p23 is a co-chaperone for the heat shock protein, hsp90. This protein binds hsp90 and participates in the folding of a number of cell regulatory proteins, but its activities are still unclear. We have solved a crystal structure of human p23 lacking 35 residues at the COOH terminus. The structure reveals a disulfide-linked dimer with each subunit containing eight \( \beta \)-strands in a compact antiparallel \( \beta \)-sandwich fold. In solution, however, p23 is primarily monomeric and the dimer appears to be a minor component. Conserved residues are clustered on one face of the monomer and define a putative surface region and binding pocket for interaction(s) with hsp90 or protein substrates. p23 contains a COOH-terminal tail that is apparently less structured and is unresolved in the crystal structure. This tail is not needed for the binding of p23 to hsp90 or to complexes with the progesterone receptor. However, the tail is necessary for optimum active chaperoning of the progesterone receptor, as well as the passive chaperoning activity of p23 in assays measuring inhibition of heat-induced protein aggregation.

p23 is a ubiquitous, highly conserved protein which functions as a co-chaperone for the larger molecular chaperone, hsp90. These two proteins plus the chaperone, hsp70, and additional co-chaperones form a complex pathway for protein folding and processing in the eukaryote cell. p23 was first identified as an hsp90-binding protein (4–6) and is thought to modulate hsp90 activity during the last stages of the chaperoning pathway (7, 8, 15). However, p23 may also assist more directly in the chaperoning process since it can interact passively with denatured proteins to maintain them in a folding-competent state (16, 17). Thus, it may have dual roles to modulate the activity of hsp90 and to interact with unfolded protein substrates.

To better understand the chaperoning activities of p23, we have determined the 2.5-Å crystal structure of a human p23 fragment lacking 35 residues of a flexible COOH-terminal tail (hereafter referred to as p23-C35). The resolved structure contains the hsp90 binding surface of p23, but is deficient in passive chaperoning activity. The structure provides a starting point for understanding the passive (hsp90/ATP-independent) versus active (hsp90/ATP-dependent) chaperoning functions for this simplest of molecular chaperones.

EXPERIMENTAL PROCEDURES

Protein Preparation and Crystallization—All p23 forms were expressed in *Escherichia coli* BL21(DE3)pLysS and purified by chromatography on DEAE-cellulose and phenyl-Sepharose as described previously (8). Pooled fractions were dialyzed and concentrated by chromatography on DEAE-cellulose. Human hsp90b and hsp70 were expressed in Sf9 cells and purified as described previously (8). Hop and Ydj-1 were expressed in *E. coli* and purified as described previously (8). Crystals of p23-C35 were grown using the hanging drop vapor diffusion method. VDX crystallization plates (Hampton Research, Inc.) contained 1.0 ml of 1.9–2.1 M (NH\(_4\))\(_2\)SO\(_4\), 0.1 M Bicine, pH 9.0 (HCl), as the reservoir solution. Hanging drops typically contained 2 \( \mu \)l of reservoir solution and 2 \( \mu \)l of protein solution (about 20 mg/ml protein in 10 mM Tris-HCl, pH 7.5). Rod-shaped crystals grew to a useful size (typically, 0.4 mm \( \times \) 0.05 mm \( \times \) 0.05 mm) in 4–7 days. Notably, crystals did not form in the presence of dithiothreitol, and higher protein concentrations consistently yielded larger and fewer crystals per drop. All crystals used for x-ray studies were transferred into 2.45 \( \mu \)l (NH\(_4\))\(_2\)SO\(_4\), 0.1 M Bicine, pH 9.0 (HCl), 20% glycerol for 5–10 min prior to flash-cooling in a liquid \( N_2 \) stream or in liquid CHF\(_3\) for storage and transport. Initial x-ray characterization of these crystals using a sealed-tube source and image plate detector indicated a solvent content of \( (V_m = 2.6 \text{ dalon}^{-1}, 53\% \) solvent) most consistent with a dimer in the asymmetric unit of space group P4\(_2\)2\(_2\) or P4\(_2\)2\(_2\) with unit cell dimensions \( a = b = 61.8 \AA, c = 162.9 \AA\).

