 7.4) in the presence or absence of bovine serum albumin (15 μM), HSP90 (0.075 or 0.15 μM), and CDDP (0.15, 1.75 and 1.5 μM). Light scattering of CS was monitored over 90 min by the optical density at 500 nm in a Pharmacia Ultrospec 3000 UV-Vis spectrophotometer equipped with a temperature control unit using semi micro-cuvettes (1 ml) with a path length of 10 mm. In this study, 1 arbitrary unit denotes an absorbance of about 0.15 at 500 nm.

CDDP-binding sites of HSP90

Purified HSP90 (5 mg ml^{-1}) was digested mildly by 0.5 μg/ml trypsin (treated with TPCK) at 37 °C for 60 min. The trypsin digests were applied to a CDDP-Sepharose column and were eluted as described above. After detection of the elutants on SDS/PAGE (12.5 % gel), the remaining elutants were combined and purified by the reverse phase column (Wakosil 5C_{18}) which was connected to an HPLC. Peptides were purified from the column with a linear gradient of 0–64 % acetonitrile at a flow rate of 0.5 ml/min, and 0.5-ml fractions were collected as previously described [33]. The amino acid sequence of the purified peptides was determined with a 491 Procise protein sequence system (Perkin–Elmer).

RESULTS

CDDP affinity-column chromatography

We have previously reported that HSP90 is specifically induced in rat kidneys with CDDP treatments [23]. The physiological meaning of the protein induction by CDDP has not yet been adequately understood. In the present study, we investigated the interaction between CDDP and HSP90 *in vitro*. To determine whether CDDP interacts with HSP90 directly, we observed the interaction between HSP90 and CDDP using a CDDP-affinity column. Bovine brain cytosols were applied to the affinity column and/or the control CH-activated Sepharose 4B column. To avoid non-specific binding proteins, those columns were washed thoroughly with a buffer containing 0.15 M NaCl. The proteins were eluted with a linear gradient of CDDP and detected on SDS/PAGE. Although no proteins bands were detected in the elutants from the control column, the 90-kDa proteins were detected as major protein bands in the elutants from the CDDP-affinity column on silver-stained gels (Figure 1A and 1C). We could not detect other proteins except for the 90-kDa protein in the elutants. We investigated whether the 90-kDa protein is HSP90. On immunoblotting using anti-HSP90 antibody, HSP90 was clearly detected in the eluted fractions from the affinity column with a linear gradient of CDDP (Figure 1D) but not in the elutants from the control column (Figure 1B). We could not find HSP70 in the elutants from the affinity column (Figure 1D). These results indicated that HSP90, but not HSP70, in the cytosol can interact with CDDP. In order to determine whether the association between HSP90 and CDDP is direct or indirect association (mediates other proteins), we analysed the interaction using a purified HSP90 alone under the same conditions. On SDS/PAGE, we could confirm that purified HSP90 was also able to bind to the CDDP-affinity column and could be eluted by increasing the CDDP concentration (Figure 2). Purified HSP90 and HSP90 in the cytosols were eluted from the column at the same CDDP concentrations (about 0.5–2 mM) (Figure 1). These

