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# Interaction of vanadate oligomers and permolybdate with the 90-kDa heat-shock protein, Hsp90

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The 90-kDa heat-shock protein (Hsp90) is a molecular chaperone that aids the folding of nuclear hormone receptors and protein kinases. Hsp90 · protein complexes can be stabilized by molybdate and by other transition metal oxyanions such as vanadate. Our earlier findings [Csermely, P., Kajtár, J., Hollósi, M., Jalsovszky, G., Holly, S., Kahn, C. R., Gergely, P. Jr, Sőti, C., Mihály, K. & Somogyi, J. (1993) *J. Biol. Chem. 268*, 1901−1907] showed that vanadate and molybdate can induce a large conformational change of Hsp90. Here we provide direct evidence for the binding of vanadate and molybdate to Hsp90 by demonstrating that surface-plasmon-resonance measurements indicate binding of various vanadate oligomers to Hsp90, $^{51}$V-NMR measurements show an extensive interaction of decavanadate with the chaperone, and permolybdate treatment of Hsp90 induces a marked mobility shift of the protein and its tryptic fragments. Our results indicate the flexibility of molybdate/vanadate-binding sites of Hsp90, which are able to accomodate various species of these transition metal anions.

*Keywords:* molecular chaperone; molybdate; $^{51}$V-NMR; surface plasmon resonance; steroid receptor.

Molecular chaperones are highly conserved, ubiquitous mediators of the folding, assembly, transport and disposal of other proteins [1], which may have played a major role in the development of modern enzymes [2]. The 90-kDa heat-shock protein (Hsp90) is a cytoplasmic chaperone that helps the folding of nuclear hormone receptors and various protein kinases [3−5].

Vanadate and molybdate have been reported and are widely used to stabilize Hsp90 · protein complexes [6−8]. Our earlier studies demonstrated that both anions induce a large conformational change of Hsp90 by increasing its $\beta$-sheet content [9]. The present studies provide direct evidence for the binding of vanadate and molybdate to Hsp90 using surface plasmon resonance (SPR), $^{51}$V-NMR and permolybdate labeling. Our data indicate a low specificity and a relatively low affinity of Hsp90 for various species of vanadate and molybdate, similarly to the low specificity and low affinity binding to the Hsp90 nucleotide-binding site(s) [10−14].

## MATERIALS AND METHODS

**Chemicals.** The chemicals used for PAGE, Q2 FPLC, and Econo-Pac HTP cartridges were from Bio-Rad. Butyl-Sepharose 4B and DEAE-Sepharose Fast Flow were purchased from Pharmacia LKB Biotechnology Inc. Coupling chemicals and SPR sensor chips were purchased from Pharmacia Biosensor AB. Bovine Hsp70 was obtained from StressGen, and p23 was a kind gift of David Toft (Mayo Clinics, Rochester MN, USA). The HPLC-purified Hsp90 peptides, YCVQQL and RLVTSPCCIVTSTYGWTANM, were synthesized by G. Orosz (Dept Organic Chemistry, Eötvös University, Budapest, Hungary). Geldanamycin was from GIBCO-BRL. Tosylphenylchloromethylketone-treated trypsin was obtained from Wortington. All the other chemicals used were from Sigma Chemicals Co. or from Fluka AG.

**Isolation of Hsp90.** The 90-kDa heat-shock protein was purified from rat liver using consecutive chromatographies on Butyl-Sepharose 4B, DEAE-Sepharose Fast Flow and Econo-Pac HTP as described earlier [10]. To increase the purity of the preparation further, Q FPLC chromatography was performed. Pooled Hsp90 fractions of the HTP column were dialyzed against 20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM dithiothreitol. The Hsp90 sample was loaded onto a BioRad Q2 FPLC column and eluted by a linear gradient from 0 to 0.5 M NaCl using a BioRad BioLogic apparatus at a flow speed of 1 ml/min. 0.25-ml fractions were collected and analyzed by SDS/PAGE. The purest fractions were collected, dialyzed against 10 mM Hepes, pH 7.4, 0.1 mM EDTA, 0.1 mM dithiothreitol, and concentrated by ultrafiltration. EDTA was included in the final buffer to prevent the slow, but regularly observed, calcium-induced proteolysis of highly purified Hsp90 preparations (Schnaider, T., Sőti, C. and Csermely, P., unpublished results). The purity of the final Hsp90 preparations was more than 95% as judged by silver staining of SDS/polyacrylamide gels. Protein concentrations were determined according to Bradford [15].

