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Cloning, overexpression and biophysical characterization of grd/grl/wrt domains from *Caenorhabditis elegans* in *Escherichia coli*

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2 Abstract

Hedgehog related genes have been shown to play a major role in development in all deuterostomes. In *C.elegans*, such genes have been found where the similarity is restricted to the C-terminal domain. This work has focused on the hedgehog related *C.elegans* proteins called ground (grd), ground-like (grl), and wart (wrt) which appear to form a unique structural family. These proteins are cysteine rich and have conserved cysteine patterns which, together with the thought that they are secreted, are expected to be in disulfide form. Since the extracellular environment is very oxidizing and due to the conserved cysteine pattern, disulfide bonds are thought to play a big part in the folding and stabilization of these proteins. The stability of the protein and the formation of a disulfide bond are related through a thermodynamic cycle, which insures that the stabilization of the protein by the disulfide is reflected by the identical stabilization of the disulfide by the protein. Practically, there are numerous parameters that can be used to try to achieve the correct disulfide bonds and folding, when doing *in vitro* trials, some of which were used in this project. *C.elegans* proteins grd-5, grd-13, grl-24, wrt-3 and wrt-5 were studied in this project. All of the proteins were expressed and purified with success, with the exception of grl-24. All constructs formed inclusion bodies. Some refolding attempts were performed on grd-13 and wrt-3. The presence of a disulfide bond in refolded grd-13 was demonstrated using chemical fragmentation. In general, these attempts did not give correctly folded proteins but provide a foundation to continue experiments aimed at producing a native-like protein for structural and functional studies.

3 Introduction

In all deuterostomes there is a family of proteins related to hedgehog proteins found initially in *Drosophila*. They are all paracrine factors that are secreted. For theses signaling molecules, disulfide bonds are important for the structure, and perhaps the function. When doing *in vitro* refolding experiments redox buffers are often used, they help when forming the disulfide bonds. This project is designed as a first step towards understanding how the proteins are involved and functions in the signaling system.

3.1 Proteins

In *Drosophila*, Hedgehog genes are involved in the development of the fruit fly. The hedgehog genes comprise one family of proteins involved in cell-cell signaling. The classical hedgehog protein consists of two domains. The N-terminal domain "hedge" is the biologically active signal. The C-terminal "hog" domain has autoproteolytic activity and is responsible for the activation and release of the signal (4).

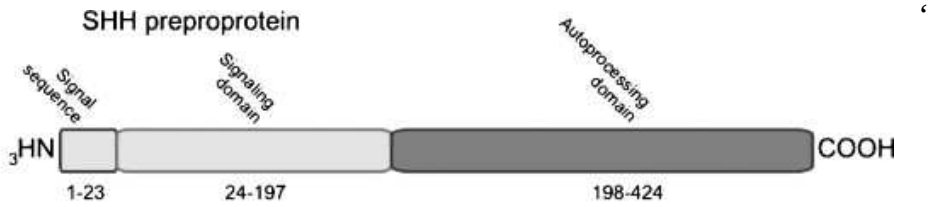


Fig 1 (1) Hedgehog realted protein Schematic picture of the domain organization in the hedgehog related genes

In *Caenorhabditis elegans*, there are several hedgehog related genes, where the similarity is restricted to the C-terminal domain called hog. It is the N-terminus of these *C.elegans* genes that divide them into different families called wart (wrt), ground (grd) and ground-like (grl). Wrt and grd can exist with or without a hog domain, while grl has never been observed to exist together with a hog domain (4,5).

Cysteines appear to be important for these wrt/grd/grl proteins, as evidenced by conserved patterns of cysteine amino acids (4,5). The Hog domain has a conserved Cys-Phe sequence at the position of the cleavage site. The different wrt proteins have eight conserved cysteines. The grd proteins typically have four cysteines (with a few exceptions) (5). In grd proteins, it appears that

the cysteines are organized into one doublet and two singlets, as can be seen by sequence alignments.

The grl proteins share some features with grd & wrt, like a central core motif and secondary structure, the central core motif have no known function (see Fig. 2) (2). A big difference is in the cysteine pattern where one of the cysteines in the doublet is lost. Based on sequence comparisons, the cysteines are lost or gained pairwise between the different proteins in the grl and grd families, but there is always cysteines present. This is a sign that they might form disulfide bond. Also relevant is the fact that the proteins are secreted, which due to the often oxidizing nature of the extracellular environment, adds to the possibility of disulfide bond formation.

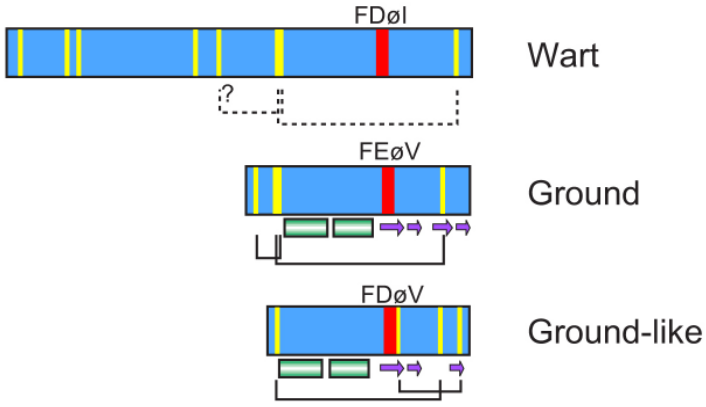


Fig 2 Conserved cysteine pattern and secondary structure(2) The conserved cysteine patterns in the different domains of wrt, grd, and grl. Predicted secondary structure is also shown.

Some structural predictions can be made by examining alignments. The first half of the protein shows high scores for two α helices (2). The second half shows high scores for β strands (3 or 4) that could form a β sheet. At the beginning of the first α helix there is a conserved cysteine.

Figure 3 shows an alignment of the protein sequences used in this project with the cysteines highlighted.

```
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                 5          15         25         35         45         55         65
Wrt-3        DYCGSDQVPY GMEVHHSGVV RLMCSKPNCY DKNYSDCPER AESRHGCQKS NQWVGGFEKN IEGDLYTMCC
wrt-5        DYCGDHKVPF GMEVHKNGNV NILCSRPSCH EKKYAECPER ~ATSTTCSTN SSWVGGVTQH SDGSLRLMCC

             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                 75         85         95        105        115        125        135
Wrt-3        EFEGLEKYAK VRYSDVRIRR GEFFEGEEKE NDDGDVVKFD VIKDIRMHKD DEGQAYYNLT VLSFNCESIP
wrt-5        EYDLLPTYST IQYEKLTIRT GEYFEGD~EQ MEGDVVTAFD LIGNIEQVKE PDGKYSYNLL IYRYHCGNIP

             ....|....| ....|....|
                145        155
Wrt-3        DVKPAWYQKS QWPYFQFAKN
wrt-5        DTPPAWYMKK QWPYWEK---


             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                 5          15         25         35         45         55         65
Grd-5        ---------- --QDNCYIND SGFTCCNKEL ESAMKGAMGG D-----DLLG SADSIQKGAE GSLGGKFETV
Grd-13       AYDDLPKPAD PYQPPGTEPK TESTCPDPYK KIITQLRAEL GKDVSS--IK FTNQLGSRVQ KAFGSAHEIT

             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|...
                 75         85         95        105        115        125        135
Grd-5        VALDDFAYKS HFKEGKSCKI EK~NGQYALA WQP------- ---------- ---------- ---------- ---
Grd-13       MGPSEATLKT NFN-GTICRH ASTDGFHYIV YPTPGQYNIN NAAVEEYFEK FAEFAALGKS ANIADLPKDP RNV


             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                 5          15         25         35         45         55         65
grl-24       QDDNFEGERC NDVILYDIIK KASKKTDDPV IIRRTSMDTM QNVFPLARSM GCICTDRNFQ FPDFTNHRYC

             ....|....| ....
                 75
grl-24       SVRVSNFRCH AIVF
```

Fig 3 Proteins used in this project Sequence alignments between wrt-3 and wrt-5, grd-5 and grd-13. Grl-24 by itself. Cysteines highlighted.

