Supporting Information
for

Chembiochem. 2015 Feb 9;16(3):393-396. doi: 10.1002/cbic.201402427. Epub 2015 Jan 23.
Shear-stress-mediated refolding of proteins from aggregates and inclusion bodies.
Yuan TZ, Ormonde CF, Kudlacek ST, Kunche S, Smith JN, Brown WA, Pugliese KM, Olsen TJ, Iftikhar M, Raston CL, Weiss GA.

This PDF includes:

General Materials and Methods
Table S1
Table S2
Figure S1
Figure S2
References

General Materials and Methods

Expression and purification of hen egg white lysozyme, caveolin-ΔTM, and HIV gp41

ORFs encoding hen egg white lysozyme (HEWL), caveolin-ΔTM (residues 1-104 with the following sequence fused to its *N*-terminus: MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRIRENLYFQG), the catalytic domain of PKA (residues 1-406 with the following peptide fused to its *N*-terminus: HHHHHHGS), and HIV gp41 (as described in reference[1]) were subcloned into the pET28c vector (GE Healthcare). Each protein was overexpressed in BL21 *E. coli* by induction when the cells reached an OD_{600} of 0.8 through addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM)[2]. The 1 l HEWL culture volume was centrifuged at 6000 rpm to collect the bacterial pellet. The pellet was reconstituted in lysis buffer (Table S2) and sonicated in 30 s continuous bursts with 1 min cooling on ice for eight cycles (20 watts). HEWL and caveolin-ΔTM were purified under denaturing conditions, and gp41 was purified under non-denaturing conditions. For specific expression and purification conditions, see Table S2. The egg whites were obtained from chicken eggs, and diluted 2:3 in PBS, heat-treated at 90 °C for 20 min, and dissolved in 8 M urea overnight at 4 °C. The His_6 tag was cleaved from HIV gp41 with Tobacco Etch Virus protease, which was then removed by immobilized metal chromatography (IMAC)[3]. Additional buffers and expression conditions are listed in Table S2. All protein concentrations were determined by bicinchoninic acid assay kit (Pierce).

Protein refolding and characterization

Commercial, lyophilized HEWL protein (Sigma) was reconstituted in PBS to provide the 'active' HEWL sample. Recombinantly expressed HEWL was pre-treated by 1:100 rapid dilution in PBS, and then refolded by VFD treatment. All samples were treated at 22 °C within a 16 cm long, 10 mm diameter glass test tube. When operated in confined mode, the VFD was set to a 45° tilt angle, and 1 ml was spun at 5 krpm, unless otherwise noted. The continuous mode experiment was conducted by flowing the rapidly diluted protein through the inlet port to the base of the sample at a flow rate of 0.1 ml/min. The caveolin-ΔTM VFD refolding was performed in confined mode (1 ml, 5 krpm, 22 °C). For comparison, caveolin-ΔTM was also refolded using conventional dialysis over 4 days (using a 1:500 dilution into 50 mM Tris-HCl, 1 mM EDTA, 4 °C, pH 8.5).

Circular dichroism (CD) spectra of HEWL were collected immediately following VFD refolding in PBS (20 nm/min, 4 accumulations), and caveolin-ΔTM CD spectra were collected in 10 mM sodium phosphate, pH 7.5 (10 nm/min, 8 accumulations)[4]. The lysozyme activity assays used the EnzChek assay kit (Invitrogen) after rapid dilution from of the protein solution described into PBS (1:100) according to manufacture instructions, except for decreasing the 37 °C incubation time from 30 to 10 min. Lysozyme activity was interpolated through fit to a calibration curve using active, commercial, hen egg lysozyme (#L7651, Sigma Aldrich) with Prism 6 software (GraphPad, Figure S1). The lysozyme activity was measured with various levels of urea, Tris, and NaCl to verify that the activity assay was not affected by such additives (Figure S2).

ELISA binding assays

The dose-dependent ELISA was conducted by coating gp41 (100 µl of 10 µg/ml in 50 mM sodium carbonate pH 9.6 for 4 h at 4 °C) on a Nunc Maxisorp 96-well microtiter plate. After removing the coating solution, a blocking solution of 0.2% non-fat milk in PBS was applied. Caveolin-ΔTM, anti-His mouse monoclonal antibody (Sigma, H1029), and anti-mouse HRP-conjugated polyclonal antibody (1:2000, Sigma, A5906) were diluted in 100 µl PT buffer (1:1000, PBS, 0.05% Tween-20) and incubated for 1 h at 4 °C with four wash steps using PT buffer (200 µl). The ELISA was developed by the addition of 1% w/v o-phenylenediamine dihydrocholoride in citric acid buffer (0.02% w/v H_2O_2, 50 mM citric acid, 50 mM Na_2HPO_4, pH 5.0), and the absorbance of the solution was measured at 450 nm using a microtiter plate reader.