**Preparation of vanadate oligomers.** Preparation of vanadate oligomers has been performed as described earlier [16]. Briefly: 50 mM stock solutions of oligovanadates were prepared by boiling freshly made aqueous solutions of $Na_3VO_4$ at pH 10 for 15 min. Decavanadate solutions were always freshly pre-

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*Abbreviations.* Hsp90, 90-kDa heat-shock protein; SPR, surface plasmon resonance.

pared by adjusting the pH of a 50 mM oligovanadate solution to 4.0, and re-adjusting to 7.4.

**SPR measurements.** SPR of carboxymethyldextran-coupled Hsp90 was measured using a BIA-Core apparatus as described earlier [17]. Carboxymethyldextran-coated sensor chip was activated with 200 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 50 mM *N*-hydroxysuccinimide. Rat Hsp90 (30 μg/ml) was coupled to the chip in 10 mM sodium formate, pH 4.0, for 7 min at 25 °C. The remaining active sites of the resin were inactivated by incubation with 1 M ethanolamine/HCl pH 8.5, for 7 min at 25 °C. The usual change in the angle of the SPR minimum of the reflected light after coupling of Hsp90 was approximately 1°, i.e. 10000 resonance units, corresponding to 10 ng Hsp90/mm$^2$ sensor chip [18]. Binding of vanadate oligomers to Hsp90 was analyzed in 10 mM Hepes, pH 7.4, 0.15 M KCl and 0.001% Tween-20. Binding experiments were repeated with 'mock-coupled' sensor chips, where in the coupling procedure Hsp90 was omitted. Sensorgrams were corrected to the small residual binding observed in these control experiments. Sensorgrams were analyzed by the BIA evaluation program 2.1 to obtain $K_d$ values using a single exponential kinetic model. Due to the complexity of the vanadate oligomer solutions the calculated $K_d$ values represent only an apparent $K_d$, giving qualitative but not quantitative information. Experiments were repeated at a flow rate of 10 μl/min to verify the effect of possible rebinding. Rebinding and mass-transfer limitation was verified by the repetition of the experiments at lower coupling ratios of Hsp90. The identical results showed that these phenomena did not occur under the conditions used.

**$^{51}$V-NMR measurements.** NMR measurements were performed on a Varian VXR-400 spectrometer using the pulsed-Fourier-transform technique. Chemical-shift values are given with reference to $VOCl_3$ (0 ppm) as standard. Spectra were recorded at 25 °C in 10-mm cells in 3 ml 50 mM Hepes, pH 7.4, 0.1 mM EDTA and 10% $D_2O$. 12000 transients were collected with an acquisition time of 15 ms.

**Binding of permolybdate to Hsp90.** Permolybdate has been generated and its binding to Hsp90 was studied as described by Meshinchi et al. [19]. To 1 μg of Hsp90 sodium molybdate and hydrogen peroxide were added sequentially to 20 mM each and the sample was incubated for 60 min at 0 °C or 37 °C as indicated. The total volume of the sample was 20 μl, and contained 50 mM Hepes, pH 7.4 and 0.5 mM EDTA. The reaction was stopped by boiling for 4 min in the presence of 5 μl Laemmli buffer [20] containing 0.1 M dithiothreitol. Samples were analyzed by SDS/PAGE [20], or on urea/PAGE [21]. Protein bands were visualized by Coomassie Blue staining.

**Trypsin treatment of Hsp90.** Tryptic fragments of Hsp90 were obtained using the method of Lees-Miller and Anderson [22]. Permolybdate treatment was stopped by the addition of 40 mM dithiothreitol and samples containing 5.4 μg Hsp90 were treated with 8 ng trypsin for 10, 30 or 60 min at 37 °C. The reaction was stopped by the addition of 0.1 μg trypsin inhibitor and by boiling for 4 min in the presence of 5 μl Laemmli buffer [20] containing 0.1 M dithiothreitol. Samples were analyzed by 11% SDS/PAGE. Protein bands were visualized by Coomassie Blue or by silver staining.

## RESULTS

**Binding of vanadate to Hsp90 as assessed by SPR.** Binding of vanadate to Hsp90 was demonstrated by SPR. This method, which measures the changes in the refractive index of carboxymethyldextran-immobilized molecules and of the compounds associated with them is an excellent tool to obtain online data on binding and dissociation of various complexes. The method is widely used to analyze protein-protein and protein-DNA interactions. Its major setback is its sensitivity for changes in the refractive index of the buffer, which can be minimized by dissolving the additives in the same running buffer.