3.2 Disulfide bonds & folding

One important aspect when it comes to protein structure is the presence of disulfide bonds. There are several different factors that affects the making and breaking of a disulfide bond. Those factors are among others, the redox potential of the environment and the stability of the disulfide bond and the stability of the protein containing it. These features are linked thermodynamically, which means that the stability of the protein and the stability of the disulfide (redox potential) stem from the same origin (3).

The formation of the correct disulfide bond *in vivo* is thought to be a matter of trial and error. When disulfide bonds are formed, they may not be the native one, in which case then the disulfides will reshuffle until it forms the native disulfide, that will say the one with the lowest Gibbs energy (6). If reshuffeling does not occur, the polypeptide is trapped in a covalent intermediate which can be difficult to escape from. Protein disulfide isomerase (PDI) catalyzes the formation of disulfide bonds. PDI have not been shown to direct the protein to the correct disulfide bonds, only to speed up the trial and error process (7,8).

The formation of a correct disulfide bond is not always easy – especially if the protein contains many cysteines and the process is truly random. The number of possible conformations of disulfide bonds that can be formed from a given number of cysteine residues can be calculated by Eq 1, where n is the number of cysteines and p is the number of bonds.

$$\frac{n!}{p!(n-2p)!2^p} \qquad \text{Eq 1}$$

If there are only two cysteines it is easy because there is only one possible bond. For example if there are eight cysteines that should form 4 specific disulfide bonds in the native protein, there is only one correct pairing of the 8 cysteines out of 105 possible.

One problem that is often encountered while trying to form intramolecular disulfide bonds *in vitro* is the formation of intermolecular dimers and oligomers. The formation of these dimers and higher order oligomers can be decreased by using very diluted protein samples (9).

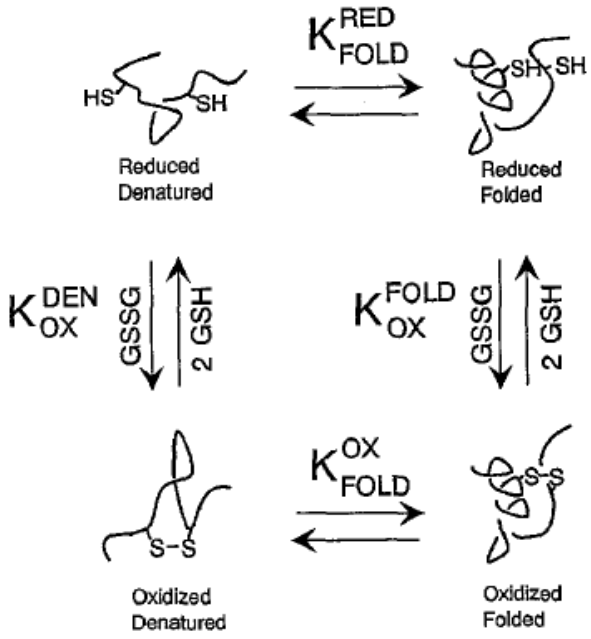


Fig 4 Thermodynamic cycle A thermodynamic cycle that shows how the stability of the protein is affected by presence or absence of a disulfide bond and how the folding affects the disulfide bond. ΔG around the cycle must always sum up to 0. GSSG is oxidized glutathione , GSH is reduced glutathione. Figure taken from **(3).**

Figure 4 shows that the formation of a disulfide bond stabilizes and promotes the folding of the protein and the folding of the protein promotes the formation of a disulfide bond. When a disulfide bond is formed it organizes the unfolded protein to favor a particular folded state (when looking at entropy). The folding of the protein favors the formation of a disulfide bond. Since it is a thermodynamic cycle the ΔG calculated around the cycle must be zero. Gibbs free energy is determined by the equilibrium constant.

$$\Delta G = -RT \ln K_{eq} \qquad \text{Eq 2}$$

Entropy greatly affects the oxidation potential of the thiol/disulfide in unfolded proteins. It can be related to the number of amino acids between the cysteines. The covalent loop formed when a disulfide bond is formed will have to pay a penalty for creating geometric and entropic constraints. The entropy directly affects the redox potential. Conformational entropy for an unfolded protein increases when a disulfide bond is broken, and this decreases the stability of the protein. There is an equation (eq 3) that can be used to calculate how a cross-link affects the entropy, where n is the number or residues between the cysteines that are involved in the disulfide bond (10).

$$\Delta S_{conf} = -2.1 - (3/2)R \ln n \qquad \text{Eq 3}$$

3.3 Redox buffers

A redox buffer is an aqueous solution of a thiol/disulfide pair of known redox potential and predetermined concentrations of the oxidized and reduced components. One of the most common redox buffers is prepared using glutathione (E°' = -0.24 V) (11), which is readily available in pure form in both the reduced and oxidized states. Another fairly common redox buffer is prepared from dithiothreitol (DTT, E°' = -0.33V) (12). DTT is commonly used for more stable disulfides as DTT is significantly more reducing than glutathione. Dithiothreitol also has the advantage of not remaining as an intermolecular disulfide due to the strong tendency to form intramolecular disulfides, so there will not be that many mixed disulfides present, that can disturb the folding.

The redox potential of a solution at non-standard conditions can be calculated by using the Nernst equation (Eq. 4) (13). E is the observed redox potential. E^o is the redox potential at the standard state (concentration 1M, 298K, 1atm) R is the ideal gas constant, T is the temperature in Kelvin, F is the Faraday constant and Q is the ratio between the reduced and the oxidized state of the redox buffer.

$$E = E^\circ - \frac{RT}{nF} \ln Q \qquad \text{Eq 4}$$

When two cysteines are close to each other in space they may form a disulfide bond. When glutathione is the reducing agent, the reaction will look like equation 5.

$$P(SH)_2 + GSSG \leftrightarrow P(SS) + 2GSH \qquad \text{Eq 5}$$

Here, $P(SH)_2$ is a protein containing two cysteines in thiol form, GSSG is the oxidized form of glutathione, P(SS) is the internally oxidized form of glutathione, and GSH is the reduced form of glutathione. The stability of the disulfide bond in comparison to the stability of the GSH^2/GSSG pair is represented by the equilibration constant K_{ox}, which is calculated by equation 6. This equilibrium constant defines the oxidation potential of the dithiol $P(SH)_2$.

$$K_{ox} = \frac{[P(SS)][GSH]^2}{[P(SH)_2][GSSG]} \qquad \textbf{Eq 6}$$

If for example a glutathione buffer with the redox potential of -263mV is to be prepared, the concentrations of GSH and GSSG must to be calculated. Here it becomes clear that one solves for the ratio $[GSH]^2/[GSSG]$. Notice that a given ratio of [GSH]:[GSSG] will have different redox potentials depending on the total concentration of glutathione in the solution. To prepare a buffer, a total glutathione concentration (C_T) must be selected such that C_T = GSH + 2GSSG, which then allows a unique solution to be calculated.

$$-0{,}263 = -E^0 - \frac{RT}{nF}\ln\frac{[GSH]^2}{[GSSG]} \qquad \textbf{Eq 7}$$

The program Solver in Microsoft Excel was used to simplify this calculation. Equation 6 was entered and GSH and GSSH were set to be variables to achieve the redox potential of -263mV with the constraint of a given range for the total glutathione concentration. The concentrations of GSH and GSSG were then obtained from the Solver routine. In this case the result was, GSH 18.5mM, GSSG 0.1mM.