Structure Determination and Refinement—All x-ray data sets used in the structure determination were collected from flash-cooled (\( \sim \)180 °C)
native crystals or from derivatives preformed and stored in solidified CHF$_3$. All x-ray data sets were collected using a Quantum 4 CCD detector (Area Detector Systems Corp.) on the F$_1$ beamline at the Cornell High Energy Synchrotron Source (CHESS), Ithaca, NY. Although two native data sets were collected, these were found to be of lower quality than, and non-isomorphous to, any of the eight derivative data sets that were collected. Statistics for the crystal structure determination are summarized in Table I. The structure was solved using a (non-derivatized) IrCl$_3$-soaked crystal as the native which proved to be isomorphous to a K$_2$Pt(NO$_2$)$_4$ derivative to high resolution (2.5 Å) with good phasing power. A KAuCl$_4$ derivative also was found to have useful phasing.

The enzyme was prepared at 0.15 M HEPES, pH 7.5, with or without the addition of 0.75 M p23 or p23 deletion mutants. Aggregation induced by incubation at 43 °C was monitored by measuring the optical density at 390 nm.

**Progestosterone Receptor Complex Assembly**—Progestosterone receptor (PR) from chick oviduct cytosol was adsorbed onto PR22 antibody-protein A-Sepharose and was assembled into complexes as described by Kosano et al. (8). The incubation contained 0.05 mM progesterone and protein composition.

**Analytical Centrifugation**—Sedimentation equilibrium was performed in the Beckman Prep-Scanner Analytical Ultracentrifuge using double sector cells in an (AnH) rotor at 17 °C. The samples were centrifuged at 16,000, 18,000, and 30,000 rpm and analyzed after 12 h at each speed. The p23 protein was dialyzed exhaustively in buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl$_2$, 50 mM KCl) prior to analysis. For the reduced samples, dithiothreitol was added to a final concentration of 4 mM. The enzyme was prepared at 0.15 M HEPES, pH 7.5, with or without the addition of 0.75 M p23 or p23 deletion mutants. Aggregation induced by incubation at 43 °C was monitored by measuring the optical density at 390 nm.

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Structure and Activity of p23

TABLE I

|                  | IrCl₃ (native) | K₃Pt(NO₃)₄ | K₅AuCl₄ |
|------------------|---------------|------------|---------|
| Wavelength (Å)   | 0.9159        | 0.9159     | 0.9159  |
| Resolution range (Å) | 15–2.49 (2.62–2.49) | 15–2.08 (2.19–2.08) | 15–2.95 (3.11–2.95) |
| Total reflections | 97860 (11297) | 210538 (8985) | 26897 (3305) |
| Unique reflections | 11743 (1651)  | 18587 (1968) | 6929 (848)  |
| Signal strength, (IO/Io) | 7.7 (8.7)    | 4.6 (5.7)  | 4.3 (1.8)  |
| Redundancy       | 5.3 (6.6)     | 11.3 (4.6) | 3.9 (3.9)  |
| Completeness (%) | 99.4 (98.8)   | 94.1 (70.9) | 95.1 (82.6) |
| Rmerge            | 0.050 (0.074) | 0.084 (0.113) | 0.116 (0.328) |

* Values in parentheses are for the highest resolution shell.

** Bond lengths and angles are root-mean-square deviations from ideal values.

Rmerge = \( \sum_i \sum_j S_{ij} ||F_{ij,\text{calc}}| - |F_{ij,\text{obs}}|| / \sum_i \sum_j S_{ij} ||F_{ij,\text{obs}}|| \), where the outer sum \((n)\) is taken over the unique reflections, the inner sum \((i)\) is taken over the set of independent observations of each unique reflection, and \(S_{ij}\) is the mean value of \(S_{ij}\) for all \(i\) measurements.

