Selective Inactivation of Parvulin-Like Peptidyl-Prolyl *cis/trans* Isomerases by Juglone†

Lars Hennig,‡ Claudia Christner,§ Marc Kipping,§ Birte Schelbert,§ Karl Peter Rücknagel,§ Susanne Grabley,▽ Gerd Küllertz,§ and Gunter Fischer§,*

Max-Planck Research Unit, Enzymology of Protein Folding, Kurt-Mothes-Str. 3, D-06120 Halle/Saale, Germany, Institute of Biology II, Albert-Ludwigs-University Freiburg, Schänzlestr. 1, D-79104 Freiburg i. Br., Germany, and Hans-Knöll-Institute of Natural Products Research e.V., Beutenbergstr. 11, D-07745 Jena, Germany

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ABSTRACT: In contrast to FK506 binding proteins and cyclophilins, the parvulin family of peptidyl-prolyl *cis/trans* isomerases (PPIases; E.C. 5.2.1.8) cannot be inhibited by either FK506 or cyclosporin A. We have found that juglone, 5-hydroxy-1,4-naphthoquinone, irreversibly inhibits the enzymatic activity of several parvulins, like the *E. coli* parvulin, the yeast Ess1/Ptf1, and human Pin1, in a specific manner, thus allowing selective inactivation of these enzymes in the presence of other PPIases. The mode of action was studied by analyzing the inactivation kinetics and the nature of products of the reaction of *E. coli* parvulin and its Cys69Ala variant with juglone. For all parvulins investigated, complete inactivation was obtained by a slow process that is characterized by pseudo-first-order rate constants in the range of 5.3×10^{-4} to 4.5×10^{-3} s^{-1}. The inactivated parvulin contains two juglone molecules that are covalently bound to the side chains of Cys41 and Cys69 because of a Michael addition of the thiol groups to juglone. Redox reactions did not contribute to the inactivation process. Because thiol group modification was shown to proceed 5-fold faster than the rate of enzyme inactivation, it was considered as a necessary but not sufficient condition for inactivation. When measured by far-UV circular dichroism (CD), the rate of structural alterations following thiol group modification parallels exactly the rate of inactivation. Thus, partial unfolding of the active site of the parvulins was thought to be the cause of the deterioration of PPIase activity.

Peptidyl-prolyl *cis/trans* isomerases (PPIases; E. C. 5.2.1.8) are enzymes that accelerate the *cis/trans* isomerization of peptide bonds preceding prolyl residues (*1, 2*). They catalyze rate-limiting steps in the in vitro and in vivo folding of proteins (*3*−*7*). Currently, the enzyme class of PPIases can be subdivided into three families according to amino acid sequence homology and to the characteristics of inhibition by microbial drugs. Two of these families were detected as primary targets of immunosuppressive compounds: the cyclophilins, which are reversibly inhibited by cyclosporin A (CsA), whereas FK506, rapamycin, and many other α-dicarbonyl amides bind to and inhibit most of the FKBPs.[1] Because there is no cross inhibition between both enzyme families, members can be differentiated by applying specific inhibitors.

To date, no inhibitor has been described for the parvulins, the most recently discovered third family of PPIases (*8*−*10*). Beside the prototypical enzyme, the 10.1 kDa parvulin of *Escherichia coli*, a number of prokaryotic and eukaryotic proteins possessing the parvulin domain have been identified by database searches (*10*, *11*).

Although there is a growing number of observations of PPIases participating in intracellular signal transduction (*12, 13*), no individual member of the cyclophilins and FKBPs has been proven to be required for cell viability. In contrast, some of the parvulins are suggested to serve nonredundant biological functions. As an example, the human nuclear parvulin Pin1 (*14, 15*) was reported to be essential for the regulation of mitosis by interacting with the NIMA kinase pathway, because molecules with side-chain phosphorylated −Ser(Thr)-Pro- moieties were shown to represent specific substrates for this enzyme (*16*). The yeast Ess1/Ptf1 is structurally and functionally related to Pin1 (*11, 17*). Its genetic inactivation results in growth arrest that is recognizable by a multibudded phenotype. In addition, a number of known deletion mutants of bacterial parvulins, which do not share −Ser(Thr)PO_3H_2−Pro- specificity with eukaryotic parvulins, show perturbations of protein secretion or periplasmic protein folding (*10, 18*).

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* Corresponding author. Phone: +49345 5522801. Fax: +49345 5511972.

‡ Institute of Biology II.

§ Max-Planck Research Unit Enzymology of Protein Folding.

▽ Hans-Knöll Institute of Natural Products Research.

[1] Abbreviations: DMSO, dimethyl sulfoxide; FKBPs, FK506 binding proteins; IPTG, isopropyl β-D-thiogalactopyranoside; NH−Np, 4-nitroanilide; PPIase, peptidyl-prolyl *cis/trans* isomerase; rhCyp18, recombinant human cytosolic cyclophilin with a molecular mass of 18 kDa; TFA, trifluoroacetic acid; DTT, dithiothreitol; DTPA, diethylenetriame-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; DTNB, 5, 5′-dithiobis(2-nitrobenzoic acid); ROS, reactive oxygen species; MALDI−PSD-MS, MALDI post-source decay MS; GSH, reduced glutathione; GdnHCl, guanidine hydrochloride.

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In addition to gene disruption experiments, selective inhibitors may provide valuable tools to further elucidate the physiological function and enzymatic mechanism of the parvulins. Taking into account the pharmaceutical importance of the known PPIase inhibitors CsA and FK506, we performed a screening of effectors of the enzyme activity of *E. coli* parvulin. To identify lead structures, a collection of pure secondary metabolites was tested. As a result, the natural compound juglone was found to specifically inactivate PPIase activity of the parvulin from *E. coli*, the homologous PPIase Ptf1/Ess1 from yeast, and the human parvulin Pin1. Juglone (5-hydroxy-1,4-naphthoquinone) is a brown dye isolated from fruit shells and leaves of walnut trees (*Juglans*) (*19, 20*).

The finding that juglone is able to discriminate between different PPIase families encouraged us to investigate the mechanism of parvulin inactivation in detail. Pointing to the possible targeting and subsequent covalent modification of cysteine residues by juglone, the enzymatically active parvulin variant C69A was included in these studies.