4 Materials & Methods

4.1 Cloning

The pET-46 Ek/LIC overexpression vector uses the Ligand Independent Cloning (LIC) technique to insert the gene into the vector. LIC does not require any restriction enzymes or ligation reactions. Instead primers are designed to use in PCR which creates the desired gene with a specific overhang complementary to the overhang in the pET vectors (14). The primers used for the PCR were designed after guidelines from the Ek/LIC protocol (Table 1).

Genes containing the wrt, grd, or grl sequences were cloned from sequencing vectors as identified from references (5) and (15) supplied as *E. coli* stab cultures or phage aliquots. Five different cDNAs were used for this project: yk1580h06(grd-5), yk1620b07(grd-13), yk123d11(grl-24) yk348a9(wrt-3), yk1139h04.3(wrt-5). A sample of the stab culture was grown in SOC containing ampicilin (100µg/ml) at 37ºC overnight. The cell culture was transferred to an Eppendorf tube and centrifuged at 13000 rpm in an Eppendorf centrifuge for 5 min. The cell pellet was prepared for PCR by washing it in 1xPBS and centrifuging at 13000 rpm for 5min. Aliquotes of phage were used directly in PCR without prior processing.

That pellet was washed once again in 1xPBS. The starting material (the sequencing plasmids containing the desired genes) from either the cell pellet or virus solution were used directly as template in PCR reactions which were prepared according to the protocol for the PFU DNA polymerase (Promega).

Primers

Grd-5 Forward 5´ - GAC GAC GAC AAG ATG CAG GAC AAT TGC TAC ATC AAT G-3´
Reverse 5´ - GAG GAG AAG CCC GGT TTA TGG CTG CCA TGC AAG - 3´

Grd-13 Forward 5´ - GAC GAC GAC AAG ATG GCC TAT GAT GAT CTC CCA AAA C-3´
Reverse 5´ - GAG GAG AAG CCC GGT TCA AAC GTT GCG TGG AT-3´

Grl-16 Forward 5´ - GAC GAC GAC AAG ATG TGT ATC GGA GGT GCC GGA AGC -3´
Reverse 5´ - GAG GAG AAG CCC GGT CAT TAA TCC TCC CAG GTA AGC-3´

Grl-24 Forward 5´ - GAC GAC GAC AAG ATG CAA GAT GAT AAT TTT GAA GGT GAA C-3´
Reverse 5´ - GAG GAG AAG CCC GGT TCA TTA AAA GAC AAT TGC ATG ACA C- 3´

Wrt-3 Forward 5´ - GAC GAC GAC AAG ATG GAT TAC TGT GGA TCC GAT C-3´
Reverse 5´ - GAG GAG AAG CCC GGT TCA GTT CTT TGC AAA TTG AAA G-3´

Wrt-5 Forward 5´ - GAC GAC GAC AAG ATG GAC TAC TGT GGG GAT CAC -3´
Reverse 5´ - GAG GAG AAG CCC GGT TCA TTA TTA TTT TTC CCA ATA CGG CCA C-3´

Quick change mutagenesis primer

Grd-5 Forward 5´ - CCT ACA AAT CTC ATT TCA AGG AGG GAA AAT CTT GCA AGA TCG - 3´
Reverse 5´ - CGA TCT TGC AAG ATT TTC CCT CCT TGA AAT GAG ATT TGT AGG - 3´

Table 1 Primers The primers used for amplification and quick change

The PCR products (10 µl + 10 µl 6xSB + 40 µl H_2O) were run on a low melting 1% DNA-gel (with 0.5mM ethidium bromide) in 1xTBE buffer (0.89M Tris-base, 0.89M Boric acid, 0.02M EDTA, pH 8.0) at 100 V, for 40 min. DNA bands were visualized by exposure to UV light. The band containing the PCR product was cut out by hand and purified by Wizard SV gel and PCR clean-up system (Promega), according to protocol.

The purified PCR product was then ligated into a pET-46 Ek/LIC vector by following the manufacturers protocol. The complete vector containing the wrt, grd, or grl genes was then transformed into Giga single cells (Novagen) for plasmid preparation. These cells were grown on a LB/amp plate overnight at 37 ºC. Colonies were selected and grown in LB/amp (100µg/ml), 37ºC with shaking overnight. Cells were harvested (13000rpm) and plasmid extracted and purified using the Wizard plus SV minipreps DNA purification system (promega). Aliquotes of the plasmid with the appropriate T7 sequencing primers were sent for sequencing to Uppsala genome center (16).

4.2 Quick change:

The Grd-5 plasmid had an incorrect base apparently introduced during the PCR protocol. This unwanted point mutation was corrected using the Quick change II site-directed mutagenesis kit (Stratagene). Complementary primers were designed that had the correct base mismatch in the middle (see Table 1). The protocol was followed and the corrected plasmid was transformed into XL1-Blue super competent cells, colonies selected, and plasmid prepared. Aliquotes of the plasmid with the appropriate T7 sequencing primers were sent for sequencing to Uppsala genome center (16).

4.3 Expression:

Plasmids containing the wrt, grd or grl genes were transformed into BL21(DE3)Star cells (Stratagene) for expression. Colonies were picked and grown in LB/amp (100 µg/ml) at 37 ºC while shaking. For a test expression, a sample of overnight growth was diluted 50 times with LB to a final volume of 10ml, and grown in 37 ºC with shaking to OD 0.7. Negative controls (no induction) were taken at this time. The cultures were induced with 1mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and allowed to shake for 3h at 37 ºC. Aliquotes were run on an SDS-PAGE gel (NOVEX 4-12% Bis-Tris Mini Gel, Invitrogen) with 1x NuPAGE MES buffer system, to test for expression. The gels were stained by Coomassie Brilliant Blue R 250 (0.25g were dissolved in 40% MeOH and 7% HOAc) and then destained using 10 % (v/v) aqueous

acetic acid. For the samples that showed expression, this was repeated in a larger scale. Glycerol stocks of the BL21(DE3)Star cells containing the expression plasmid were prepared by taking 1 part of cell culture and 2 parts of 70% glycerol. Following expression, the cells were harvested by centrifugation at 7000 rpm for 20 min at 4ºC.

4.4 Purification:

The cell pellets were resuspended in NPI-10 binding buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole, pH 8.0). Hen egg lysozyme (0.1 mg/ml) was added to the cell pellets and the suspension was incubated on ice for 30 min. After the incubation period, the cells were sonicated (Branson S-250 analog sonicator, Sonifier) with a 13mm step horn, using maximum power 5 seconds on, 5 seconds off in a cycle for 2 min. The lysed cells were then centrifuged at 15000 rpm for 40 min at 4ºC to pellet cell debris. Preliminary experiments indicated that the proteins were expressed as inclusion bodies in all cases and were present exclusively in the pellet at this stage. The pellet was resuspended in NPI-10 binding buffer containing 8M urea to dissolve the inclusion bodies and centrifuged again. Now the protein was found in the supernatant.