SPR measurements have been successfully applied to characterize Hsp90-protein and Hsp90-ATP interactions [14, 17, 23]. Fig. 1 A shows the changes of Hsp90 SPR during a 5-min pulse of 5 mM oligovanadates or decavanadate. Calculation of the apparent binding affinities of the oligovanadate mixture and decavanadate solutions (Fig. 1B, C) gave a binding affinity for oligovanadate species of 0.8−7 mM, while the apparent $K_d$ for decavanadate fluctuated around 3.7 ± 0.9 mM. The increased apparent $K_d$ of oligovanadates at higher monomer concentrations is probably due to looser binding of vanadate oligomers to Hsp90 than monovanadate, since at vanadate monomer concentrations above 5 mM tetravanadate becomes the dominant vanadate species in neutral solutions. In contrast, decavanadate is always a dominant species of decavanadate solutions in the vanadate monomer concentration range of 0.5−10 mM (Table 1, [16, 24]). This may explain the relative stability of the apparent $K_d$ of decavanadate at various vanadate concentrations.

Since $Mg^{2+}$ were shown to interact with the ADP/ATP-binding site of Hsp90 [12] and Hsp90 is a $Ca^{2+}$-binding protein [5] we investigated the effects of $Mg^{2+}$ and $Ca^{2+}$ on binding of vanadate oligoanions. However, addition of 5 mM $MgCl_2$ or $CaCl_2$ did not change the binding of oligovanadate or decavanadate (data not shown).

The maximal change in the deflection of the reflected light was 93 ± 16 RU or 664 ± 174 RU (0.0093 ± 0.0016° or 0.0664 ± 0.0174°) after the addition of 2 mM oligovanadate or 10 mM decavanadate, respectively. Vanadate concentrations were selected at the upper limit of the concentration range where monovanadate and decavanadate are the dominant species in the vanadate oligomer mixture. Under these conditions the oligovanadate solution contains approximately 50% each of monovanadate and tetravanadate while the decavanadate content of the decavanadate solution is around 70−80% (Table 1, [16, 24]). The maximal SPR signal under these conditions corresponded to 93 pg and 664 pg oligovanadate and decavanadate/mm$^2$ sensor chip, or to 7 mol and 52 mol vanadate monomer/mol Hsp90, respectively. The magnitude of vanadate-induced SPR changes suggests a stoichiometry of the vanadate · Hsp90 complex between 1:2 and 1:7 for oligovanadate (assuming that the 7 mol vanadate monomer comes from the exclusive binding of 2 tetravanadate or 7 monovanadate groups, respectively) and around 1:5 for decavanadate (taking into account that each molecule decavanadate is formed by ten monovanadate units).

Changes in SPR may arise by changes in the refractive index of Hsp90 itself after vanadate addition (a phenomenon likely to occur due to the vanadate-induced conformational change of the protein [9]), or from the increased binding of ions, buffer components or Tween-20 to Hsp90 after vanadate addition; these components of the SPR signal seem to be minor in our experiments since molybdate, which induces a similar conformational change of Hsp90 to that of vanadate [9], did not induce any significant change in the SPR signal of Hsp90 up to 10 mM (data not shown).

Analysis of the Hill plots of vanadate-Hsp90 and molybdate-Hsp90 interactions yields an apparent Hill coefficient lower than 1, which suggests the interaction of Hsp90 with multiple species of both anions (Table 2).

In contrast to the readily observable characteristic yellow colour of decavanadate species in decavanadate solutions, a blue colour was not observed during our experiments which indicates that despite its ability to reduce cytochrome *c* [Nardai, G., Sass,