EXPERIMENTAL PROCEDURES

Materials. Juglone, DTNB, and DTPA were purchased from Aldrich (Steinheim, Germany). Acetonitrile, DTT, and TFA were products of ROTH (Karlsruhe, Germany). DMSO, ethanol, and methanol were obtained from MERCK (Darmstadt, Germany). All solvents were of HPLC grade purity. The peptide Suc-Ala-Phe-Pro-Phe-NH−Np was purchased from Bachem (Heidelberg, Germany), and Suc-Ala-Glu-Pro-Phe-NH−Np was synthesized by Dr. M. Schutkowski. FK506 was a gift from Dr. A. Lawen, and cyclosporin A was kindly provided by AWD (Dresden, Germany).

Prime Zyme DNA polymerase was obtained from Biometra (Göttingen, Germany), TPCK-trypsin from Worthington (Freehold, NJ, USA), and pancreatic α-chymotrypsin from MERCK (Darmstadt, Germany). Thrombin and subtilisin were purchased from Sigma (St. Louis, MO). Human Cyp18 was produced recombinantly in *E. coli*. Ptf1/Ess1 (recombinant from *E. coli*) was kindly provided by Dr. J. Rahfeld. The overexpression clone of human Pin1 was constructed using a plasmid of Kun Ping Lu (Boston). The purified recombinant Pin1 was kindly provided by S. Hottenrott, human FKBP12 (recombinant from *E. coli*) by T. Zarnt, *Legionella pneumophila* FKBP25 (Mip protein) (recombinant from *E. coli*) by B. Schmidt, and authentic *E. coli* trigger factor by G. Stoller.

Methods. Molecular Cloning Techniques. All plasmids were constructed by standard recombinant DNA techniques (*21*) and their authenticity was confirmed by DNA sequencing (*22*) using T7 Sequencing Kit of Pharmacia (Freiburg, Germany).

Escherichia coli DH5α was used for plasmid construction and nucleotide sequencing. *Escherichia coli* M15 [pREP4] was purchased from Qiagen (Hilden, Germany) and used as host for the overexpression of mutated and recombinant parvulin. pSEP38 (*9*) encoding the gene of parvulin (*par*A) on a 1134 bp insert was used as template to amplify a 535 bp fragment with the primers EP1 (5′-ACGACCATGGCAAAAAACAGCAGC-3′) and EP2 (5′-GAGCTGGATCCGGGCGACCTGG-3′) by polymerase chain reaction (PCR). EP1 includes the 5′-sequence of *par*A and a recognition site for *Nco*I, whereas EP2 contains the sequence ∼200 bp downstream of the gene and a site for *Bam*HI. The PCR product was subcloned into pQE60 (Qiagen) using *Bam*HI and *Nco*I, thus giving rise to pSEP612. The following four primers were necessary to construct directed point mutations in *par*A: MP1: 5′-GCAGGATCCGATGACGATGACAAAGCAAAAACAGCAGCAGCAC-3′; MP2: 5′-CTAAGCGTGCCATGCGATTGAGCAG-3′, MP3: 5′-CGGGCGAGCTCGGTAAAGCTA-3′; and MP69: 5′-AAAGTGGTTTTCTCTGCTCCGGTACTGGAGCC-3′.

The sequence of MP1 is based on the first six amino acids of *par*A and encodes an additional cleavage site for enterokinase and a recognition site for restriction endonuclease *Bam*HI. The primer MP2 complementary to the region ∼160 bp downstream of *par*A contains eight mismatched nucleotides at its 3′ end. The Primer MP3, containing a recognition site for restriction endonuclease *Sac*I, is derived from the sequence ∼240 bp downstream of *par*A. The primer MP69 carries the nucleotide sequence for the desired point mutation C69A. The PCR was performed according to Mikaelian & Sergeant (*23*). Plasmid pPSM69 was constructed by inserting the modified *par*A DNA as a 532 bp *Bam*HI/*Sac*I fragment into pUC18. The following subcloning of the *par*A mutated in position 69 into pQE30 (Qiagen) resulted in expression plasmid pQSM69. Consequently, overexpressed protein of the parvulin mutant C69A carried a $(His)_6$-tag, whereas the wild-type protein did not.

Protein Expression and Purification. The *E. coli* K-12 strain DH5α carrying the appropriate plasmid was grown at 37 °C to late stationary phase in YT-broth (1.6% bactotryptone, 1% yeast extract, and 0.5% NaCl) containing ampicillin (100 μg/mL) and kanamycin (25 μg/mL). IPTG (1 mM) was used to induce overexpression of the lacZ-promoted parvulin. The purification of the overexpressed wild type protein has been described previously (*9*). To purify the mutant parvulin C69A, cells were harvested by centrifugation (6000 × *g*, 4 °C, 10 min). The pellet was resuspended in 35 mM HEPES (pH 7.8) and cells were disrupted in a SLM Aminco FRENCH pressure cell with 20 000 psi of pressure followed by stirring for 30 min at 25 °C with 0.1% (v/v) benzonase. After centrifugation for 40 min in a Beckman L8 60M ultracentrifuge (4 °C, 20 000 × *g*), the pH of the supernatant was adjusted to 8.0. The protein solution was passed through a Ni−NTA column, equilibrated with 50 mM phosphate buffer (pH 8.0) containing 200 mM sodium chloride (buffer A), and washed with 50 mM phosphate buffer (pH 6.0) containing 300 mM sodium chloride and 10% (v/v) glycerin. Bound protein was eluted with 0.5 mL of imidazole in buffer A. Fractions containing parvulin C69A as judged by SDS−PAGE were pooled, concentrated on a Filtron OMEGACELL (5000 Da), and applied to a HiLoad 16/60 Superdex 75 gel filtration column equilibrated with 10 mM HEPES (pH 7.8), containing 150 mM potassium chloride, 1.5 mM magnesium chloride and 0.5 mM DTT. Fractions that contained protein purified to homogeneity according to silver-stained SDS-polyacrylamide gels were pooled, dialyzed against 35 mM HEPES (pH 7.8), and stored at −80 °C until use.