The expressed proteins were purified from the supernatant, taking advantage of the histidine tagged N-terminus by using a 5ml Ni-NTA superflow cartridge (Qiagen) with an ÄKTA purification system (GE health care). First the Ni-NTA superflow cartridge was equilibrated with 10 column volumes (CV) of NPI-10 binding buffer containing 8M urea. Then the sample was loaded into the column, and then NPI-10 buffer containing 8M urea (2 column volumes, CV) was flushed through to wash out nonspecifically bound proteins at a flow rate of 5 ml/min. The bound protein was eluted using a gradient from NPI-20 (50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, 8M urea, pH 8.0) to NPI-250 (50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole, 8M urea, pH 8.0) over 10 CV. Column effluent was monitored by absorbance at 260 and 280 nm. During the gradient, fractions of 5 ml were collected. Fractions containing the proteins were identified by SDS-PAGE analysis and pooled.

The pooled samples were diluted 5 times to decrease the amount of imidazole (to a final volume of 100 ml,and a concentration of 50mM), and then loaded back onto a clean Ni-NTA column. Once the protein was bound, a gradient from 8M urea to buffers without urea was applied, this was to try to refold the proteins. Once the urea was removed, an imidazole gradient from 20mM to 250mM was applied, to elute the proteins.

To remove imidizole following the Ni-NTA column step, pooled fractions containing the protein were concentrated by ultrafiltration (Amersham) using a YM-3 membrane (3kDa cutoff membrane, Millipore). The concentrated sample was then diluted with a potassium phosphate buffer (50mM, pH 7) and concentrated again. This was repeated until the concentration of imidazole had been reduced from 250mM to < 1mM.

The protein concentration of the purified samples was determined by Beer's law using a UV-spectrophotometer (AVIV, model 14DS UV-VIS). The absorbance was measured at 280 nm. Extinction coefficients used for calculation was determined *via* a "Protein calculator" based on the method of Gill and von Hippel (17,18).

Protein	Extinction coefficient reduced (M^{-1} cm^{-1})	Extinction coefficient oxidized (M^{-1} cm^{-1})	MW (Da)	# Cys
Grd-5	9530	9770	9119	4
Grd-13	8960	9080	15383	2
Grl-16	38160	32220	37504	7
Grl-24	2560	2800	9856	5
Wrt-3	31150	31630	18738	8
Wrt-5	38120	38600	17844	8

4.5 Refolding

4.5.1 Air oxidation

Ni-NTA purified samples of Grd-13 and Wrt-3 were reduced with a 50 times molar excess of DTT, for 30 min. The samples were desalted using a PD-10 column (GE healthcare) with phosphate buffer and 0.5 ml fractions were collected. The fractions from the void volume with high concentrations of protein and no DTT were pooled. Protein concentrations were determined by UV-spectroscopy at 280 nm. Protein solutions(with a concentration of 3.3 mg/ml) were left to oxidize in air and sampled by taking aliquots after 0h, 2h, 4h, 8h, 16h, 32h, 64h and 128h. The samples were immediately quenched with H_3PO_4 (20µl to 40µl sample) to prevent further oxidation or disulfide exchange. Reversed phase HPLC (RP-HPLC) was used to separate the reduced proteins from the oxidized variants. An RP-18 column (Chromolith) with a gradient from 5% acetonitrile to 95% acetonitrile during 90 minutes was used. Column effluent was monitored at 214 and 280nm.

4.5.2 DTT redox buffer

First a test was performed to determine the kinetics of DTT in the air oxidation. Then, two versions of this auto oxidation experiment were performed without the protein present. First a stock solution was prepared. (50mM DTT, 8M urea, 100mM Tris and 2mM EDTA). In the first experiment the stock solution was diluted 10 times with water, creating time point zero. 10 µl diluted samples were taken and quenched with 5 µl H_3PO_4 after various time intervals. In the second experiment, a part of the stock solution was left to oxidize. When 10µl samples were taken, they were diluted 10 times with water and left to equilibrate for 1h and then quenched with 50µl H_3PO_4.

Grd-13 refolding was initiated by using the second version of the experiment (where the aliquot is diluted 10 times). All the samples were separated and analyzed by RP-HPLC. The gradient started with an isocratic segment (15% buffer B) for 5 min and then a gradient from 32% to 40% buffer B over 28 min.

4.5 CNBr cleavage at methionine residues:

A stock solution of 50 µM CNBr in 0.1M HCl was prepared with caution in the fume hood as this compound is extremely toxic. Three samples were prepared, one negative control with 10 µg of protein in 0.1M HCl to test for non-specific cleavage by solvent and two samples with 10µg of protein in 0.1M HCl and different concentrations of CNBr (5mM, 12mM). The protein was added to the CNBr mixtures as a 0.065mM solution. Since the protein concentration and the CNBr concentrations are known the molar ration for the CNBr added can be calculated. With 5mM concentration of CNBr there is an approximate 77 times molar excess of CNBr. With 12.5mM concentration of CNBr there is a molar excess of approximately 192 times. The samples were incubated overnight (wrapped in aluminum foil to minimize light catalyzed free radical reactions) and then frozen using a mixture of ethanol and dry ice. Samples were lyophilized, resuspended in H_2O and lyophilized again to remove all traces of the volatile acid and CNBr. This was repeated at least 3 times. The samples were then suspended in water and aliquotes were taken and prepared for SDS-PAGE gel. (5µl sample, 5µl 4xSB, 6µl H_2O). For each of the three samples, a sample with 5mM DTT and a sample without DTT were prepared. Samples were boiled at 95ºC for 10 min. The gel was run for 40 min, then stained with Coomassie Brilliant blue for 2h and then destained with 10% acetic acid for 1h.

5 Results & Discussion

5.1 Expression

The cloned grd, grl, and wrt genes were expressed to see if the cloning was correct and that the right protein was expressed. Grl-24 was the only protein was not express in any of the trials. Based on SDS PAGE analysis, grd-5, grd-13, wrt-3 and wrt-5 were all expressed.

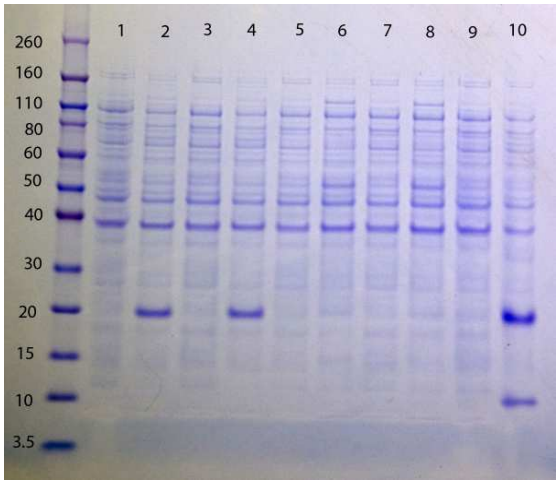


Fig 6 Test expression of grd-13 (lanes 1-4), grl-24 (lanes 5-8), and wrt-3 (lanes 9-10). Every odd numbered lane are uninduced and every even numbered lane are induced with IPTG for 3 hrs.

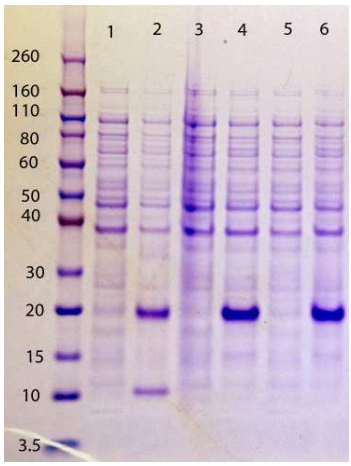


Fig 7 Test expression for wrt-3 (lanes 1-1) and wrt-5 (lanes 3-6). Odd numbered lanes are uninduced, even numbered lanes are induced with IPTG. All samples are treated the same with the exception that the even numbered lanes are treated with IPTG.