Rfree = \( \sum_i S_{ij} ||F_{ij,\text{calc}}| - |F_{ij,\text{obs}}|| / \sum_i S_{ij} ||F_{ij,\text{obs}}|| \), where \(i\) is a test reflection and the numerator is the root-mean-square difference, the denominator is the root-mean-square lack of closure for the derivative and \(F_{ij,\text{calc}} = F_{ij}\) when \(F_{ij,\text{calc}}\) and \(F_{ij}\) are the calculated protein and heavy-atom structure factors, respectively.

Rfree = \( \sum_i S_{ij} |F_{ij,\text{calc}}| - |F_{ij,\text{obs}}|| / \sum_i S_{ij} |F_{ij,\text{obs}}|| \), where the outer sum \((n)\) is taken over the set of \(n\) reflections that are not used in refinement calculations.

\( \Delta \bar{B} \) for mainchain and sidechain atoms (Å²) = 3.16/4.89

FIG. 2. p23-C35 dimer structure and topology. The core of the monomer folding domain is comprised of two opposing \(\beta\)-strands. The larger sheet is formed by \(\beta\)-strands 8, 1, 2, 7, and 6 on the near surface of the right-most molecule (from left to right). The second sheet is formed by \(\beta\)-strands 3, 4, and 5 on the far surface of the same molecule (from right to left). Turn elements are colored magenta and the two residues colored orange (Leu\(^{55}\) and Thr\(^{90}\)) are isolated hydrogen-bonded bridging residues. The Cys\(^{58}\)-Cys\(^{38}\) intermolecular disulfide is shown in green. The NCS axis relating the two chains of the dimer runs through the dimer interface approximately in the figure plane. The program DSSP (38) was used to assign secondary structural elements. The figure was generated using the program RIBBONS (39).

RESULTS

To gain some perspective on structural features that relate to p23 activities, we have mapped the regions of highest conservation in p23. Fig. 1 illustrates a sequence alignment of human p23 with six sequences representing the existing family of p23 proteins in the GenBank data base. These include p23 from chicken (4), *Saccharomyces cerevisiae* (12–14), and *Schizosaccharomyces pombe* (28) which have been shown to interact with hsp90. A p23-related protein was identified from data base analysis of *Caenorhabditis elegans* but this has not been studied. *tsp23* is a related human gene of unknown function (29), and B-ind1 is a mouse protein of unknown function. Only the most highly conserved residues present in at least five members are shaded. These p23 homologues are notably similar in the region of residues 86–108 (human p23 numbering). There is considerable divergence in the COOH-terminal sequences, but a common characteristic is the high proportion of acidic residues.

Initial attempts to crystallize full-length p23 failed, but well diffracting crystals were produced after removal of 35 residues from the very acidic COOH terminus (Table I). p23-C35 crystallizes as a dimer; each monomer consists of eight \(\beta\)-strands within a compact globular \(\beta\)-sandwich structure comprised of two opposing sheets (Fig. 2). The two molecules of the dimer are related by an approximate 2-fold NCS axis and are covalently linked through an intermolecular disulfide bond. The monomer topology resembles that of the immunoglobulin Fc or light chain fold, but differs in the spatial arrangement of the constituent \(\beta\)-strands. The larger of the two opposing \(\beta\)-sheets is formed by \(\beta\)-strands 8, 1, 2, 7, and 6 while \(\beta\)-strands 3, 4, and 5 form the smaller (see Fig. 1 for numbering). The two sheets pack together with alternating hydrophobic side chains on each \(\beta\)-strand contributing to a tightly packed hydrophobic core centered on a ring stacking interaction between Phe\(^{19}\) and Phe\(^{38}\) (Fig. 3). The amino terminus is contained within the five-stranded sheet and the carboxyl terminus of the resolved
structure ends at residue Glu^{110} near the dimer interface. An extensive, but well structured loop from Gly^{80} to Glu^{110} contains the most highly conserved region of the molecule. The last 15 amino acids of p23-C35 (i.e. residues 111–125) are apparently unstructured and were not observed in the electron density.