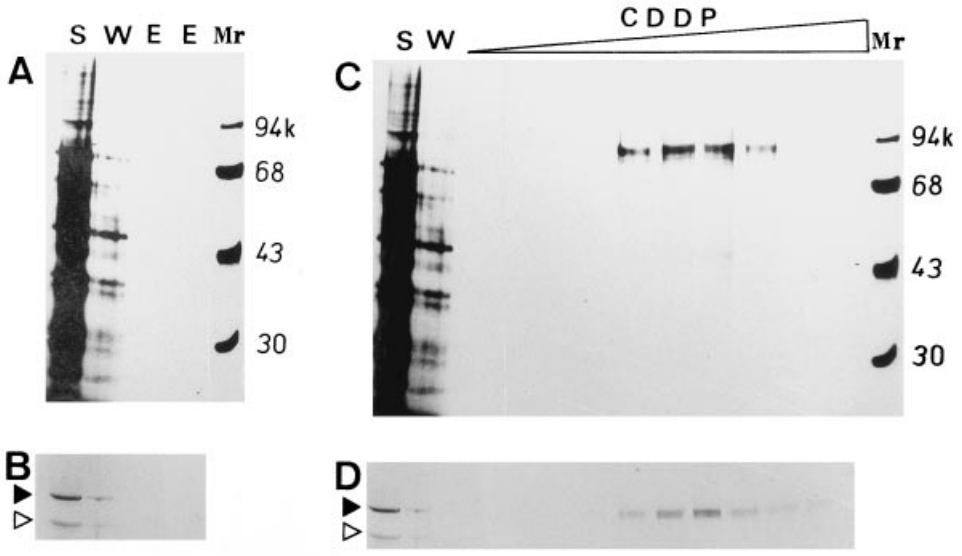

Figure 1 CDDP affinity-column chromatography using cytosol

Bovine brain cytosols were applied to the control CH-activated Sepharose 4B column or the CDDP-affinity column. After washing, the proteins were eluted with 3.33 mM CDDP from the control column or eluted with a linear gradient of CDDP (0–3.33 mM) from the affinity column. The elutants from those columns were electrophoresed on SDS/PAGE (9% gel) followed by staining with silver (**A** and **C**) or immunoblotting using anti-HSP90 antibody or anti-HSP70 antibody (**B** and **D**). **A** and **B**, control CH-activated Sepharose 4B column chromatography. **C** and **D**, CDDP-affinity column chromatography. In **A** and **C**, S, W, E, E and M_r denote applied samples, washed proteins, eluted proteins with 1.0 M NaCl, eluted proteins with 3.33 mM CDDP, and molecular standard proteins, respectively. In **B** and **D**, HSP90 and HSP70 are indicated by a closed triangle and an open triangle, respectively.

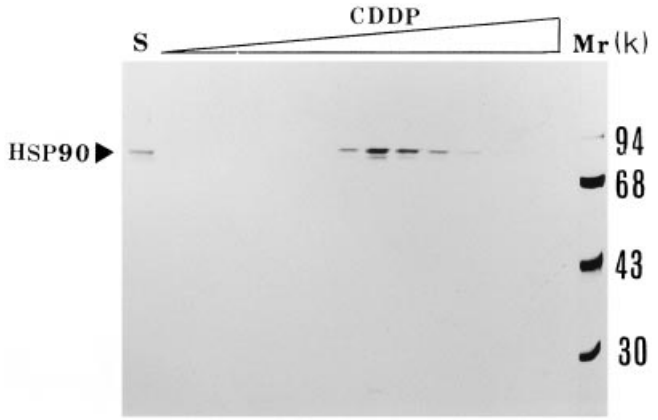

Figure 2 CDDP affinity-column chromatography using purified HSP90

Purified bovine HSP90 was applied to the affinity-column, and the elutants were electrophoresed on SDS/PAGE (9% gel), followed by staining with silver under the same conditions as in Figure 1. S and M_r (k) in the panels denote applied samples and molecular standard proteins, respectively. HSP90 is indicated by a closed triangle.

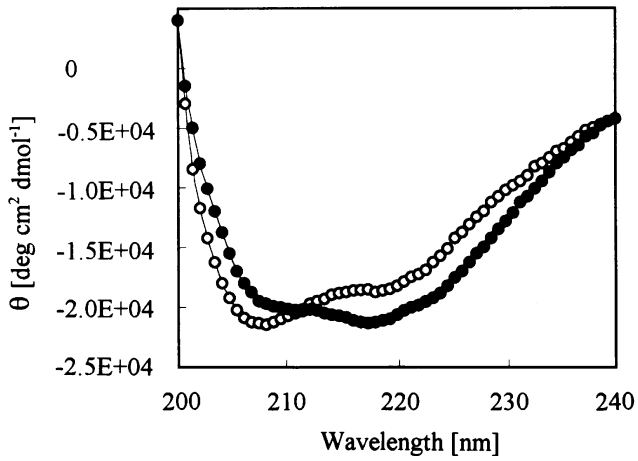

Figure 3 Effect of CDDP on the CD spectrum of HSP90

The CD spectrum of HSP90 was measured in the absence (○) or presence (●) of CDDP as described in the methods section. θ denotes the mean residue ellipticity.

results indicated that CDDP has a high affinity for HSP90 and this affinity is highly specific.