The computer program UN-SCAN-IT (Silk Scientific, Inc.) was used to digitize the gel bands and obtain a chromatogram-like representation for the differnet lanes (Fig. 8). From the graph of the lanes containing protein standards, a calibration curve could be obtained (Fig. 9). A fourth-order polynomeal equation was fitted to the scanned band positions as a function of migration distance using the IGOR program. The resulting paramaterized equation was used to calculate the molecular weights for the expressed proteins.

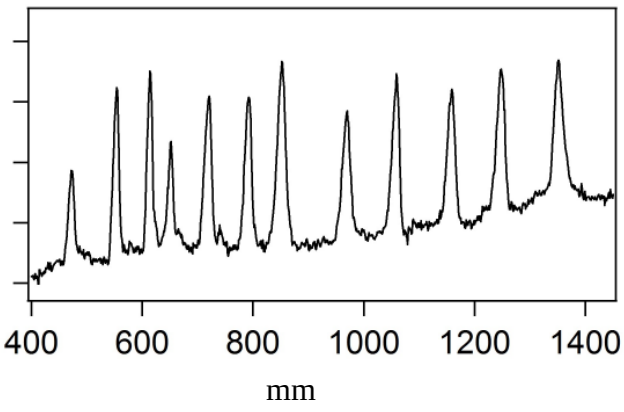


Fig 8 Chromatogram of the protein ladder, values from UNSCANIT

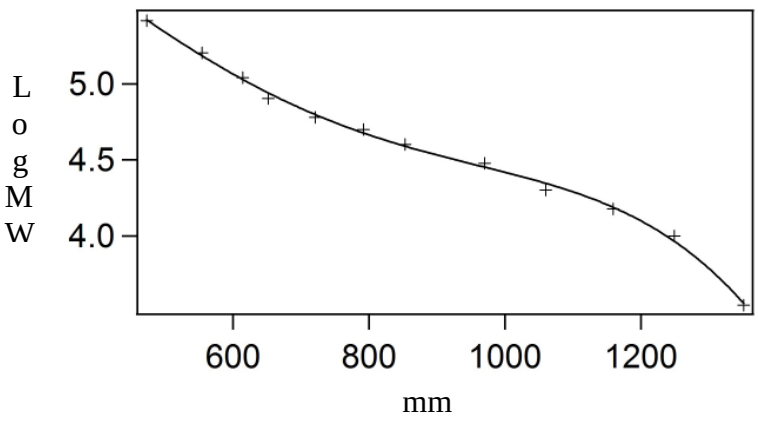


Fig 9 Curve fitting to the values from the protein ladder.

Protein	MW calculated UNSCANIT (Da)	MW calculated actual values (Da)
Grd-13	22021	15383
Wrt-3	23341	18738
Wrt-5	8mM)22475	17844
Grd-5	13163	9119

Table 3 shows the molecular weights calculated from UNSCANIT values. (mean value from the two double samples)

Table 3 shows the calculated values from UNSCANIT and the actual calculated values. The values from UNSCANIT were some kDa bigger than real values. It could be that the proteins have a conformation or charge distribution that makes them act bigger than they are on SDS PAGE.

5.2 Purification

A Ni-NTA column was used to purify the proteins by following protocol. The Ni-NTA column was first run on ÄKTA with buffers containing 8M urea to keep the proteins, expressed as aqueous insolubile inclusion bodies in solution. The fractions containing the protein were pooled and bound to a freshly equilibrated Ni-NTA column again. This time buffers without urea were used. Between these steps some of the protein was lost (Table 4). Several samples of grd-13 from different stages of purification (See Fig. 12) were loaded on a SDS gel to see the purity. The pooled protein fractions, now in buffer without urea, were concentrated and the imidazole concentration decreased by ultrafiltration. After this step, the protein concentration was measured by UV at 280nm.

Grd-13 and wrt-3 partially precipitated while stored in the freezer, so they had to be centrifuged to remove the precipitated proteins and the concentration remeasured. We were able to obtain the concentrations grd-13 (248 µM), wrt-3 (196 µM), wrt-5 (138 µM). Then they were stored at 4 C.

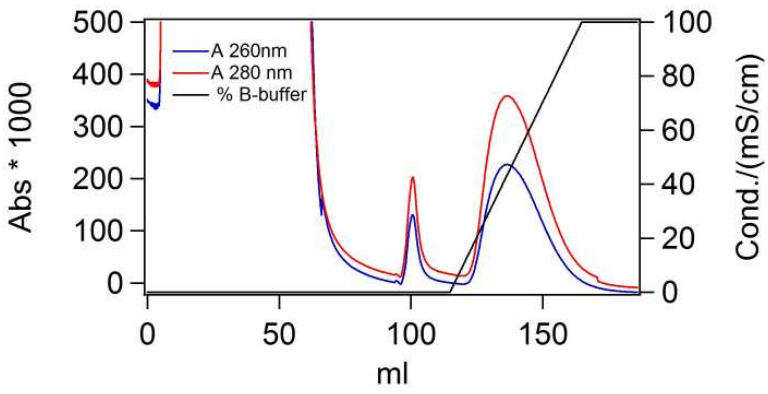


Fig 10 Representative chromatogram (grd-13) from Ni-NTA purification in the first cycle under denaturing conditions.

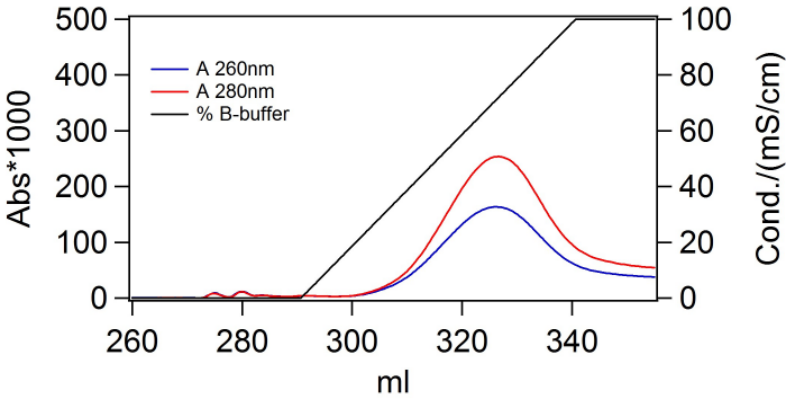


Fig 11 Chromatogram from Ni-NTA purification(grd-13). The second cycle under native condition.

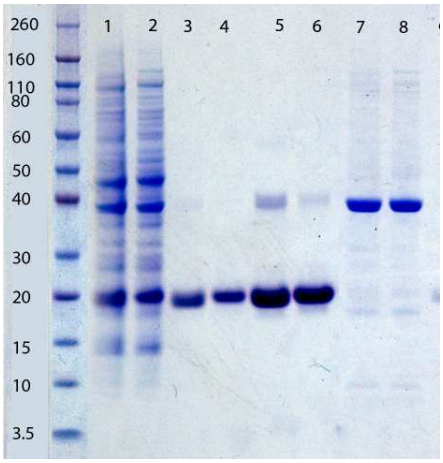


Fig 12 SDS PAGE of the Purification Process Samples after different stages of purification of Grd-13. Even numbered lanes are treated with DTT (to break any disulfide bonds), odd numbered lanes are not. Lanes 1,2 are flow through from the Ni-NTA column (cycle 1). Lanes 3,4 are from the small peak of of purification in Ni-NTA. Lanes 5,6 are from the large peak. Lanes 7,8 are from the pellet before dissolved and put on Ni-NTA column.