Shear stress-mediated refolding of His-PKA bound IMAC resin and activity assays

The catalytic subunit of PKA was overexpressed in BL21 *E. coli* through induction with IPTG (1 mM) after cells reached an optical density of 0.8 (600 nm). This experiment utilized the residual pellet from a 12 l culture, a waste product more typically discarded. After dissolution in lysis buffer, sonication was applied as described above. His-PKA was then denatured in 6 M guanidine-HCl, 20 mM sodium phosphate, 500 mM NaCl and incubated with Ni^{2+}-charged Profinity IMAC resin (Bio-Rad) for 2 h at room temperature (1 ml of a 1.72 mg/ml His-PKA to 50 µl or 250 µl bed volume IMAC). A control experiment used uncharged IMAC resin instead. The IMAC-His-PKA solution was then diluted to 1 M guanidine-HCl with binding buffer containing 1 mM imidazole, or with the elution buffer containing 500 mM imidazole as a control. This diluted solution was immediately treated in the VFD (1 ml, 5 krpm, 20 min). After transferring to a 1.5 ml eppendorf tube, the resin was washed by aliquoting 1 ml wash buffer,

inverting the tube three times, and centrifuging the tube at 2000 $\times g$ for 2 min to separate the beads from the supernatant. This process was repeated two additional times before elution with elution buffer containing 500 mM imidazole. For protein quantification only, samples containing 500 mM imidazole were diluted 1:100 in wash buffer to prevent residual imidazole from interfering with the bicinchoninic acid assay[5].

The activity of the catalytic domain of PKA was determined using the following assay. As functional PKA depletes ATP during phosphorylation of the peptide Kemptide, the ADP-dependent activity of the enzyme lactate dehydrogenase could be monitored through observing loss of its co-factor, NADH[6]. Thus, PKA activity could be monitored as loss of NADH by measuring absorbance at 340 nm (for a 300 µl assay volume, 10 mM ATP, 0.5 mM NADH, 1 mM phosphoenolpyruvate, 0.0153 U/µl lactate dehydrogenase, 0.0269 U/µl pyruvate kinase, 0.67 mM kemptide, 100 mM MOPS, 9 mM $MgCl_2$, pH 7.0). Kemptide was synthesized by conventional solid-phase peptide synthesis. All other reagents were purchased from Sigma-Aldrich.

Fixed speed, variable time

90 °C treated egg white					Native egg white				
Time (m)	Speed (rpm x 1000)	Activity (U/mg)	Std. Dev.	%	Time (m)	Speed (rpm x 1000)	Activity (U/mg)	Std. Dev.	%
0	5	25.17	11.56	1.0%	0	5	2649.35	432.80	100.0%
2.5	5	238.78	36.62	9.0%	2.5	5	2482.34	298.14	93.7%
5	5	364.93	27.52	13.8%	5	5	2725.92	756.38	102.9%
10	5	125.62	15.14	4.7%	10	5	2064.42	168.00	77.9%
15	5	159.23	109.97	6.0%	15	5	2250.25	275.44	84.9%
30	5	0.00	0.00	0.0%	30	5	2158.75	212.99	81.5%

Fixed time, variable speed

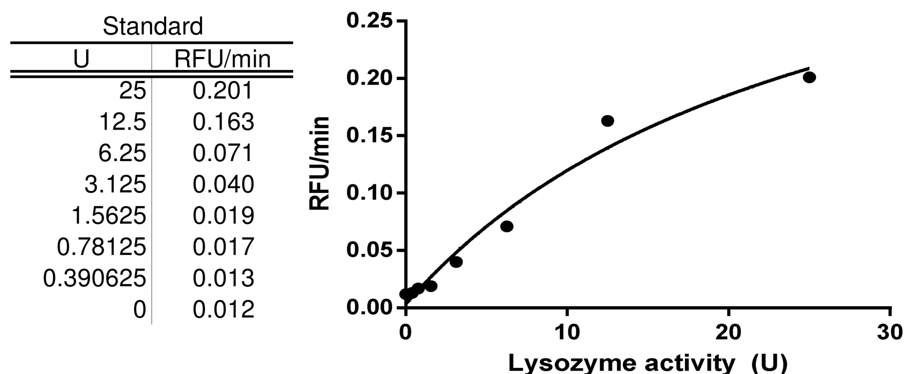
90 °C treated egg white					Native egg white				
Time (m)	Speed (rpm x 1000)	Activity (U/mg)	Std. Dev.	%	Time (m)	Speed (rpm x 1000)	Activity (U/mg)	Std. Dev.	%
5	0	16.11	10.34	0.6%	5	0	2479.12	211.10	100.0%
5	1	15.90	6.87	0.6%	5	1	1997.07	121.39	80.6%
5	2	15.32	6.11	0.6%	5	2	1680.03	82.96	67.8%
5	3	113.93	25.07	4.6%	5	3	1609.84	44.08	64.9%
5	4	70.14	17.69	2.8%	5	4	1653.37	120.44	66.7%
5	5	353.62	118.90	14.3%	5	5	1561.79	83.94	63.0%
5	7	288.21	81.54	11.6%	5	7	1568.70	264.62	63.3%
5	9	87.48	37.36	3.5%	5	9	1654.56	314.61	66.7%

Table S1. Recovery of lysozyme activity from boiled egg whites after VFD refolding. The lysozyme activity per mg of total protein following VFD refolding of boiled egg whites (treated at 90 °C for 20 min) or native egg white with fixed VFD speed at 5 krpm or fixed 5 min refolding time at the indicated VFD speeds (190 µg/ml total protein, PBS, 15 mM, GSH 0.5 mM GSSG, 22 °C).