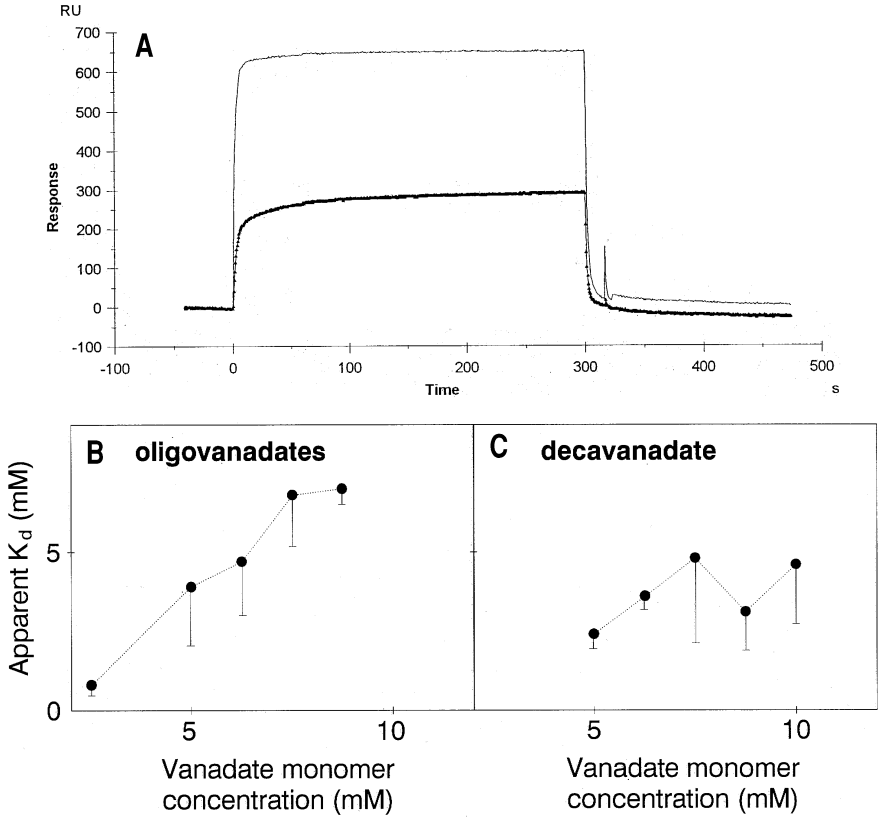


**Fig. 1. SPR analysis of Hsp90-vanadate interactions.** Immobilization of rat liver Hsp90, SPR measurements, and calculation of apparent dissociation constants were performed as described in Materials and Methods. (A) Sensorgram of oligovanadate (▲) and decavanadate (−) binding at vanadate monomer concentrations of 5 mM. Curves are representatives of three experiments. (B) Apparent dissociation constants of oligovanadates. (C) Apparent dissociation constants of decavanadate. Data are means ± SD of three experiments.

**Table 1. Composition of monovanadate and decavanadate solutions at pH 7.4.** For details of the preparation of the two solutions, see Materials and Methods. Data show the concentration of various vanadate species at different total vanadate concentrations and were adapted from Fig. 2 of our previous study [16].

| Solution | Total vanadate concentration | Concentration of | | | |
|---|---|---|---|---|---|
| | | monomer | dimer | tetramer | decamer |
| | mM | | | | |
| Monovanadate | 1 | 0.8 | 0.1 | 0.1 | |
| | 2 | 1 | 0.2 | 0.8 | |
| | 5 | 1 | 0.6 | 3.4 | |
| | 10 | 1 | 0.8 | 8.2 | |
| Decavanadate | 1 | 0.1 | 0.05 | 0.05 | 0.8 |
| | 2 | 0.3 | 0.1 | 0.2 | 1.4 |
| | 5 | 0.7 | 0.2 | 0.8 | 3.3 |
| | 10 | 0.7 | 0.2 | 1.9 | 7.2 |

**Table 2. Apparent Hill coefficients of Hsp90-molybdate and of Hsp90-vanadate interactions.** Apparent Hill coefficients were determined using the equation $\log (y/1-y) = n_H \times \log c$ where $y$ is the measure of the interaction, $n_H$ is the apparent Hill coefficient, and $c$ is the concentration of molybdate or vanadate [33, 34]. Apparent Hill coefficients and correlations were calculated from a linear fit to the Hill plot of 3−10 measurements.

| Ion | Interaction | $n_H$ | Correlation | Reference |
|---|---|---|---|---|
| Molybdate | competition with azido-ATP | 0.71 | 0.91 | [9] |
| | inhibition of Hsp90-associated Ca-ATPase activity | 0.33 | 0.95 | [11] |
| Vanadate | competition with azido-ATP | 0.63 | 0.91 | [9] |
| | inhibition of Hsp90-associated Ca-ATPase activity | 0.49 | 0.99 | [11] |

B., Eber, J., Orosz, G., Schnaider, T., Sőti, C. and Csermely, P., unpublished results], Hsp90 induced no major reduction of vanadate species to tetravalent vanadyl ions.

**Characterization of binding of various vanadate oligoanions to Hsp90 by $^{51}$V-NMR.** Since SPR cannot discriminate between the binding of various vanadate oligoanion species to Hsp90, the relative affinity of vanadate oligoanions to Hsp90 has been characterized further using $^{51}$V-NMR spectroscopy, a powerful tool to monitor the association of vanadate oligoanions to proteins [16, 25].