Table 4 Final concentrations after purification. The amount of protein after each step ofthe Ni-NTA purification

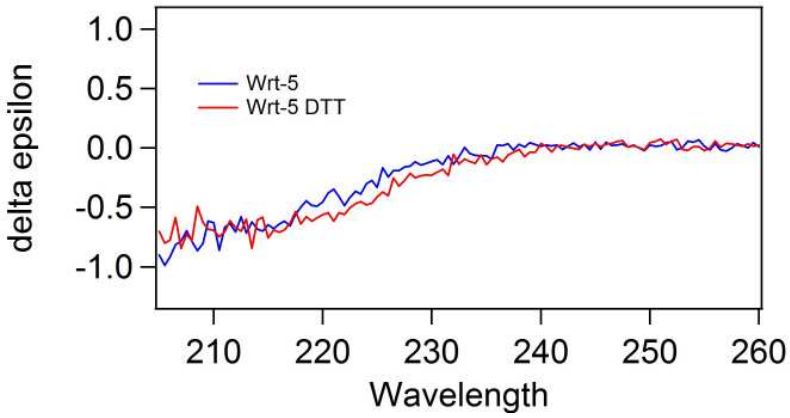
Protein	After first Ni-NTA (mg)	After second Ni-NTA (mg)
Grd-13	62	43
Wrt-3	71	43
Wrt-5	97	25

Fig 12 shows that the samples were > 95% pure after the final purification step. This is shown by the single bands in lanes 3-6. The amount of protein obtained after each step of the purification process is shown in table 4. Note the considerable loss between the first and second step of the purification.

5.3 Circular Dichroism Spectroscopy

CD spectra were taken of the purified wrt-3, wrt-5 and grd-13 proteins to determine if the proteins hade the predicted fold or if they were unfolded, and in need of refolding. The CD spectra shown in Figs. 18-20 are CD spectra to show the result of the refolding experiments.

Fig 18 CD spectrum wrt-5, blue line refolded sample, red line reduced sample.



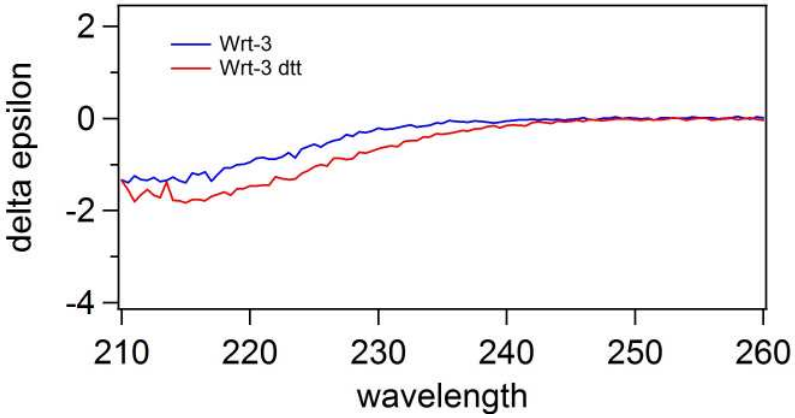
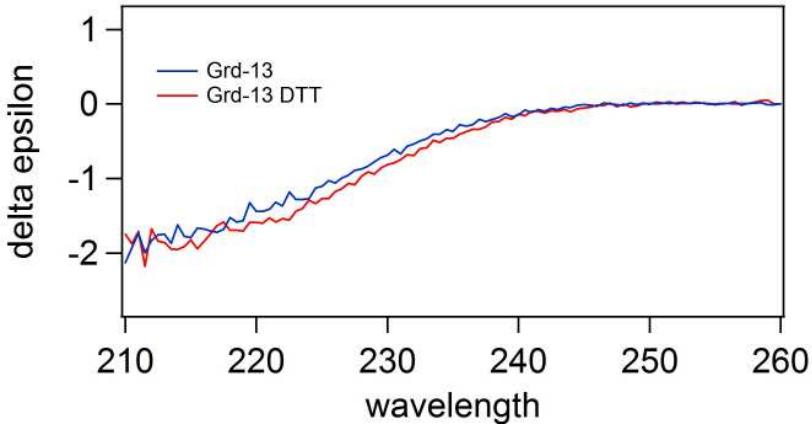


Fig 19 CD spectrum wrt-3, blue line refolded sample, red line reduced sample.

Fig 20 CD spectrum Wrt-5, blue line refolded sample, red line reduced sample.



Fore each protein, one sample was reduced with 6M urea and one sample with 50mM DTT. The 6M urea and 50mMDTT samples showed the same results. The reduced samples were compared with untreated protein samples. All the different proteins show a difference between the untreated and the treated samples. The delta epsilon value starts decreasing at a higher wavelength for the reduced (hopefully unfolded) samples than for the untreated samples (hopefully folded). This indicates that there is some folding present in the intreated samples. If the samples contained only correctly folded protein the delta epsilon should start decreasing at a higher wavelength (19). The results show a mix between correctly and uncorrectly folded proteins. This can be an indication that 6M urea or 50mM DTT may not be enough to fully reduce the protein samples. Due to some problems with the CD instrument, together with the high buffer background, values at wave lengths lower than 210 nm could not be obtained. It is in that region that the difference between unfolded and folded is showing the most.

5.4 Refolding

Since the CD spectra showed that the proteins were not in a homogenous, correctly folded state, some refolding experiments were made to try to get them correctly folded.

5.4.1 Air oxidation

Aliquotes from the refolding experiments were analyzed by reversed phase HPLC which can distinguish different disulfide bonded pairings and will be used to check for sample homogeneity (from this perspective). The chromatograms (Figs. 14 and 15) showed that something happened to the protein during the oxidation. But it did not shift in retention time when compared to fully oxidized Grd-13 and Wrt-3. Since the peak is also growing broader and not higher, this indicates that it is not likely a homogenous product after oxidation. It seams to be a mixture of many types of misfolded proteins – perhaps oligomers.

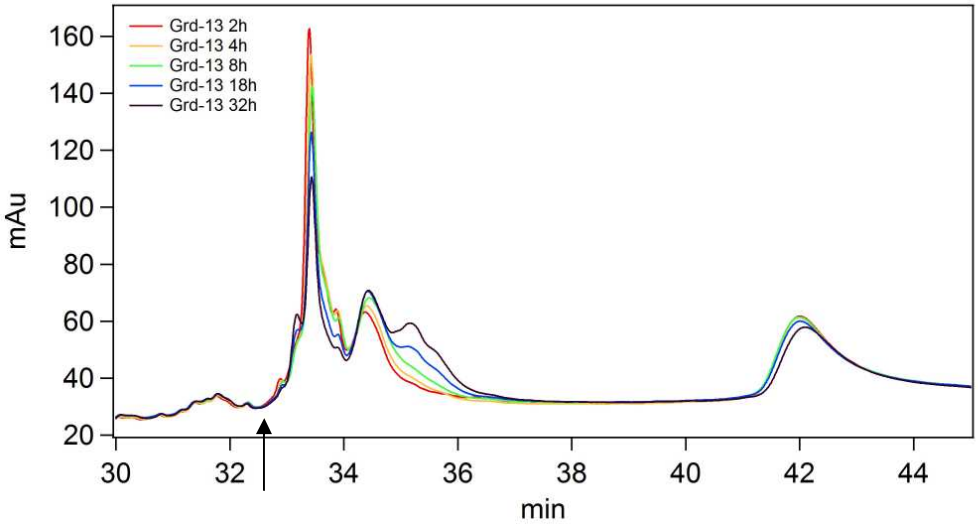


Fig 14 Chromatograms for the air oxidation experiment with Grd-13. Arrow indicates retention time for fully reduced Grd-13.