The dimer interface buries 556 Å² of surface area per monomer or about 9% of the solvent accessible surface of the dimer.
While the intermolecular disulfide linkage between the Cys58 side chains is the most obvious feature of dimer association (Fig. 4), hydrogen bonding, and electrostatic interactions confer additional stabilization. Electrostatic interactions between Glu82, Arg88, and Arg93 side chains are particularly extensive and only partly conform to the NCS of the dimer; Arg93 clearly shows different density in the two molecules of the dimer and therefore was not restrained by NCS during refinement. However, only three of the 11 residues at the dimer interface are conserved (Arg88, Lys91, and Glu92) and notably, Cys58 is not among these. Indeed, intermolecular disulfide bond formation may be an artifact induced by crystallization at pH 9.0. This conclusion is supported by the observation that addition of reducing agents inhibits crystal formation. Lys33 in the dimer interface is also not conserved. In contrast, Arg88 and Glu92 are strictly conserved in all but two sequences where the latter is conservatively replaced by Asn or Gln, suggesting that this salt-bridge is a key element of dimer stabilization. Lys91 is also strictly conserved except in one instance where it is replaced by Gln which would still allow for a hydrogen bonding interaction.

To test whether p23 exists as a dimer in solution we analyzed p23 by analytical ultracentrifugation. Full-length p23 was centrifuged under conditions similar to those used for activity assays (10 mM Tris, 50 mM KCl, 5 mM MgCl2, pH 7.5), with or without 1 mM dithiothreitol (Table II). This revealed a molecular mass range of between 19,000 and 23,000 under all conditions, which is very close to the calculated mass of 18,697 for p23 monomer. To confirm that the disulfide formed by Cys58 is not essential, we have prepared the C58K mutant where Cys58 is replaced by lysine. This mutant is fully active in the functional assays described below (results not shown). Thus, at least in vitro, p23 exists mainly as a monomer in solution and the disulfide-linked dimer observed in the crystal structure would appear to be either a minor form of the protein or an artifact of crystallization.

The locations of highly conserved residues in the p23 monomers are indicated in Fig. 5. Of the first 110 residues of human p23, 27 are conserved in at least four other sequences (see Fig. 1). Of these, 15 are relatively buried (and mostly hydrophobic) residues while Gly43 allows a β-hairpin between β-strands 4 and 5. Of the remaining 11 residues, Pro61 is the only prominent conserved side chain on one face of the monomer (Fig. 5b). In contrast, the opposite face of the molecule (Fig. 5a) presents an array of conserved residues which may define a surface region important for p23 binding to hsp90 or for its passive chaperoning activity. On this surface, the exposed aromatic side chains of Phe103 and Trp106 are particularly notable. In addition, there is a solvent-accessible cavity surrounded by conserved residues (Lys95, Thr90, Arg88, Pro87, Trp8, and Phe103), the floor of which is further defined by conserved asparagine residues (Leu89 and Leu99). The electrostatic potential surface of the “conserved” face of the monomer (Fig. 5c) shows a pronounced separation of charge on either side of the putative protein-binding region and highlights the depth of the apolar cavity. The opposite face (Fig. 5d) shows no such features. Thus, the most conserved block of amino acids, residues 86–92 (WPRLTKE), which might be considered a signature for this family of proteins, largely defines the solvent-accessible surface of a cavity having polar side chains for walls and an apolar floor. This pocket could easily accommodate a side chain from a protein ligand.

To gain understanding on structure-function relationships, we have compared activities of full-length p23 with those of the crystallized protein (p23-C35) and with p23-C50. The latter construct was produced to correspond with the observed crystal structure (residues 1–110). Fig. 6 illustrates the binding of these proteins to hsp90. Hsp90 was incubated at 30°C in the presence or absence of ATP to convert it to the ATP-dependent conformation. It was then incubated with p23, p23-C35, or p23-C50 and complexes were assessed by co-immunoprecipitation and gel electrophoresis. All three proteins bind hsp90, and this interaction is dependent upon the conversion of hsp90 to its ATP-bound state as shown previously (15, 30). We have also tested the passive chaperoning activity of these three proteins through their ability to prevent aggregation of denatured citrate synthase (17). At 43°C, citrate synthase denatures and its aggregation can be measured by light scattering. Under the conditions employed, aggregation is greatly reduced by a 5-fold molar excess of p23 (Fig. 7). p23-C35 has about one-half the activity of full-length p23 while p23-C50, which was also tested at higher concentrations, is inactive in this assay. Thus, the folding domain which is resolved in the crystal structure is sufficient for binding to hsp90, but the unstructured COOH-terminal tail appears to be required for the passive chaperoning activity of p23.