Fig. 2A shows a representative $^{51}$V-NMR spectrum of a mixture of 0.1 mM each of oligovanadate and decavanadate solutions. Decavanadate gave the characteristic triplet peaks at chemical shifts of −422, 496 and 513 ppm, while the additional peaks at −558 ppm and −572 ppm correspond to monovanadate and tetravanadate, respectively [16, 24]. Upon addition of 24 μM Hsp90 the decavanadate peaks disappeared partially. The mono-

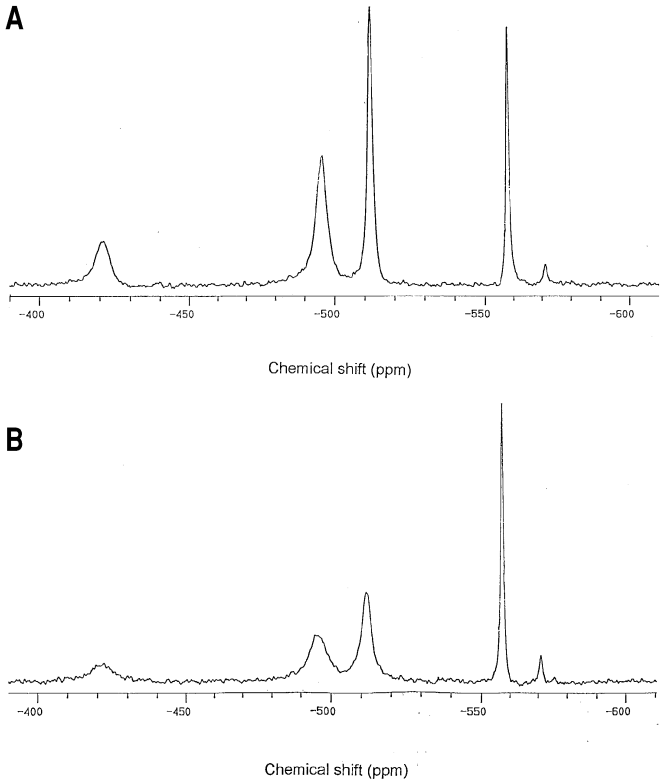


**Fig. 2. $^{51}$V-NMR analysis of vanadate binding to Hsp90.** $^{51}$V-NMR spectra were recorded as described in Materials and Methods. (A) $^{51}$V-NMR spectrum of 0.1 mM oligovanadate/0.1 mM decavanadate. (B) $^{51}$V-NMR spectrum of 0.1 mM oligovanadate/0.1 mM decavanadate containing 24 μM rat liver Hsp90. Curves are representatives of three experiments.

vanadate and tetravanadate peaks were relatively unchanged (Fig. 2B). Similar results were obtained when Hsp90 was added to separate oligovanadate or decavanadate solutions or when different concentrations of oligovanadate and decavanadate were examined in the absence and presence of Hsp90. In the presence of 5 mM $CaCl_2$ or $MgCl_2$ the $^{51}$V-NMR spectra also behaved similarly (data not shown). In one of the three experiments analyzing the effect of Hsp90 on the NMR spectrum of 0.1 mM oligovanadate/0.1 mM decavanadate; in addition to the large and characteristic decrease of the decavanadate triplet, a small decrease in the monovanadate and tetravanadate peaks could be detected. This phenomenon was probably caused by the fast interconversion of the different vanadate species [26] (data not shown).

**Binding of permolybdate to Hsp90.** Molybdate is generally used to stabilize Hsp90 · steroid-receptor complexes [6, 7, 27]. For a long time molybdate was thought to interact with the steroid-receptor component of the receptor · Hsp90 complex. However, the discovery that other Hsp90 complexes can be stabilized by molybdate [8] and our previous observations that molybdate induces a conformational change of Hsp90 in the absence of steroid receptors [9] raised the possibility of a direct molybdate-Hsp90 interaction. In our present experiments we have analyzed the binding of molybdate to Hsp90 by using the characteristic mobility shift observed after the addition of molybdate in the presence of hydrogen peroxide [19]. Under these conditions diperoxomolybdate is probably formed [28], which we call permolybdate throughout this paper.

Fig. 3A shows the upward shift in the position of the Hsp90 band after the simultaneous addition of molybdate and hydrogen

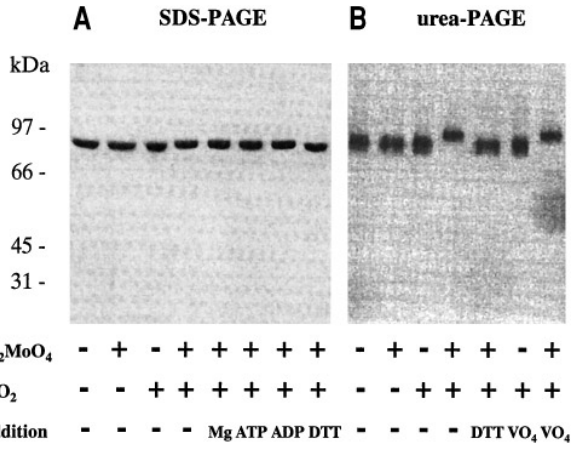


**Fig. 3. Mobility shift of Hsp90 after permolybdate treatment.** Permolybdate treatment of 1 μg rat liver Hsp90 was performed as described in Materials and Methods. $MgCl_2$ (Mg), ATP, ADP, dithiothreitol (DTT) and sodium oligovanadate ($VO_4$) were added to 5, 2.5, 2.5, 100 and 1 mM, respectively. (A) Permolybdate labeling analysed by SDS/PAGE; (B) permolybdate complex of Hsp90 analysed by urea gel electrophoresis. Gel pictures are representatives of three experiments.