Conformational change in HSP90 on binding with CDDP

It has been reported that HSP90 has an average composition of 36% α-helix and 46% β-strand structure [34] under normal conditions. A conformational change in the protein has been caused by ATP with decreasing α-helix and increasing β-strand structure [34]. Based on the available information, we analysed whether CDDP may give rise to a conformational change (secondary structure) in HSP90 using a far-UV CD spectrum. As shown in Figure 3, our data (HSP90 alone) were in good agreement with the CD spectrum data of HSP90 [34]. The binding of CDDP to HSP90 resulted in the conformational changes in

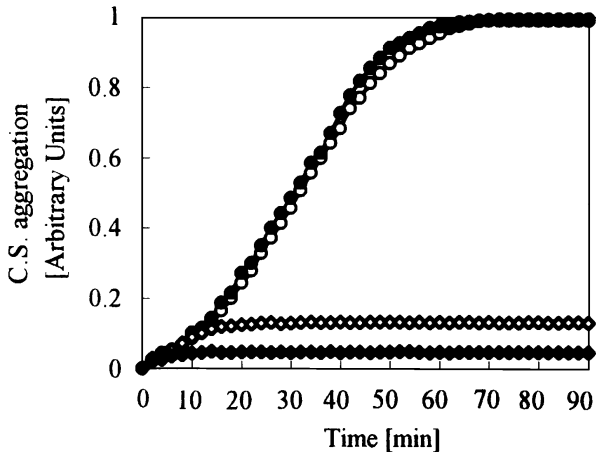

Figure 4 Chaperone activity of HSP90 using citrate synthase

Thermal aggregation of CS (0.075 μM) in the absence of additional components (○), in the presence of a 200-fold molar ratio of albumin (●), an equimolar ratio of HSP90 (◇), and a two-fold molar ratio of HSP90 (◆) was monitored at 500 nm (see the Materials and methods section).

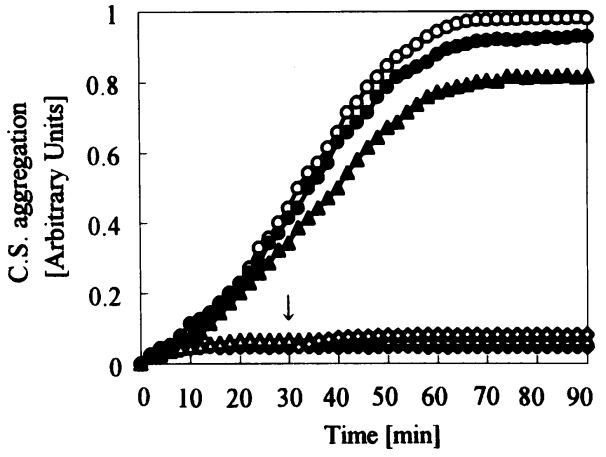

Figure 6 Influence of ATP on the chaperone activity of HSP90

The influence of ATP, HSP90 and CDDP on the thermal aggregation of CS was monitored as described in the Materials and methods section. Influence of 5 mM ATP/$MgCl_2$ on thermal aggregation of CS and HSP90 (△) or ATP/$MgCl_2$ was added (↓) after 30 min of thermal aggregation of CS and HSP90 (◇). Thermal aggregation of CS in the absence (○) or presence (▲) of 5 mM ATP and 5 mM $MgCl_2$ with 1.5 μM CDDP and 0.15 μM HSP90. ○, ◆, same as in Figure 4.