The identity of purified proteins was routinely checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) (*24*) followed by silver staining and analysis with matrix-assisted laser desorption time-of-flight mass

spectrometry (MALDI-TOF-MS). The protein concentration was determined by the method of Gill & Hippel (*25*).

The *E. coli* parvulin variant C69A displayed a catalytic activity of 44% of the wild type protein when assayed with Suc-AFPF−NH−Np as the substrate in the protease-coupled assay.

PPIase Assay and Inactivation Studies. Parvulin activity was measured according to the method of Janowski et al. (*26*) to avoid unwanted cleavage of the protein. For the assay, a 60 mM substrate solution of Suc-AFPF−NH−Np for *E. coli* parvulin and Suc-AEPF−NH−Np for Ptf1/Ess1 and Pin1 in 0.5 M LiCl/trifluoroethanol was used, thereby ensuring a proportion (∼50%) of peptide molecules in *cis*-conformation. The kinetics of the decrease of *cis* content to ∼10% upon substrate dilution into aqueous buffer was determined by the change in absorbance at 330 nm in a Hewlett-Packard 8452A diode array UV−vis spectrophotometer. The assays were performed at final concentrations of 8.5 nM *E. coli* parvulin and *E. coli* parvulin variant C69A, 12.0 nM Pin1, and 0.1 μM Ptf1/Ess1, respectively, and 120 μM peptide in 0.035 M HEPES and 4 μM BSA (pH 7.8) at 10 °C. For inhibition experiments, the enzymes were incubated at 10 °C, usually with a 10-fold molar excess of juglone. Activity measurements of other PPIases were made by the protease-coupled assay developed by Fischer et al. (*27*) with α-chymotrypsin (final concentration 830 μg/mL) as isomer-specific protease and the peptide Suc-AFPF−NH−Np (50 μg/mL) as substrate. The test was performed by observing the released 4-nitroaniline at 390 nm with a Hewlett-Packard 8452A diode array UV−vis spectrophotometer at 10 °C. The total reaction volume was adjusted to 1200 μL by mixing appropriate amounts of 35 mM HEPES (pH 7.8) with enzyme and effector solutions diluted with the same buffer. The reactions were started by addition of 2−3 μL of a peptide stock solution (10 mg/mL in DMSO). Juglone was freshly diluted from a 6 mM stock solution in methanol, FK506 from a 12.2 mM stock solution in ethanol, and CsA from a 5 mM stock solution in 50% methanol and 50% ethanol. The amount of organic solvent was kept constant within each experiment, usually <0.1% (v/v). Inhibitors were incubated for the specified times with the enzymes at 10 °C in 35 mM HEPES (pH 7.8) prior to the determination of PPIase activity. Routine controls were conducted to exclude possible effects of any additives on the rate of the uncatalyzed *cis/trans* isomerization or on the activity of the used protease.

The pseudo-first-order rate constant k_{obs} for *cis/trans* isomerization in the presence of PPIase and the first-order rate constant k_o of the uncatalyzed *cis/trans* isomerization were used to determine the specificity constant k_{cat}/K_m from the equation $k_{cat}/K_m = (k_{obs} - k_o)/[\text{PPIase}]$ (*28*). Pseudo-first-order rate constants *k* for the parvulin inactivation by excess juglone were calculated by fitting the data to the integrated equation of a pseudo-first-order reaction. Under the condition of approximately equal concentrations of enzyme and inhibitor, second-order rate constants were determined by applying the integrated equation of a bimolecular reaction. Inactivation data of the *E. coli* parvulin variant C69A obtained under five different subsaturating conditions (8.3−64 nM) were analyzed according to the Kitz−Wilson equation for an irreversible inhibition: $1/k = (K_i/k_{inact})(1/[\text{juglone}]) + (1/k_{inact})$ (*29*). The value of K_i corresponds to the dissociation constant of the product and k_{inact} to the limiting rate of formation of inactivated enzyme.

Derivatization of Cysteine Residues of Parvulin with DTNB. The amount of free thiols was determined spectroscopically after 10 min of incubation of protein in 35 mM HEPES (pH 7.8) containing 1 mM EDTA and 150 μM DTNB at 25 °C. The increase in absorption at 412 nm ($\epsilon_{412\ nm} = 14\,150\ M^{-1}cm^{-1}$ for the TNB chromophore) was corrected for the increase in absorption of a buffer control (*30*).

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) of Juglone-Modified Parvuline. Parvulin (10 μM) was incubated with the given concentrations of juglone already described above for 4 h. To remove remaining juglone, the incubation solution was applied onto a Vydac protein/peptide reversed-phase 214TP column equilibrated with 0.1% TFA in water. The protein was eluted at 1 mL/min using a linear gradient of 39−44% acetonitrile containing 0.1% TFA.

Determination of Parvulin Modification by Mass Spectrometry. Parvulin samples from RP-HPLC were analyzed by MALDI-TOF-MS. Sinapinic acid and α-cyano-4-hydroxycinnamonic acid were used as matrix materials. Mass spectra were obtained using a REFLEX MALDI-TOF mass spectrometer (Bruker-Franzen, Germany) either in the linear mode or, for post-source decay (PSD) spectra, in the reflector mode.

Amino Acid Sequence Analysis. A sample of RP-HPLC purified modified parvulin was dried under a steady stream of nitrogen and redissolved in water. The pH was adjusted to 8.5 by adding ∼1/10 volume of 1 M ammonium hydrogen carbonate. The protein was incubated with 5% (w/w) trypsin for 2 h at 37 °C. The resulting peptides were separated on a ET 125/8/4 Nucleosil 500−5 C3−PPN column (Macherey-Nagel, Germany) using water and acetonitrile each containing 0.1% TFA in a gradient from 1 to 80% acetonitrile in 70 min at a flow rate of 1 mL/min. Amino acid sequences were determined with an Applied Biosystems sequencer 476A according to the manufacturer's instructions.