peroxide, but not in the presence of either molybdate or hydrogen peroxide alone. The decreased mobility of the permolybdate adduct of Hsp90 is even more pronounced on urea/polyacrylamide gels (Fig. 3B). Permolybdate labeling gave a similar mobility shift when performed at 0 or 42°C instead of 37°C (data not shown).

$MgCl_2$, ATP or ADP did not influence the permolybdate-induced mobility shift of Hsp90 (Fig. 3A). On the contrary, dithiothreitol efficiently prevented the reaction of permolybdate with Hsp90, suggesting the involvement of free SH groups of the chaperone, in agreement with other permolybdate-labeling studies [19, 28, 29]. Pervanadate does not induce a mobility shift of Hsp90 and vanadate cannot compete with the permolybdate-induced shift of the chaperone (Fig. 3B). Higher concentrations (up to 10 mM) of oligovanadate or decavanadate were unable to prevent the permolybdate-induced mobility shift of Hsp90. 1 μM of the Hsp90-specific drug, geldanamycin was also ineffective as a competitor of permolybdate. SDS treatment of Hsp90 did not induce a further increase of the permolybdate-induced mobility shift of the protein (data not shown).

Characterization of the permolybdate-induced mobility shift of Hsp90 indicates that there is a relatively rapid increase up to 1 mM molybdate followed by a slower change in the mobility shift at higher molybdate concentrations (Fig. 4A). In contrast, relatively high concentrations of hydrogen peroxide (> 10 mM) are required for a maximal effect (Fig. 4B). A further increase in the hydrogen peroxide concentration (100 mM to 1 M) resulted in the reduction of the molybdate(VI) species to molybdate(III) (data not shown). Using 20 mM each of molybdate and hydrogen peroxide the reaction was complete within 20 min (Fig. 4C).

Tryptic digestion of permolybdate-labeled Hsp90 gave a different pattern from the fragment composition of unlabeled Hsp90 (Fig. 5). The most profound differences were found at fragments of 75 and 35−37 kDa, corresponding to the middle and C-terminal regions of Hsp90 [22]. Geldanamycin did not change the permolybdate-induced shifts in various tryptic fragments of Hsp90 (data not shown).

## DISCUSSION

SPR measurements indicate the binding of multiple vanadate species to Hsp90 with a stoichiometry higher than one to one.