Finally, we tested the three p23 constructs for their ability to interact in chaperoning complexes with the progesterone receptor (PR) and to increase the proportion of PR that is capable of binding hormone. A cell-free system with purified proteins was used for this purpose (8). This chaperoning process requires the cooperative actions of five proteins: hsp70, hsp40 (Ydj-1), Hop, hsp90, and p23. Forms A and B of the PR from chick oviduct cytosol were isolated on antibody resin and incubated under conditions optimal for chaperone complex formation. Following incubation, the samples were chilled and incubated with [3H]progesterone for 4 h at 4°C. The resin-bound PR complexes were then isolated and analyzed for protein composition (Fig. 8a) and for bound hormone (Fig. 8b). While some hsp90 complex was formed in the absence of p23 (Fig. 8a, No p23), the amount of hsp90 binding was enhanced considerably by the presence of p23 (p23 WT). p23-C35 and p23-C50 also enhanced hsp90 binding to PR (p23 C35 and p23 C50). However, these three p23 proteins differed in their effects on the hormone binding activity of PR complexes. When compared at three protein concentrations (Fig. 8b), p23-C35 was as effective as full-length p23 and increased the hormone binding activity of PR by about 2-fold. However, p23-C50 showed only a slight increase compared with samples lacking p23. These results show that the enhancement of PR activity promoted by p23 can be separated into two aspects: the binding of p23 to the complex, presumably by binding hsp90, and an influence of p23, directly or indirectly, on PR structure, which is facilitated by the COOH-terminal tail region.

**DISCUSSION**

The 2.5-Å crystal structure of a p23 mutant lacking 35 COOH-terminal residues reveals a disulfide-linked dimer; each monomer contains a compact folding domain in the N-terminal 80 residues of the sequence. The COOH-terminal 30 residues in the resolved crystal structure contain a highly

**TABLE II**

| Velocity | p23 WT | p23 WT + 1.0 mM DTT |
|----------|--------|---------------------|
| rpm      |        |                     |
| 18,000   | 22,785 | 885                 |
| 30,000   | 19,562 | 1083                |

.* * *
conserved peptide sequence, residues 86–92 (WPRLTKE), which is essentially a signature for this family of proteins. This region largely defines the solvent-accessible surface of a cavity having polar side chains for walls and an apolar floor which, we speculate, may accommodate a side chain from its binding partner, hsp90, or from other passively chaperoned substrates.