Proteolytic Degradation. For proteolytic degradation studies, samples of 10 μM juglone-treated or untreated parvulin were incubated in a 1-mg/mL solution of α-chymotrypsin in 35 mM HEPES buffer (pH 7.8) at room temperature. Reactions were terminated after 0.5, 1, and 5 min by adding 9 μL of 882 μM eglin (Sigma) in 50 mM sodium citrate buffer (pH 2.5). As a control, the same reactions were performed with addition of eglin to completely inhibit α-chymotrypsin. Reaction mixtures were analyzed on a 17.5% SDS-polyacrylamide gel followed by Western blotting. Proteins were transferred to nitrocellulose and the Western blots were probed with a polyclonal antibody raised against recombinant wild type *E. coli* parvulin. Probed proteins were detected by a goat antirabbit antibody linked to horseradish peroxidase (Sigma) and visualized by incubation with 0.3% 4-chloro-1-naphthol and H_2O_2 in 50 mM Tris-HCl, 150 mM NaCl, 0.2% Tween 20, and 10% methanol (pH 7.5).

Spectroscopic Examinations. For recording UV−vis spectra the Specord S10 spectrophotometer (Carl Zeiss Jena, Germany) was used. The cell (1 cm) was thermostated by circulating water through the cuvette holder. Data sampling and analysis were performed with the Aspect Plus software,

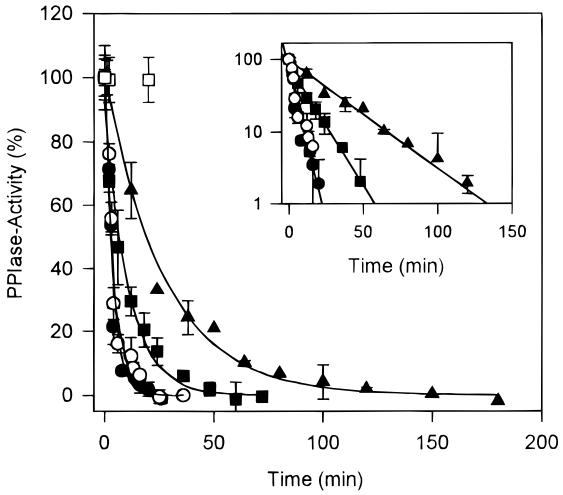


FIGURE 1: Kinetics of the inactivation of parvulins by juglone. Concentrations of 10 μM *E. coli* parvulin (●), 10 μM *E. coli* parvulin variant C69A (○), 10 μM human Pin1 (■) and 50 μM yeast Ptf1/Ess1 (▲) were each incubated at 10 °C with a 10-fold molar excess of juglone. The open squares (□) show the time course of activity of *E. coli* parvulin in the presence of both 5.7 μM juglone and 1 mM DTT. Remaining PPIase activity was followed by analyzing withdrawn aliquots as described in *Materials and Methods*. For wild type, C69A *E. coli* parvulin, Pin1, and Ptf1/Ess1, each point represents the mean of three independent experiments. A semilogarithmic plot of the data (see inset) reveals apparent first-order kinetics. Calculated rate constants are $(4.5 \pm 0.1) \times 10^{-3}$ s^{-1} (wild type *E. coli* parvulin), $(4.2 \pm 0.1) \times 10^{-3}$ s^{-1} (*E. coli* parvulin C69A variant), $(1.5 \pm 0.2) \times 10^{-4}$ s^{-1} (human Pin1), and $(5.3 \pm 0.1) \times 10^{-4}$ s^{-1} (yeast Ptf1/Ess1).

Vers. 1.2 (Carl Zeiss Jena, Germany). Circular dichroism (CD) was measured on a JASCO J-710 spectrophotometer (Jasco, Japan) in 5 mM potassium phosphate (pH 7.8). The resulting spectra were the average of eight scans, recorded in a 0.1-cm cell at 10 °C. For subtracting the buffer baseline and smoothing, the software provided by Jasco was used.

Determination of the Partial Pressure of Oxygen in Solutions. To reduce the partial pressure of oxygen, buffers were deaerated under reduced pressure followed by bubbling argon through the liquids immediately prior to the experiments. The partial pressure of oxygen was adjusted to values between 100 and 70% of air saturated buffer as measured in a Clark electrode (Rank Brothers, Great Britain) according to the manufacturer's instructions.

Computational Methods. Kinetic analysis and presentations of experimental data were performed using SigmaPlot Scientific Graphing System Vers. 2.0 (Jandel Corp., Chicago, IL, USA). For sequence alignments and secondary structure prediction we applied the CLUSTAL W program (*31*) as well as MAXHOM and PHDsec programs on the PredictProtein mail server at the EMBL, Heidelberg, Germany (*32, 33*).

RESULTS

Juglone irreversibly and specifically inactivates enzymatic activity of E. coli parvulin, yeast Ptf1/Ess1, and human Pin1. Figure 1 depicts the effect of juglone on the enzymatic activity of three different parvulins and the *E. coli* parvulin variant Cys69Ala. Under the experimental conditions described in Figure 1, both wild type *E. coli* parvulin and the Cys69Ala variant were completely inactivated within 30 min. Similarly, no remaining activity was detectable for human Pin1 after 60 min and for yeast Ptf1/Ess1 after 150 min of incubation. When measured in terms of remaining enzyme activity under pseudo-first-order conditions, the inactivation of the parvulins obeyed first-order kinetics, as demonstrated by the linearity of the semilogarithmic plot over four half-times of the reaction. Fitting the data according to a first-order equation by nonlinear regression yielded rate constants of $(4.5 \pm 0.1) \times 10^{-3}$ s^{-1} for wild type *E. coli* parvulin, $(4.2 \pm 0.1) \times 10^{-3}$ s^{-1} for the protein variant Cys69Ala, $(1.5 \pm 0.2) \times 10^{-3}$ s^{-1} for human Pin1, and $(5.3 \pm 0.1) \times 10^{-4}$ s^{-1} for yeast Ptf1/Ess1. The concentration-independent parameters of irreversible inhibition of *E. coli* parvulin variant C69A were obtained by applying the Kitz-Wilson equation. The respective plot was linear ($R = 0.992$, $P = 0.0009$), resulting in K_i of (55.9 ± 20.4) nM and k_{inact} of $(5.4 \pm 0.2) \times 10^{-3}$ s^{-1}. The presence of either 50 μM of the peptide substrate Suc-Ala-Phe-Pro-Phe-NH−Np or 10 mM H−Ala-Pro-OH during incubation did not significantly influence inactivation rates, indicating a lack of direct involvement of active site residues in the rate-limiting step of inactivation. Similarly, the insensitivity of the inactivation rate for pH variations within a range of 4 to 10 is attributable to a lack of general acid/base catalysis in approaching the transition state of inactivation. Both wild type enzyme and the Cys69Ala *E. coli* parvulin variant, which has a single Cys (Cys41) retained in the protein chain, displayed similar inactivation kinetics, thus justifying the use of the protein variant as a simplified model for exploring the mechanism of inactivation. Lowering the partial pressure of oxygen to 70% of saturation level did not affect inactivation rates. After completion of the inactivation reaction, both dialysis and gel permeation chromatography on Sephadex G-75 of the reaction mixture failed to restore any PPIase activity. Excess DTT did not reactivate juglone-inactivated *E. coli* parvulin. However, DTT protected the enzyme when present in excess during the inactivation (Figure 1). The metal-chelating agent DTPA (0.5 mM), which was reported to protect bacteria from the toxicity of juglone by interfering with metal ion-catalyzed ROS generation (*34*), did not affect the kinetics of inactivation at 5.7 μM juglone (data not shown).