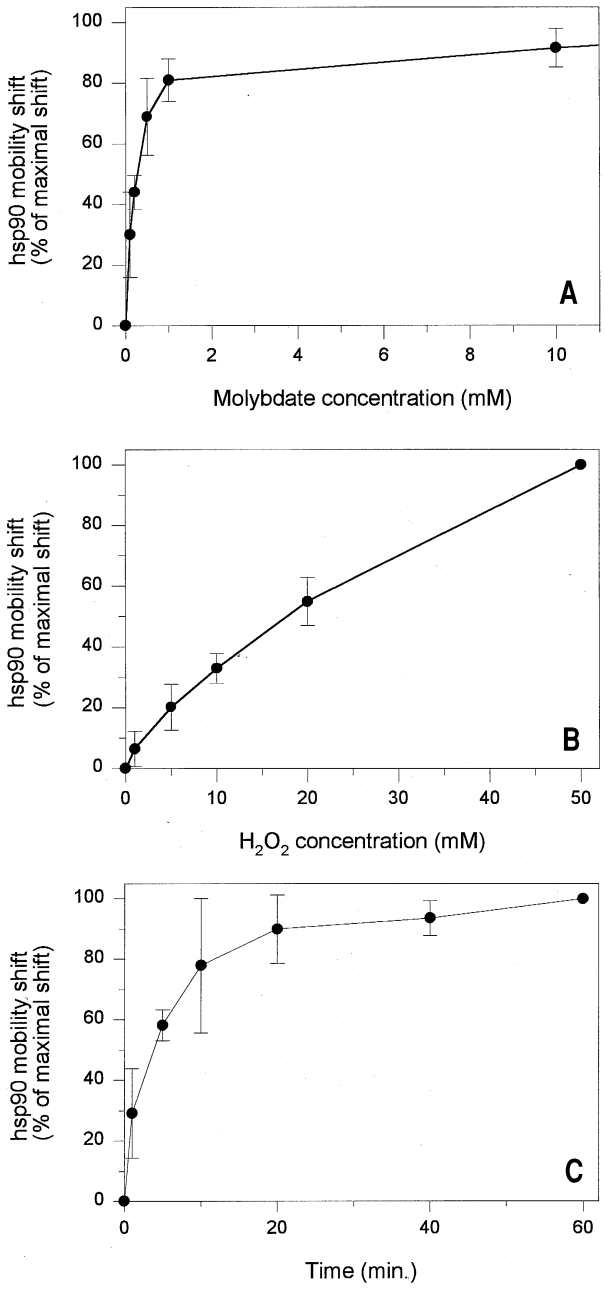


**Fig. 4. Characterization of permolybdate modification of Hsp90.** Permolybdate treatment of 1 μg rat liver Hsp90 has been performed as described in Materials and Methods. (A) Molybdate-concentration dependence of permolybdate labeling; (B) hydrogen-peroxide-concentration dependence of permolybdate labeling; (C) time dependence of permolybdate labeling. Data are means ± SD of three experiments and were normalized to the extent of mobility shift observed at 20 mM molybdate concentration (A), at 50 mM hydrogen peroxide concentration (B) and after incubation for 60 min (C).

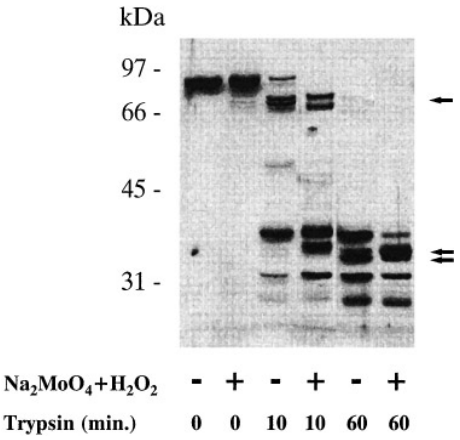


**Fig. 5. Tryptic fragments of permolybdate labeled Hsp90.** Permolybdate treatment of 5.4 μg rat liver Hsp90 and tryptic digestion of Hsp90 were performed as described in Materials and Methods. Arrows point to tryptic fragments most affected by permolybdate. The silver-stained gel is representative of three experiments.

Comparing the changes in the apparent dissociation constants (Fig. 2B and C) with the composition of the vanadate solutions at the respective vanadate monomer concentrations [16, 24] we can conclude that the affinity of vanadate-Hsp90 interactions decreases in the presence of mono-, deca- and di-/tetra-vanadate species. The relatively high affinity of monovanadate binding to Hsp90 is in good agreement with the high potency of vanadate to inhibit the binding of azido-ATP to Hsp90 [9], to induce a conformational change of the protein [9] and to inhibit the Hsp90-associated ATPase/GTPase activity [11]. From our present results it is likely that the monomeric vanadate species is responsible for most of the above effects.

Monovanadate is generally considered as a phosphate analog; therefore, one of its binding sites might be at the ADP/ATP-binding site of the Hsp90 N-terminus [12]. On the other hand, decavanadate is thought to bind to sites with multiple positive charges [25]. The highly charged middle region of Hsp90 contains several lysine/arginine clusters, best represented by a bipartite nuclear localization signal at amino acids 270–281 of the mouse Hsp90 homolog [5, 11]. This cluster and the adjacent positively charged amino acids might accomodate one or more decavanadate species.

The extreme line broadening of the decavanadate NMR signals accompanying the binding of decavanadate to protein species was reported for sarcoplasmic reticulum Ca-ATPase [16] and tubulin [25]. A major cause of this line broadening might be an accelerated exchange between bound and free forms of decavanadate, a phenomenon likely to occur at the observed low affinity binding of decavanadate to Hsp90. However, other possible explanations, such as the restricted mobility of the bound vanadium atoms and a distortion of the symmetry around the vanadate nucleus due to binding [26] may also contribute to the diminished decavanadate NMR signal in the presence of Hsp90.