HSP90. By binding of CDDP to HSP90, decreasing of the mean residue ellipticity [θ] near 208 nm and increasing of [θ] near 218 nm indicated the decreasing α-helix and increasing β-strand structure of HSP90, respectively, in the CD spectrum (Figure 3). These results indicate that the binding of CDDP to HSP90 is not a transient association and may cause a conformational change in the protein with a significant meaning.

Measurement of protein aggregation

One of the characteristic features of molecular chaperones is their ability to suppress the aggregation of proteins under stressed conditions. To analyse the functional properties of HSP90, we studied its action in protein folding and unfolding reactions *in vitro*. As an assay system, the thermal unfolding and aggregation of mitochondrial CS was used, because CS is inactivated and aggregates rapidly upon incubation at 43 °C [32]. As shown in Figure 4, spontaneous aggregation of CS has been caused at 43 °C and the reaction reached a plateau at 60 min. Although there was no effect on the reaction in the presence of a 200-fold molar excess of albumin, an equimolar amount of HSP90 suppressed the reaction and a two-fold molar excess of HSP90 inhibited the aggregation of CS almost completely. HSP90 apparently interacts transiently with these highly structured early unfolding intermediates. As a consequence, CS is effectively stabilized in the presence of HSP90. The spontaneous aggregation of CS was not affected in the presence of CDDP itself, and also CDDP did not cause any optical changes in HSP90 in the light scattering analysis. Interestingly, CDDP reduced the chaperone activity of HSP90 (Figure 5) in a dose-dependent manner. A molar ratio of 1:1 (HSP90:CDDP) and 1:5 (HSP90:CDDP) caused about 20 and 60 % suppression of the chaperone activity of HSP90, respectively. The chaperone activity was suppressed almost completely at a molar ratio of 1:10 (HSP90:CDDP). The CDDP concentration (1.5 μM) is lower than that of the GA concentration (18 μM) which inhibits the ATPase activity of HSP90 almost completely [21].

Next, we investigated the influence of Mg/ATP on this assay system. As shown in Figure 6, Mg/ATP has a slight influence on the suppression of CS aggregation by HSP90. Addition of Mg/ATP to a preformed HSP90/CS complex also partly affected the chaperone activity of HSP90. Addition of ATP to the HSP90/CS complex (after 30 min of thermal aggregation of CS and HSP90) shows little influence. These results were in good agreement with data from yeast HSP90 [20]. On the contrary, the chaperone activity of HSP90, which was suppressed completely by CDDP, was slightly affected (about 20 % of the chaperone activity of HSP90 was recovered) in the presence of Mg/ATP.

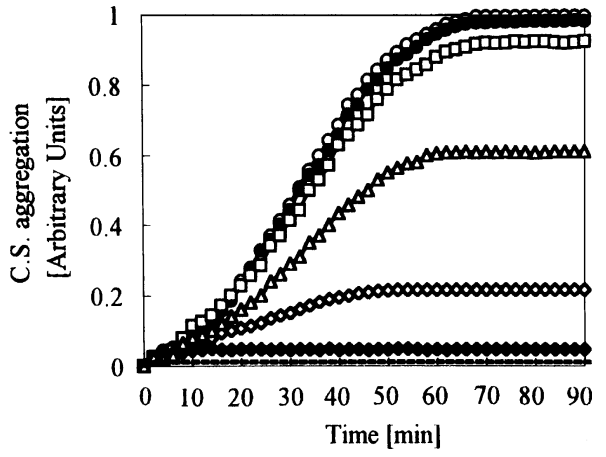

Figure 5 Effect of CDDP on the chaperone activity of HSP90

The effect of HSP90 and CDDP on the thermal aggregation of CS was monitored as described (Materials and methods section). Thermal aggregation of 0.15 μM HSP90 in the presence of 15 μM CDDP (– – –). Thermal aggregation of CS (0.075 μM) in the presence of 15 μM CDDP (●) or 0.075 μM CS and 0.15 μM HSP90 in the presence of 0.15 μM CDDP (◇), 0.75 μM CDDP (△), and 1.5 μM CDDP (□). ○, ◆, same as in Figure 4.