Table 1: Specificity of PPIase Inhibitors[a]

enzyme[b]	percentage of remaining activity after enzyme incubation with: CsA	FK506	juglone
hCyp18	*1.7 ± 0.5*	98.9 ± 15.2	95.6 ± 4.6
hFKBP12	106.9 ± 4.8	*0.0 ± 1.6*	102.0 ± 0.9
L.p. FKBP25 (Mip)	100.7 ± 10.3	*78.2 ± 11.2*	97.8 ± 15.8
E.c. Trigger factor	103.5 ± 6.4	94.0 ± 4.1	100.6 ± 3.5
E.c. Parvulin	98.0 ± 13.9	102.3 ± 7.4	*0.0 ± 2.5*
S.c. Ptf1/Ess1	100.9 ± 15.7	97.2 ± 1.8	*0.0 ± 2.6*[c]

[a] After incubation of the respective PPIase with 1.3 μM CsA, 70 nM FK506, and 5.7 μM juglone, respectively for 45 min; activities were determined as described in *Experimental Procedures*. The values given are percentages of activity relative to control. [b] Concentrations of enzymes were 15 nM, 24 nM, 67 nM, 28 nM, 6 nM, and 0.1 μM for hCyp18, hFKBP12, Mip, trigger factor, parvulin, and Ptf1/Ess1, respectively. [c] After incubation for 180 min.

To characterize whether enzyme inactivation by juglone expresses specificity with regard to different PPIases, we incubated members of the three PPIase families with juglone (Table 1). For comparison, the interaction of the reversible inhibitors CsA and FK506 was measured as well. The results show resistance of the parvulins to both CsA and FK506.

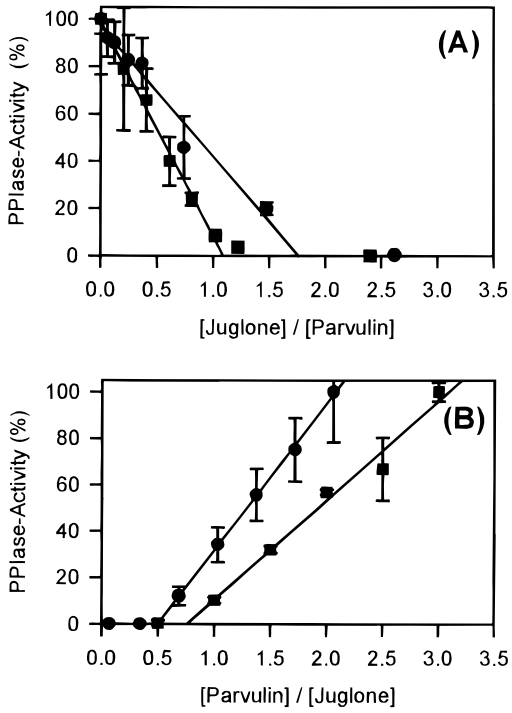


FIGURE 2: Influence of the enzyme/inhibitor ratio on the percentage of inactivation by juglone for *E. coli* parvulin and its C69A variant. Parvulin was incubated at 10 °C with juglone in 35 mM HEPES (pH 7.8). Remaining PPIase activity was determined after 4 h as described in *Materials and Methods*. The level of 100% activity was determined at zero time for each experiment. (A) Varying juglone concentration at constant concentrations of enzymes: 10 μM *E. coli* parvulin (●) and 5 μM of the protein variant C69A (■). (B) Varying enzyme concentration at a constant concentration of juglone of 13.3 μM for *E. coli* parvulin (●) and 2.5 μM for the parvulin variant C69A (■). A zero level of enzyme activity was extrapolated at a enzyme/juglone ratio of 0.58 for the wild type protein and 0.97 for the parvulin C69A variant.

Juglone exhibits specificity because PPIases except parvulins cannot be inactivated under conditions leading to full inactivation of the parvulins.

Stoichiometry of the Reaction of E. coli Parvulin with Juglone. To determine the stoichiometry of the inactivation, *E. coli* parvulin, both wild type and protein variant C69A, were coincubated with various concentrations of juglone for 4 h. Prolonging the reaction time did not lead to further changes in enzyme activity. Figure 2 shows the relationship between the molar ratio of juglone to parvulin and the PPIase activity. When the concentration of either the enzyme (Figure 2A) or the inhibitor (Figure 2B) was kept constant, about two equivalents of juglone were detected in the completely inactivated wild type *E. coli* parvulin. In contrast, about one equivalent of juglone resulted in complete inactivation of the parvulin variant C69A.