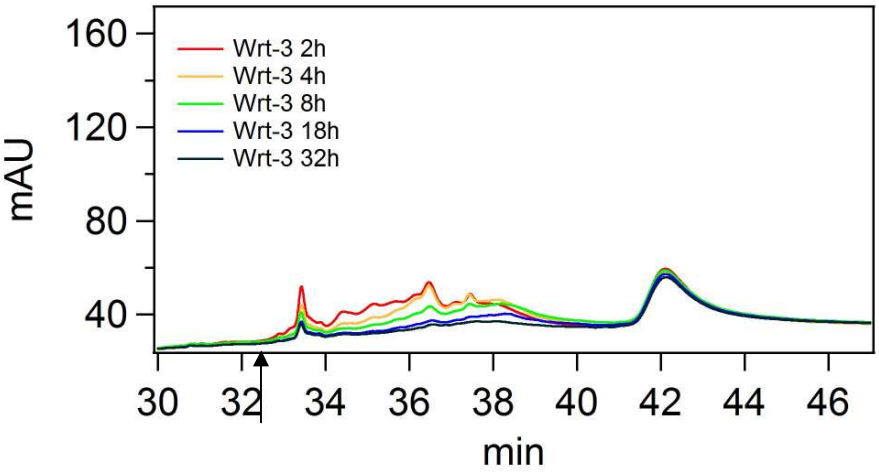


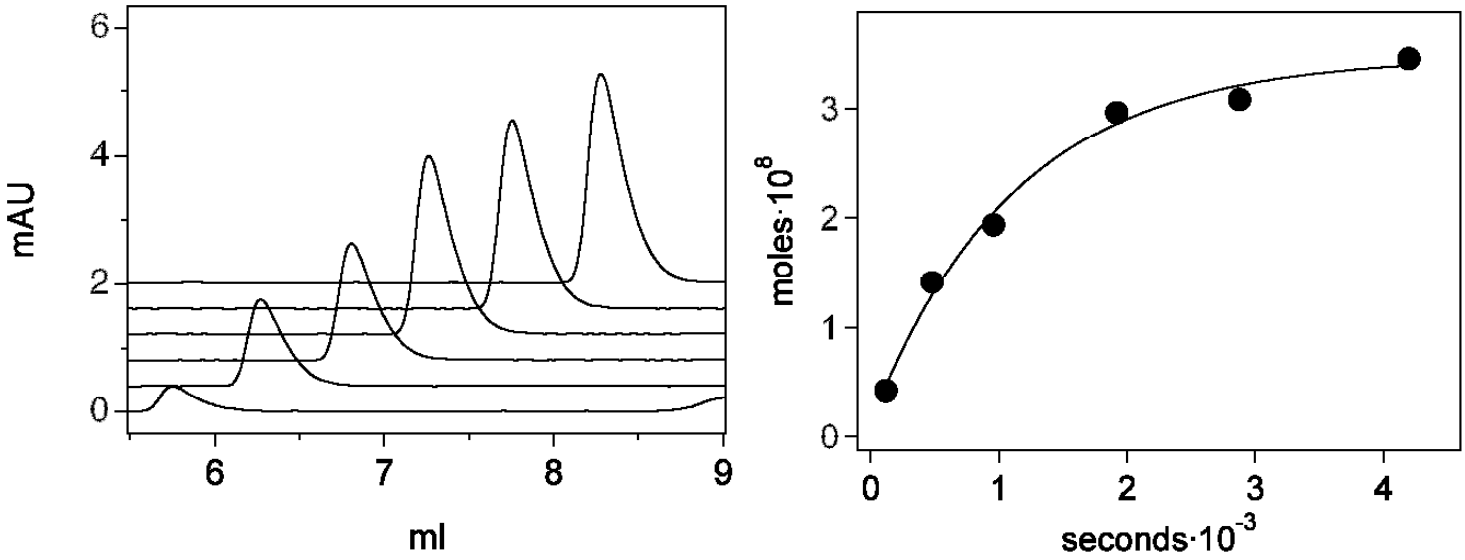
Fig 15 Chromatograms for the air oxidation experiment with Wrt-3. Arrow indicates retention time for fully reduced wrt-3.

5.4.2 DTT redox buffers

Taking advantage of the ability of thiols to air air oxidatize, an oxidation scheme was devised in which a starting concentration of reduced DTT would be allowed to oxidize in air thus creating a constantly changing redox buffer. Proteins in this buffer would then have a chance to equilibrate at these redox potentials. Since the redox potential of samples containing DTT changes over time, some calculations on DTT had to be made to get the redox potentials for the various samples. DTTox has a known extinction coefficient at 290nm. Therefore the measurements on DTT were made at that wavelength (se fig 16). The peaks for DTTox were integrated as a function of oxidation time. A plot of the amount of DTTox as a function of oxidation time is clearly a first-order reaction as evidenced by the fit of the experimental data points to a first-order equation (Eq. 7), by non-linear regression.

$$DTT_{ox} = DTT_{t=0} + DTT_{t=\infty} \times (1 - e^{(-k*t)}) \qquad \textbf{Eq 7}$$

The parameters obtained for the fit shown in Fig. 16B are $DTT_{t=0} = 1.40 \times 10^{-9} \pm 1.74 \times 10^{-9}$ mol., $DTT_{t=\infty} = 3.33 \times 10^{-8} \pm 1.81 \times 10^{-9}$ mol., and $k = 8.88 \times 10^{-4} \pm 1.47 \times 10^{-4}$ mol. sec^{-1}. By knowing the amount of DTTox the ratio between DTTox and DTT is known and the redox potentials could be calculated. (table 5)



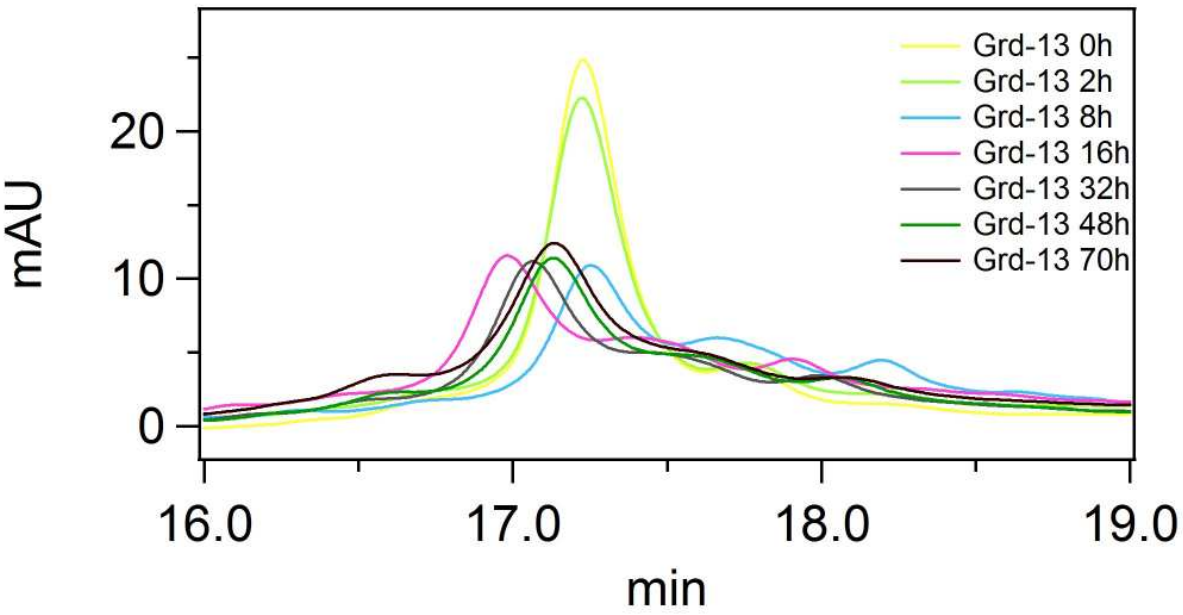


Fig 17 Grd-13 at 214nm. Treated with the DTT buffer.