The NMR peak at −558 ppm, corresponding to the monovanadate species, did not show major changes after the addition of Hsp90. This shows an apparent discrepancy between the SPR data, which suggests a binding of monovanadate to Hsp90 comparable to the binding of decavanadate under conditions of the NMR experiments (16% or 23% total vanadate monomers bound for monovanadate and decavanadate, respectively) and the NMR data, which show a small binding of monovanadate to Hsp90. However, the relatively unchanged monovanadate NMR signal might indicate a smaller exchange between bound and free forms of the tightly bound monovanadate and may arise from the presumably smaller distortion of the symmetry around the more compact monovanadate nucleus than that of the larger decavanadate species. An increased sensitivity of decavanadate for binding-induced distorsion also explain why the NMR peak at −572 ppm, corresponding to the tetravanadate species, changes slightly after Hsp90 addition despite a similar affinity of tetravanadate and decavanadate for Hsp90 (Fig. 1B, C). As an additional explanation, the presence of Hsp90 might induce an accelerated conversion of decavanadate to monovanadate and oligovanadates, resulting in an overestimation of the binding of

decavanadate and an underestimation of binding of monovanadate and oligovanadate species to Hsp90. However, this Hsp90-induced decavanadate hydrolysis might not be very dominant, since longer incubations of decavanadate solutions with Hsp90 do not induce a gradual shift from deca- to oligo- or monovanadates (data not shown).

The readily detectable shift in the mobility of Hsp90 after permolybdate labeling demonstrates the binding of this species to Hsp90. The major question of the permolybdate experiments is whether binding and covalent labeling of this species occurs at the molybdate-binding sites of Hsp90, or whether the SH-group-selective permolybdate labeling reflects the accessibility of the free SH groups of Hsp90.

The concentration dependence of permolybdate labeling of Hsp90 (Fig. 4) shows a good agreement with the hydrogen peroxide concentrations required to produce permolybdate [29]. It displays a molybdate-concentration dependence, indicating the presence of multiple high-affinity and low-affinity binding sites. The low-affinity sites are saturated at the relatively high concentrations of molybdate required to induce the stabilization of steroid-receptor · Hsp90 [6, 7, 27] and pp60v-Src · Hsp90 complexes [8]. This similarity in the characteristics of molybdate effects and permolybdate labeling would suggest a molybdate-speficic binding of permolybdate to Hsp90. However, the complete lack of the inhibition of permolybdate labeling by the analogous vanadate argues for a more general SH-group-specific interaction.

Permolybdate seems to exert the largest effect on tryptic fragments of 75 and 35−37 kDa, corresponding to the middle and C-terminal regions of Hsp90 (Fig. 5, [22]). However, two Hsp90 peptides of the middle/C-terminal region, YCVQQL and RLVTSPCCIVTSTYGWTANM, containing highly reactive cysteine residues based on prediction studies and on their ability to reduce cytochrome *c* and promote disulfide isomerization ([30], Nardai, G., Sass, B., Eber, J., Orosz, G., Schnaider, T., Sőti, C. and Csermely, P., unpublished results), did not prevent the permolybdate labeling of Hsp90 (data not shown). Similarly, SDS treatment of Hsp90 did not induce an increase in permolybdate labeling of Hsp90 despite the increased accessibility of SH groups after SDS, as judged by the Ellman method (data not shown). These observations imply that permolybdate labeling is not entirely dependent on the presence of reactive SH groups of Hsp90.

On the other hand, permolybdate labeling seems to be a fairly general phenomenon of SH-group-containing proteins, since permolybdate induced a mobility shift of the chaperones Hsp70 and p23 and reduced (but not oxidized) lactalbumin. BSA, which lacks free SH groups, was not labeled by permolybdate (data not shown). Permolybdate also reacts with the SH groups of steroid receptors [19] and with the catalytic SH group of phosphotyrosine phosphatases [29].

Summarizing our permolybdate experiments, the present data do not allow a clear distinction between the effects of permolybdate as an SH-group-interacting agent, and those of permolybdate as a molybdate analogue. Further different approaches are necessary to reveal the usefulness of permolybdate as a possible covalent label for molybdate-binding sites on various proteins.

The physiological concentrations of vanadate or molybdate are much lower than those applied in the present study. However, molybdate and vanadate are widely used as highly efficient stabilizers of Hsp90 · protein complexes, including the complexes of this chaperone with nuclear hormone receptors [31]. The effective concentrations of molybdate and vanadate are those we examined in the present report. Thus, our data provide information on the mechanism of action of molybdate and vanadate and contribute to the characterization of the biochemistry of Hsp90. In addition to molybdate and vanadate several low-molecular-mass endogenous factors were shown to act as stabilizers of Hsp90 · protein complexes. These include metal-like factors [8] and an ether aminophosphoglyceride [32]. Demonstration of vanadate and molybdate interactions with Hsp90 may provide clues to interactions of these endogenous stabilizers with the Hsp90-based chaperone machinery.

In conclusion the present studies provide evidence for the binding of vanadate to Hsp90 by means of SPR and $^{51}$V-NMR spectroscopy. Our data indicate a low specificity and a relatively low affinity of Hsp90 for various species of vanadate and molybdate, similar to the low specificity and low-affinity binding to the Hsp90 nucleotide-binding site(s) [10−14].

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