Impact of Juglone on the Amount of Free Thiols in Parvulin. DTNB was used to quantify free thiols in native *E. coli* parvulin. The linear relation between thiol content and protein concentration, which was characterized by a slope of 1.8 (Figure 3A), demonstrates that both cysteines exist mainly as free thiols in the active enzyme and are accessible to modifying reagents. Consequently, wild type *E. coli* parvulin was treated with different juglone concentrations and the amount of free thiols was determined. Figure 3B shows that the amount of free thiols decreases linearly with increasing juglone concentration. After equimolar addition

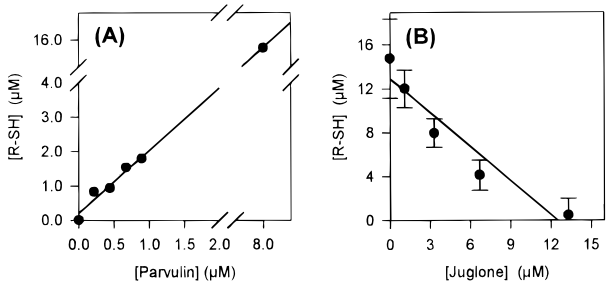


FIGURE 3: Titration with DTNB of free thiol groups of *E. coli* parvulin. (A) Concentration of thiol groups dependent on the *E. coli* parvulin concentration. The linear relationship gives a slope of 1.82 ($r^2 = 0.999$). (B) Decrease of thiol concentration of *E. coli* parvulin (8 μM) after the treatment of the enzyme with juglone under the experimental conditions of Figure 2. Linear regression of the data gives a slope of 1.03 ($r^2 = 0.917$). Data points are mean values of three experiments.

of juglone, compared with the amount of thiols in untreated enzyme, virtually no remaining free thiols were detectable. Linear regression revealed a slope of 1.0, indicating that every added equivalent of juglone masks one equivalent of thiols.

Both juglone-treated and untreated *E. coli* parvulin (wild type and parvulin variant C69A) were examined by MALDI-TOF-MS. To avoid detection of noncovalent adducts, any loosely bound juglone was removed by RP-HPLC of the proteins prior to the MS analysis.

Recorded mass spectra of wild type parvulin treated with juglone revealed two new signals of 10 272 ± 4 and 10 443 ± 8 Da, representing molecular masses of proteins associated with 1 and 2 juglone molecules, respectively (calc., 10 275 and 10 449 Da). Similarly, for the His-tagged C69A variant (molecular mass 12 058 Da), only the signal representing the molecular mass of a singly modified enzyme (exp. 12 232 ± 4 Da, calc. 12 232 Da) was seen.

We exposed these samples of juglone-treated parvulin C69A variant as well as the untreated parvulin variant to a tryptic digestion. By comparison, the HPLC chromatograms revealed additional peptides in the juglone-treated sample when recorded at 280 nm. Edman degradation identified these peptides as H38 to K45, K37 to R46, and H38 to R46. These peptides displayed increased molecular masses caused by either a single modification by juglone [+174] or by juglone addition and parallel oxidation to sulfoxide [+174, +16]. By analysis of the MALDI−PSD mass spectrum recorded for the H38−R46 fragment, Cys41 was unambiguously identified as the site of modification by juglone. These techniques did not reveal any additional modification site.

Time-Dependent UV−vis Spectra for the Reactions of Juglone with GSH and E. coli Parvulin C69A. It is well established that juglone reacts with GSH via a Michael-type addition to the 3-glutathionyl-hydrojuglone (*35*). Oxidation leads to 3-glutathionyl-juglone in a slow, subsequent process. A similar reaction has been observed for various quinones with SH-groups of enzymes (*36*). Comparison of time-dependent UV−vis spectra monitored during the reactions of juglone with GSH and the *E. coli* parvulin variant C69A (Figure 4) revealed a similar spectral pattern. The Michael addition of SH to the C3 atom of the chinoid ring became observable as reaction occurring rapidly under the experimental conditions described in Figure 4. Both reactions lead

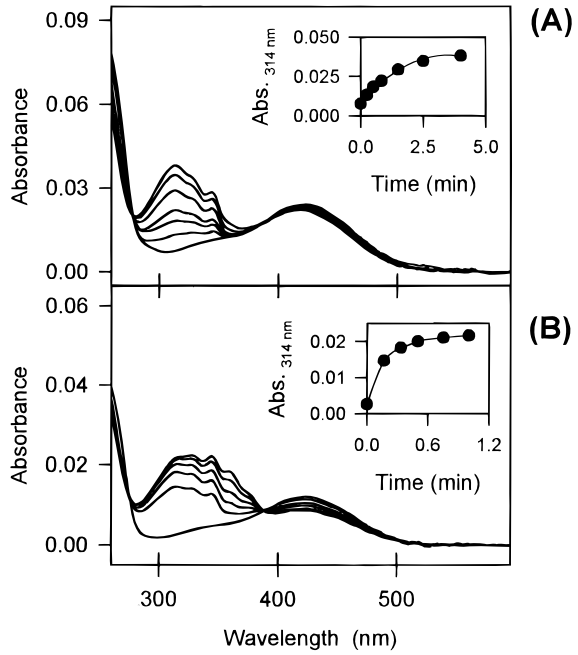


FIGURE 4: Time-dependent UV−vis spectra monitored during the reaction of juglone with GSH and *E. coli* parvulin variant C69A. GSH stock solution was added to 6.6 μM juglone in 35 mM HEPES (pH 5.3) to a final concentration of GSH of 10 μM. (B) *E. coli* parvulin variant C69A was added to 3.5 μM juglone in 35 mM HEPES (pH 5.0) to a final protein concentration of 12 μM and spectra were recorded as already indicated. The temperature was kept constant at 10 °C in both experiments. The insets represent time courses of the corresponding absorbance signal at 314 nm.