Time (h)	Redox potential (mV)
8	-361
16	-336
32	-328
48	-308
70	-261

RP-HPLC chromatograms of aliquots from the Grd-13 refolding experiment indicate that the protein is changing over time as evidenced by shifting peak positions. The sample that was shifted the most was the sample after 16h, with the redox potential of -336mV. Since the samples are oxidized in a high concentration of urea, the disulfide bonds may not stay native (intramolecular) since the protein will not fold and stabilize the bond. This is the reason why the peak is shifting even after the protein has been in the right redox buffer. From these results a redox buffer of -336mV (Table 5) would seem to be the most effective at achieving intramolecular oxidation.

5.5 CNBr analysis of Grd-13

Cyanogen bromide is one of the few chemical methods for cleaving peptide chains (after methionine) that is specific enough to be useful in most cases. Used with care, it can be used to demonstrate the presence of a disulfide bond (providing there is at least one methionine between the suspected cystine pair). The most common solvent for cleavage is 70% formic acid which fulfills the low pH requirement of the reaction and is an excellent solvent for eventual peptide fragments (20-22). 70% formic acid is very reducing so it may also reduce the disulfide bond (20). A side reaction that will often occur with 70% formic acid is formylation of the peptide, thereby increasing it's mass and modifying nucleophilic amino acid side chains. To avoid this HCl can be used (22). 70% formic acid and other solutions like guanidine hydrochloride are good solvents except when serine or threonine follow the methionine. Instead of cleaving the polypeptide, a side reaction occurs that keeps it intact. CH_3CN/HCl in different ratios has been shown to decrease the side reactions.

Serendipitously, grd-13 is an excellent protein to perform this experiment on. It has a single methionine halfway between the two cysteine residues suspected to form the disulfide bond. When the CNBr cleaves the protein and then run on a SDS-PAGE gel, two different results can occur, depending on the state of the the two cysteine residues. First if there is a disulfide bond present a band will show with the same size as the whole protein. On the other hand if there is no disulfide bond present two smaller bands will show.

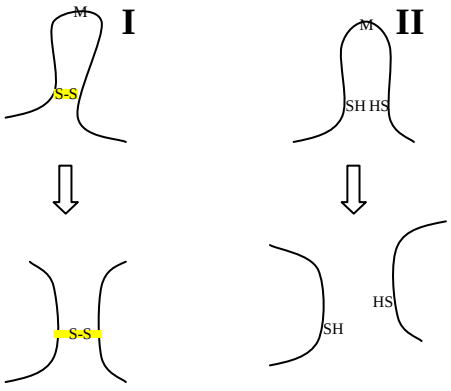


Fig 5 CNBr cleavage Shows how the two peptide fragments are held together after cleavage when a disulfide bond (yellow line) is present. II shows how the two peptide fragments are separated after cleavage when there is no disulfide bond.

The negative controls (without CNBr), both with and without DTT both showed a band with the molecular weight of approximately 21kDa (Fig. 13) Samples that had been treated with CNBr and then reduced with DTT showed a band at approximately 6kDa, approximately the size of the expected fragments N- and C-terminal to the single methionine (7.5kDa and 7.8kDa, respectively), indicating that at least some of the sample was cleaved. The CNBr treated sample without DTT showed a single band at 17kDa, approximately the size of the sum to the two fragments and approximately the actual size of the whole protein (15 kDa). Comparing the lanes containing the CNBr-cleaved material, with and without DTT indicates that there is a significant population of molecules containing a disulfide bond. If not, the CNBr treated sample without DTT should show the same band as the CNBr treated sample with DTT. The band in the negative control is somewhat larger than the band in the CNBr treated sample. One possible explanation is that the intact Grd-13 protein, due to its conformation, migrates at an apparent molecular weight larger than the expected value. When cleaved at the single methionine, this conformation is lost and the protein migrates closer to the expected value. While the SDS-containing sample buffer is expected to denature proteins allowing a uniform density of bound detergent, this may not be the case and could explain the abnormal migration.

What this experiment does not show is whether the disulfide bond is intramolecular or intermolecular since both cases would give rise to the same molecular weights. The absence of strong, unique bands at higher molecular weights makes this scenario less attractive.

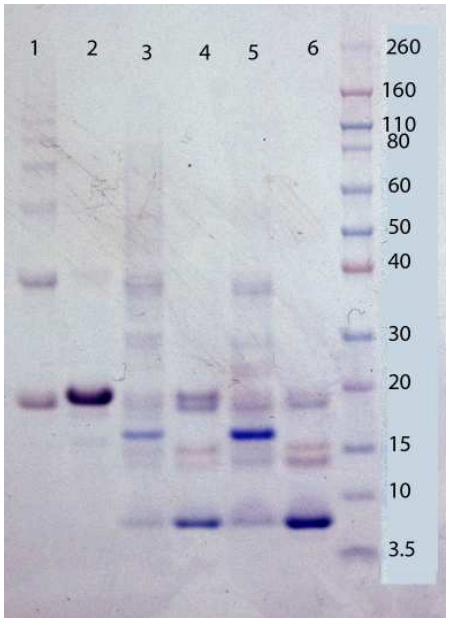


Fig 13 CNBr experiment. Even numbered lanes are treated with DTT, odd numbered are not. Lanes 1,2 negative control with only the HCl solvent. Lanes 3,4 treated with 5mM CNBr. Lanes 5,6 treated with 12,5mM CNBr.

6 Conclusions and future perspectives

All the proteins were successfully cloned into the pET-vectors as determined by sequencing. The test expressions were successful for all proteins except grl-24 which showed no detectable expression. Grd-5 was never used past this stage. Grd-13, wrt-3 and wrt-5 were all expressed in full scale and purified. CD spectra were taken for all the proteins but none of the proteins showed large amounts of secondary structures. Different techniques for refolding were attempted but none showed positive indications that it would be correctly folded. For grd-13, the DTT redox buffer refolding showed that -336mV could be a good redox potential of a DTT buffer if refolding is attempted again. The presence of a disulfide bond in grd-13 was shown by the cyanogen bromide experiment.

Ultimately, the goal is to find out how these proteins function in the signaling system of *C. elegans*. This project has contributed by cloning, expressing and purifying the proteins and starting to characterize the structure of the proteins. In the future for grl-24, different temperature and times of inducing can be applied to get it to express, then purification should not be a problem. Grd-5 should be expressed in a larger scale and then purified. The same technique that was used to get the approximate redox potential ford grd-13 could be used for the other proteins, then attempts to refold the grd-13 (-336mV) and the other proteins in that calculated redox buffer can be performed. The next step is to find a better way to detect the disulfide bonds and if the proteins are correctly folded and how they are folded. It is a matter of trial and error until we find one of the many available techniques that is good these proteins. Mass spectrometry one of these methods that definitely should be tested to determine the sequence of the protein.

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