FIG. 5. Asymmetric distribution of evolutionarily conserved residues (a, b) and corresponding electrostatic potential surface (c, d) of the p23-C35 monomer. The two views are related by a 180° rotation about a vertical axis through the molecule. Several of the conserved hydrophobic residues (Val, Leu, Phe, and Ile) are relatively buried in the protein interior and are probably important for protein stability. The remaining conserved apolar residues Ile, Leu, and Leu define the floor of a solvent-accessible cavity on the conserved face of the monomer (a and c), while P61 resides on the non-conserved face (b and d). Polar or charged residues Trp, Pro, Arg, Thr, Lys, Glu, Phe, Trp, and Asp comprise the walls of the cavity and a surface region potentially involved in binding to hsp90 or chaperoned substrates. In a and b, side chains conserved in at least five of seven p23 sequences (see Fig. 1) are colored according to residue type: Lys and Arg side chains are blue; Asp and Glu side chains are red; hydrophobic side chains (Ala, Val, Leu, Ile, Phe, Pro, and Met) are magenta; and polar side chains (Ser, Thr, His, Cys, Asn, Gln, Trp, and Tyr) are yellow. The (non-conserved) Cys sulfur atom shown in green marks the dimer interface. In c and d, blue and red colors correspond to positive and negative potential, respectively; a fully saturated color indicates a potential of ±10 kT (where k = Boltzmann constant and T = temperature. The calculation assumes a salt concentration of 0.1 M. The program GRASP (40) was used to calculate and display the images.
While p23 can form a dimer, the prevalent form in solution is monomeric. The only other passive chaperones for which structural information is available are the small heat shock protein from *Methanococcus jannaschii*, MjHSP16.5 (31), and PapD (32, 33) and FimC (34) from *E. coli.* None of these proteins are related to p23 by sequence comparison. The highly compact \(\beta\)-sandwich folding domain of the p23 monomer is reminiscent of the well known immunoglobulin fold, but differs in \(\beta\)-strand topology. A search for three-dimensional structural homologues using the Dali web server (35) reveals a core structure that is remarkably similar to that of the MjHSP16.5, but lacking structural elaborations that enable the latter to self-assemble into a large hollow spherical oligomer composed of 24 subunits. Fig. 9 shows a least-squares superposition of p23-C35 onto the MjHSP16.5 structure; 73 carbon atoms are aligned in the two core structures with a root mean square deviation of 1.27 Å. MjHSP16.5 has an extensive loop insertion (residues 84–102) and a COOH-terminal peptide (residues 137–147) which are used for dimerization and trimerization of dimers, respectively, to construct the oligomer. In contrast, p23-C35 lacks the dimerization loop, and its COOH-terminal extension from the core folding domain contains a well structured loop that defines a putative hsp90 or protein substrate-binding surface. Whether the striking similarity in the core structure of these two proteins relates to a common chaperone function remains to be investigated.

The actual functions of p23 remain unclear. Most studies have viewed p23 as a co-chaperone that in some way facilitates the activity of hsp90. However, p23 may also interact with some protein substrates directly. Yeast two-hybrid studies have shown an interaction of p23 with cytosine 5-methyltrans-
ferase (36) and with telomerase, a protein that is also chaperoned by hsp90 (10). The hepadnavirus reverse transcriptase requires chaperoning by hsp90-p23 and interaction of p23 in this complex has been shown to persist after the removal of hsp90 (37). Finally, the ability of p23 to prevent the aggregation of denatured citrate synthase (17) and β-galactosidase (16) supports a direct interaction of p23 with protein substrates. There is no evidence for a direct interaction of p23 with steroid receptors although this remains a possibility. It is equally possible that p23 stabilizes or chaperones a conformational state of hsp90 that is needed to achieve the final active form of the receptor. These two possibilities are not mutually exclusive.

The present study reveals that the ability of p23 to bind hsp90 and to prevent aggregation of denatured citrate synthase are separable activities. p23-C35 is fully able to bind hsp90, but lacks the ability to chaperone protein substrates. p23-C50 behaves similarly to intact p23 in facilitating PR reconstitution, but is only 50% active in preventing citrate synthase aggregation. Whether the COOH-terminal tail participates directly in chaperoning or is needed indirectly remains unknown. The lack of conservation in the COOH-terminal tail argues in favor of an indirect role. For example, the very hydrophilic tail may be needed to maintain solubility of complexes with denatured proteins or it may mask a hydrophobic region when p23 is free in solution. With structural information now at hand, mutational studies are needed to address these possibilities and to identify other critical surface features required for p23 activity. At present, p23 is the simplest known form of a molecular chaperone and it is now possible to intelligently address features of this protein that contribute to its activity and to study the structural basis for its action.

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Addendum—During the submission of this article, Weikl et al. (41) have presented a theoretical model of p23 structure based upon biochemical studies. Their model is qualitatively similar to our x-ray structure and biochemical data showing an unstructured COOH-terminal region needed for chaperoning, but not for hsp90 binding.

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