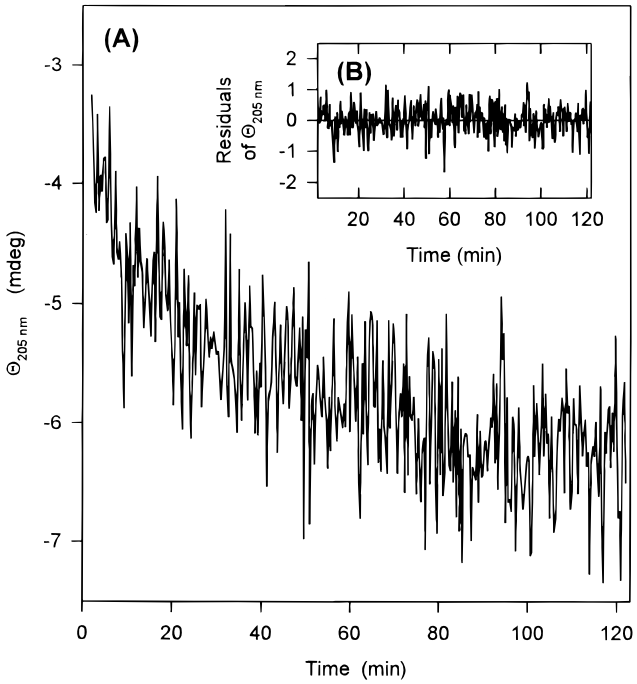


FIGURE 5: Kinetics of the decrease in ellipticity at 205 nm upon modification of *E. coli* parvulin variant C69A parvulin with juglone. The enzyme (10 μM) was added to juglone (8 μM) in 5 mM potassium buffer (pH 7.8). Zero time was set during mixing. After mixing, the solution was transferred into a silica cell that was maintained at 10 °C. The control was a sample consisting of protein and the solvent of the juglone stock solution, which showed no change of the $\Theta_{205\ nm}$ in the time interval used for the kinetic experiments. Data are the mean of six repetitive experiments. (B) The data were fitted to a second-order rate law yielding a rate constant of (3.9 ± 2.0) × 10^{-3} μM^{-1} s^{-1}. The inset shows the residuals of the fit.

to identical isosbestic points at 243, 278, and 385 nm for the time-dependent spectra, indicating an uniform reaction.

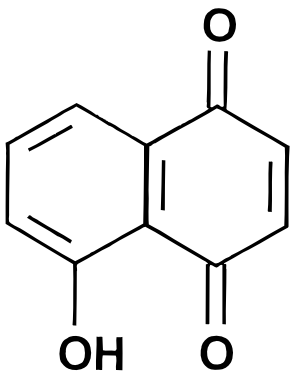
Appearance of isosbestic points is accompanied by the formation of absorbance maxima at 223, 314, and 344 nm. When the observations were carried out over a longer period of time (60 min) with both GSH and parvulin variant C69A, a slower second reaction step followed that was accompanied by a pronounced decrease of the absorbance at 344 nm. The rate of this spectral change was linearly dependent on the partial pressure of oxygen in the solution (data not shown), suggesting an oxidation of the primarily produced substituted hydrojuglone to the substituted juglone. In contrast, the rate of the fast reaction step shown in Figure 4 was independent of the oxygen partial pressure. At 10 °C and oxygen saturation, second-order rate constants of 2.4 × 10^{-2} μM^{-1} s^{-1} at pH 5.0 and 2.2 × 10^{-2} μM^{-1} s^{-1} at pH 7.8 were calculated for the Michael addition of juglone to the parvulin variant C69A. Under the same conditions (10 °C, oxygen saturation), the secondary oxidation reaction followed apearent first-order kinetics, with estimated rate constants of 2.0 × 10^{-3} (pH 5.0) and 1.6 × 10^{-3} (pH 7.8) s^{-1}.

Juglone-modified parvulin is still structured but proteolytically sensitive. SDS−PAGE revealed that in contrast to the untreated native parvulin, juglone-modified parvulin was completely degraded by excess α-chymotrypsin within a 1-min period of incubation time. The increased sensitivity to α-chymotrypsin suggests a loss of native structure of juglone-modified parvulin compared with the unmodified molecule leading to exposure of protease cleavage sites. However, the modified enzyme is still structured. To evaluate the thermodynamic stability of the enzymes, GdnHCl-induced unfolding of modified (4 h after addition of juglone) and unmodified C69A parvulins was monitored in the far-UV CD spectrum at $\Theta_{220\ nm}$. Unmodified parvulin C69A was characterized by a strongly cooperative transition with a ΔG_{fold} of −20 kJ mol^{-1} (−4.8 kcal mol^{-1}) and a midpoint of the transition at 1.25 M GdnHCl. In contrast, the modified parvulin C69A had a ΔG_{fold} of −8 kJ mol^{-1} (−2 kcal mol^{-1}) and a midpoint at 0.75 M GdnHCl. Thus, after modification, the protein has undergone limited structural alterations rendering the protein more unstable.

Modification of parvulin leads to a time-dependent far-UV CD spectrum. The far-UV CD spectra of *E. coli* parvulin (*9*) and its C69A variant are characterized by a moderate negative signal at 221 nm and a strong positive ellipticity recorded at 194 nm. After modification, an impact of juglone on the CD spectrum of the protein variant became apparent by a reduction of the amplitudes of both signals.

The changing CD spectra could be caused by either deteriorations of the native secondary structure of the parvulin variant C69A or by juglone itself when it gets into the asymmetric environment of the protein interior. Evaluation of the spectra with the programs k2d, CONTIN, and MLR according to (*37*) implicated a nearly unchanged helix content and a decreased content of β-structures. Because the SH group modification itself is very rapid, we tested whether the time course of the changing CD spectrum resembles either the modification or the inactivation. We observed the ellipticity at 205 nm as a function of time after adding juglone to parvulin C69A (Figure 5). Because of the high absorbance

Chart 1: Structure of Juglone
(5-Hydroxy-1,4-naphthoquinone)



of juglone at 205 nm, pseudo-first-order conditions could not be applied. However, kinetics can be described by the second-order rate law at lower juglone concentrations. The observed rate constant of $(3.9 \pm 2.0) \times 10^{-3}$ μM^{-1} s^{-1} was not significantly influenced by varying the partial pressure of oxygen. This rate constant agrees very well with the second-order rate constant of enzyme inactivation $(3.5 \pm 0.5) \times 10^{-3}$ μM^{-1} s^{-1} calculated from the pseudo-first-order constant under the experimental conditions of Figure 5. Taking into account the magnitude of the rate constant k and the activation enthalpy $\Delta H^{\ddagger}$ of the inactivation, the possibility of a peptidyl-prolyl *cis/trans* isomerization as rate-limiting step has to be considered. For yeast Ptf1/Ess1, human Pin1, and *E. coli* parvulin, the first-order rate constants of partial unfolding of the thiol group modified proteins were found in the range $0.5-4.5 \times 10^{-3}$ s^{-1}, and $\Delta H^{\ddagger}$ unfolding was -14.2 kcal mol^{-1} for the *E. coli* parvulin variant C69A. These values are in a range typical of prolyl isomerizations (*1*). However, neither of the PPIases 300 nM rhCyp18, 360 nM *L. pneumophila* FKBP25 (Mip), 3 μM rhFKBP12, nor 3 μM *E. coli* trigger factor, showed any influence on the inactivation rate of *E. coli* parvulin by juglone.