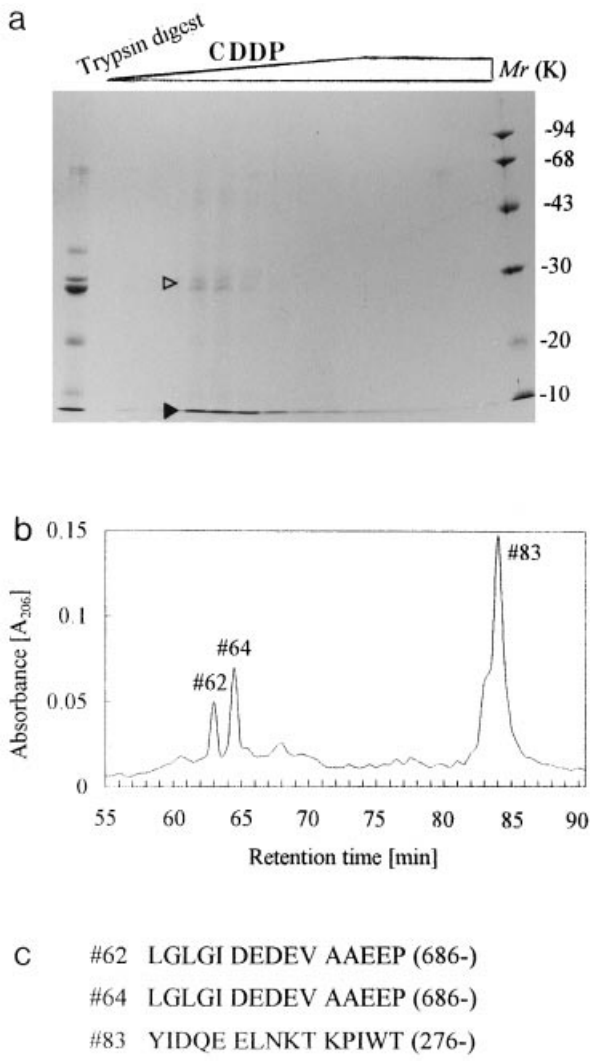

Figure 7 CDDP binding sites of HSP90

a. HSP90 was digested by trypsin, and the digests were applied to the CDDP affinity column and eluted with a linear gradient of CDDP (0–3.33 mM). The elutants were electrophoresed on SDS/PAGE (12.5% gel) and stained with Coomassie Brilliant Blue. Eluted peptides of 32/30 kDa and small peptides from the column are indicated by △ and ▲, respectively. M_r denotes molecular standard proteins. **b**. Trypsin digests of HSP90 eluted from the CDDP affinity-column were separated by reverse phase column chromatography. The purified peptides indicated in the panel, nos. 62, 64 and 83, were sequenced by a peptide sequencer. **c**. The three peptides (nos. 62, 64 and 83) were sequenced and compared with human HSP90 β [35]. Parentheses indicate the position of HSP90 β [35].

CDDP binding sites of HSP90

An important question regarding the CDDP-binding sites of HSP90 remains unanswered. To investigate the CDDP-binding domain of HSP90, we analysed the sites using trypsin digests of HSP90 and a CDDP affinity-column. As shown in Figure 7a, HSP90 was digested mildly by trypsin, and the digests (68-, 38, 32-, 30-, 22-, 12 kDa, which may be smaller than the 10-kDa peptides which appeared in the dye front on 12.5% SDS/PAGE), were applied to the CDDP-affinity column and eluted with a linear gradient of CDDP (0–3.33 mM). Among these peptides, 32- and 30-kDa peptides and smaller than 10-kDa peptides were eluted from the affinity column. These elutant peptides were combined and purified on a reverse phase column which was connected to an HPLC. Figure 7b shows a peptide map obtained from the elutant trypsin digests of HSP90 from the affinity column. Three peptides (numbers 62, 64 and 83 which has a shoulder peak in 82) were separated on the reverse phase column, and the separated peptides were sequenced by a protein sequencer (Figure 7c). Peptides 62 and 64 showed the same sequences, and the sequences were located in the COOH-terminal of HSP90 [35,36]. If these sequences were from 694 to the COOH-terminal end of HSP90 α [36] or 686 to the COOH-terminal end of HSP90 β [35], the molecular weight would be 4152 and 4137, respectively. Because of the small molecular weight, we could not detect them on SDS/PAGE, only in the same position as the dye front (Figure 7a). The difference in the retention time of peptides 62 and 64 may attributed to the difference in their molecular weight; both peptides of the NH_2-terminal were the same, but not those of the COOH-terminal. The other peptide (32 and 30 kDa), number 83 with a shoulder of number 82, gave the sequence and was located in the middle domain of the COOH-terminal of HSP90. Peptide 83 also contains two peptides; the NH_2-terminals were the same, but not the COOH-terminals of these peptides, which may attributed to the difference in the molecular weight such as between 62 and 64. Our results identify the COOH-terminal of HSP90 as the specific binding sites for CDDP.

DISCUSSION

In most eukaryotic cells, three classes of heat shock proteins, HSP90, HSP70 and small HSPs, are predominantly synthesized [1,37]. In these three classes of HSPs, the role of HSP70 and chaperonins has been well characterized [38,39]. In contrast, much less is known about the physiological and biochemical mechanisms of HSP90. Only recently, the partial crystal structure of HSP90 has been published [18,19]. The function of HSP90 is specifically involved in the folding or conformational regulation of central signal transduction molecules, including steroid hormone receptors and proto-oncogene kinase [10,34,40]. The chaperone activity of HSP90 is inhibited by ansamycin antibiotics (GA) [18]. Recently, it has been published that the *in vivo* function of HSP90 is dependent on ATP binding and ATP hydrolysis [21,22]. Furthermore, an important result has been reported that HSP90 acts as a capacitor for morphological evolution [41]. Despite their ubiquitous biological function and cellular abundance, the HSP90 family of proteins remains among the least understood of all the molecular chaperones.

We have previously reported that HSP90 is markedly expressed in both the cytoplasm and nucleus of rat kidneys with CDDP-induced acute renal failure on days 3 and 5 [23]. Degenerative changes in epithelial cells appeared in the S3 segment of the proximal tubules on day 3, and epithelial cell regeneration in the protein was found from day 5. In contrast, HSP70 was not induced in this treatment. Although HSP70 does not have an affinity for CDDP *in vitro*, HSP90 had a high affinity for the reagent in the present study. HSP70 is an abundant chaperone as well as HSP90 in many organs including the brain, liver and kidneys [27]. In these proteins, only HSP90 binds to a CDDP-affinity column preferentially, but HSP70 does not. These results indicate that HSP90 has high affinity for CDDP. On the basis of our *in vitro* results, we suggest that CDDP acts as a specific inhibitor of HSP90-mediated chaperone activity.

It has been reported that two chaperone sites in HSP90 (NH_2- and COOH-domains) have a differential structural specificity for target substrates [4,20]. The chaperone activity in the NH_2- and

COOH-domains of the protein is ATP-dependent and ATP-independent, respectively. The sequence of RGYVYQGL (VSV-G peptide, antigenic peptides designated for presentation of an MHC class I complex and a specific substrate of GRP94) binds specifically to the COOH-domain [4]. Recently, it has been reported that TPR proteins bind to the COOH-terminal 12 kDa domain of HSP90 [4]. The cochaperones of HSP90 (Hop, Hop-HSP70 and FKBP52) bound specifically to the COOH-domain and not to the NH_2-domain [20]. The chaperone activity in the COOH-domain of HSP90 is an ATP-independent mechanism. On the contrary, the 19-aa peptide GR1 (steroid receptor fragment) bound to the NH_2-domain [20]. The NH_2-domain fragment binds to partly folded proteins in an ATP-dependent manner. GA bound to the NH_2-domain, the same as the ATP-binding pocket, and inhibited the chaperone activity of HSP90 [18]. In contrast, the mechanism of the chaperone activity and its inhibitory compound in the COOH-domain were unclear. These results indicated that the two chaperone sites of HSP90 seem to act independently in a similar way in the fragment and in the wild-type protein.