DISCUSSION

It was previously found that the parvulin family of PPIases is not inhibited by known PPIase inhibitors such as CsA, FK506, and rapamycin. (*8*, *9*). Here we will report that the parvulins are covalently modified and subsequently inactivated by the brown walnut dye juglone (Chart 1). Thus juglone represents the first inhibitor of the parvulin family of PPIases described so far. Table 1 shows clearly that members of the other families of PPIases are resistant to inactivation by juglone, indicating inhibitory specificity for parvulin-like PPIases, such as *E. coli* parvulin, yeast Ptf1/Ess1, and human Pin1.

Because of its bacterio- and fungistatic effect (*38*, *39*) as well as its cytotoxicity toward eukaryotic cells (*36*), juglone became the subject of extensive studies to examine its potential pharmaceutical use. Though the inhibition of several enzymes by juglone (for example pyruvate decarboxylase, glutathion-*S*-transferase) was reported (*38*), the mechanisms of inhibition have not been established in detail. In some cases, indirect evidence indicates modification of thiols being responsible for the inhibition (*40*), whereas with other enzymes, competitive binding to the site of the coenzyme NADPH seems to occur (*41*). A further possible chemical trait is based on juglone reduction to the corresponding hydroquinone, followed by reoxidation to the quinone form with parallel formation of ROS in the presence of molecular oxygen. Thus, the observed biological effects were indirect, caused by ROS-mediated damage in biomolecules (*36*, *42*).

Our results derived from MALDI-MS and peptide sequencing following tryptic digestion demonstrate that inactivation of *E. coli* parvulin by juglone leads to covalent modification of two amino acids, including Cys41. Cys69 was identified as the second modification site because replacement of the amino acid residue Cys69 by Ala resulted in a *E. coli* parvulin variant whose inactivated adduct contained only one juglone molecule covalently bound to the side chain of Cys41.

Given the similar mechanisms of the reactions of juglone with GSH and *E. coli* parvulin variant C69A, as shown by UV−vis spectroscopy, we conclude that a Michael addition of the α,β-unsaturated carbonyl system of juglone and the sulfhydryl group of Cys41 of the protein variant occurs. The rapid Michael addition is followed by a slow oxidation of the resulting hydrojuglone−protein adduct. These redox processes are not involved in *E. coli* parvulin inactivation because changes of the partial pressure of oxygen failed to affect the rate constant of inactivation. However, enzyme inactivation proceeds 5-fold slower than the thiol group modification. Thus, a subsequent event triggered by the covalent attachment of juglone to the protein must be the cause of the loss of enzyme activity. We were able to show that SH group modification by juglone renders the enzyme sensitive to proteolysis, indicating a certain degree of destabilization of the native structure. Moreover, the conformational interconversion of unmodified and modified enzymes plays a key role for inactivation because the progress curves of far-UV CD signal changes were similar to those observed for the inactivation of the *E. coli* parvulin variant C69A by juglone. Deterioration of PPIase activity parallels exactly a decrease in the content of β-sheets in the enzyme. However, the inactive, juglone-modified enzyme still embodies an intact protein characterized by a low negative value of ΔG_{fold} as well as considerable folding cooperativity. Misplacement of catalytic residues of the enzyme may result from partial unfolding of the active site of parvulin, rendering the enzyme inactive. The molecular process involved in partial unfolding of the active site has some properties of a proline-limited event. However, PPIases failed to increase inactivation rates, thus rendering conclusions about a proline-limited unfolding still uncertain.

Based on the recently obtained crystal structure of human Pin 1, a general impression of the catalytic site of the parvulins was given (*15*). In addition to the lack of primary sequence similarity to FKBP-like and cyclophilin-like PPIases, there are marked differences in the composition of the amino acids forming the active site in that the Xaa-Pro binding pocket is surrounded by numerous functional side chains. In Pin 1, a cluster of conserved amino acids can be found in this position, including Cys113. Alignment of the amino acid sequences reveals that all investigated parvulins that are inactivated by juglone have this conserved cysteine in common (Figure 6). Referring to the results found for the modification of the homologous Cys41 in *E. coli* parvulin

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Parvulin E.c.     31   FGKLAKKHSICPSGKRGGDLGEFR.QGQMVPAFDKVVFSCPVLE
human Pin1       103   FESLASQFSDCSSAKARGDLGAFSR.GQMQKPFEDASFALRTGE
Ptf1/Ess1 S.c.   111   FEALAKERSDCSSYKRGGDLGWFGR.GEMQPSFEDAAFQLKVGE
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FIGURE 6: Alignment of a segment of sequences flanking conserved cysteines of *Escherichia coli* (E.c.) parvulin, human Pin 1, and Ptf1/Ess1 from *Saccharomyces cerevisiaea* (S.c.). The alignment was performed by CLUSTAL W and refined manually, and it represents a portion of a global alignment including all known members of the parvulin family of PPIases. Identical residues are indicated by bold faced letters. The conserved cysteine residue is underlined. Gaps are indicated by dots.

by juglone, we propose presence, reactivity, and surface accessibility of this cysteine residue as requirements for the initialization of the partial unfolding of these PPIases. In addition, juglone thus conferred specificity to the inactivation of parvulins.

Inactivation via partial unfolding of the active sites has already been shown for several enzymes such as D-glyceraldehyde-3-phosphate dehydrogenase, ribonuclease A, and creatine kinase (*43*,*44*). Loss of enzyme activity was thereby induced by applying low concentrations of denaturants, such as guanidine hydrochloride and urea. It was suggested that the active sites of these enzymes display more conformational flexibility than the proteins as a whole. In the course of our inactivation studies, we made similar observations for members of the parvulin family of PPIases using juglone, a covalent modifier, bound to the proteins instead of denaturants.

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