In the present study, mitochondrial CS was chosen as a model substrate in the analysis of chaperone activity because it is inactive and aggregates rapidly upon incubation at 43 °C [32]. HSP90 binds transiently to unfolding intermediates of the thermally unfolding CS. Upon release from HSP90, the intermediates are able to refold rapidly to the native state. HSP90 stabilizes the native CS and dramatically slows the subsequent aggregation process. HSP90 interacts with highly structured unfolding intermediates of CS in an ATP-independent manner. Taking all these results together, the chaperone sites recognizing these intermediates may be located exclusively in the COOH-terminal domain of HSP90 [20]. On binding of GA to the NH_2-domain of HSP90, GA causes the misfolding and degradation of the substrates. In contrast, the chaperone activity of the protein will also be suppressed by CDDP in a dose-dependent manner and the activity will be inhibited completely at 1.5 μM CDDP. The concentration of CDDP is lower than that of GA (100 μM: [20] or 18 μM: [21]). The chaperone activity of HSP90 which was suppressed completely by CDDP will recover by about 20% in the presence of Mg/ATP. The reason why the chaperone activity of HSP90 recovered due to Mg/ATP even when in the presence of CDDP has not yet been adequately understood.

In the present study, the COOH-domain of HSP90 was able to bind to the CDDP-affinity column. We could not confirm the NH_2-domain in the elutant from the affinity column using a trypsin digest of the protein. These results suggest that the CDDP binding domain and the intermediate binding domain may be located both in the same or closely related to the COOH-domain of HSP90. By binding of CDDP to the COOH-domain of the protein, intermediates of CS would not be able to bind to the site and may result in thermal aggregation of CS. In the chaperone site of HSP90, ATP may not influence the chaperone activity. However, ATP had a slight effect on the chaperone activity with CDDP.

To elucidate the mechanism of ATP action, recovery of the chaperone activity of HSP90 seemed necessary. For this mechanism, there are some possibilities as follows: (i) ATP induces a conformational change in HSP90 (from an open to a closed form) [32]. Like many other chaperones, HSP90 is a rather hydrophobic protein, and its hydrophobicity further increases after addition of ATP or heat treatment [32]. The secondary structure of HSP90 is also changed by CDDP. A further conformational change in HSP90, which is bound to CDDP, may be induced by ATP treatment. The additional conformational change in the HSP90/CDDP complex by ATP may cause a slight recovery of the chaperone activity or dissociation of HSP90 from CDDP. (ii) It has been shown that complete HSP90 possesses a chaperone activity *in vivo* dependent on ATP binding and ATP hydrolysis [21,22]. The CDDP binding site was located in the COOH-terminal domain of HSP90 (this study). Taking our present results together, it is difficult to explain the mechanism of ATP action against the chaperone activity of HSP90 and CDDP by the ATPase activity of HSP90; (iii) HSP90 has an ATP-binding site in the NH_2-domain [19–22]. HSP90 would have another CDDP binding site in the NH_2-domain and the CDDP binding site may locate in the same domain or be closely related to that of the ATP binding domain. Although HSP70 is also an ATP binding protein, the protein does not bind to the CDDP-affinity column. We could not find NH_2-residues in the elutant from the CDDP-affinity column using mild trypsin digests of HSP90.

The NH_2-terminal chaperone site appears to be selective concerning substrate specificity, although the interaction with a non-native polypeptide is regulated tightly by ATP binding. In contrast, the COOH-terminal site is more promiscuous in binding, with a preference for partly folded structures under stress conditions. Thus, HSP90 contains two independent chaperone sites, and the chaperone activity will be caused by different mechanisms. The CDDP-binding domain of HSP90 is quite different from the GA binding site or the ATP binding domain. The chaperone activity of HSP90 will be inhibited by different antineoplastic agents by different mechanisms.

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