Protein	Expression time (h)	Expression temp. (℃)	[IPTG] (M)	Lysis buffer
Hen egg white lysozyme	4	37	1	50 mM NaH_2PO_4, 500 mM NaCl, 10 mM imidazole, 1 mM HALT protease inhibitor (Pierce), 10 mM 2-mercaptoethanol, pH 8.0
Caveolin-ΔTM	3	37	0.5	50 mM Tris-HCl, 10 mM NaCl, 5 mM EDTA, 100 mM PMSF, pH 8.0
HIV gp41	8	22	0.5	50 mM NaH_2PO_4, 300 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM HALT protease inhibitor, pH 8.0
His-PKA	5	37	1	50 mM NaH_2PO_4, 500 mM NaCl, 10 mM imidazole, 1 mM HALT protease inhibitor, 10 mM 2-mercaptoethanol, pH 8.0

Protein	Resin	Denaturing buffer	Binding/wash buffer	Elution buffer
Hen egg white lysozyme	UNOsphere S (Bio-Rad)	20 mM Tris, 10 mM NaCl, 8 M urea, pH 7.8	20 mM Tris, 10 mM NaCl, 8M urea, pH 7.8	Wash buffer, 400 mM NaCl
Caveolin-ΔTM	Ni-NTA (Bio-Rad)	50 mM Tris, 50 mM NaCl, 8 M urea, pH 8.0	50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, 0.2% Na azide, 8M urea, pH 8.0	Wash buffer, pH 4.0
HIV gp41	Ni-NTA	-	50 mM NaH_2PO_4, 300 mM NaCl, 10 mM 2-mercaptoethanol, 20 mM imdazole, pH 8.0	Wash buffer, 250 mM imidazole
His-PKA	Ni-NTA	20 mM NaH_2PO_4, 500 mM NaCl, 6 M guanidine-HCl, pH 7.0	50 mM NaH_2PO_4, 300 mM NaCl, 1 mM imdazole, pH 7.0	Wash buffer, 500 mM imidazole

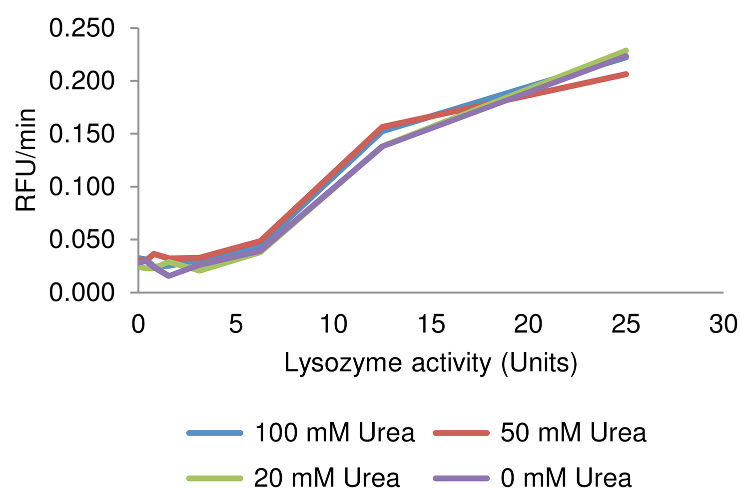
Table S2. Expression (upper) and purification (lower) conditions for recombinantly expressed proteins. The protein target used for ELISA binding studies, HIV gp41, was purified with non-denaturing conditions.



Standard

U	RFU/min
25	0.201
12.5	0.163
6.25	0.071
3.125	0.040
1.5625	0.019
0.78125	0.017
0.390625	0.013
0	0.012

Figure S1. Lysozyme activity interpolated by least-squares regression fit. The relative fluorescence units per minute was fit to standardized lysozyme activity assayed using the EnzChek Lysozyme Activity kit (Invitrogen). A calibration curve allowed interpolation of RFU/min data to lysozyme activity ($\frac{RFU}{min} = \frac{A*U}{B+U} + C$). All assays were conducted in 96-well black well microtiter plates, 100 µL reaction volumes, with 10 min incubation at 37 °C.



Rapid dilution	[Urea] (mM)	[Tris] (mM)	[NaCl] (mM)
1:80	100	0.25	5.0
1:160	50	0.125	2.5
1:400	20	0.05	1.0
PBS only	0	0	0

Figure S2. The stability of the lysozyme activity assay with low concentrations of urea, Tris, and NaCl in PBS. In this report, the lysozyme activity assay is applied to samples with a range of different additive concentrations. As shown here, such additives have little affect on the activity of lysozyme, measured by monitoring the fluorescence of fluorophore-labeled bacteria subject to lysis during lysozyme-catalyzed glycosidase activity. The relative fluorescence units were correlated with the concentration of active lysozyme in solution with varying concentrations of urea, Tris, and NaCl to simulate rapid dilution from a denaturing solution into PBS.

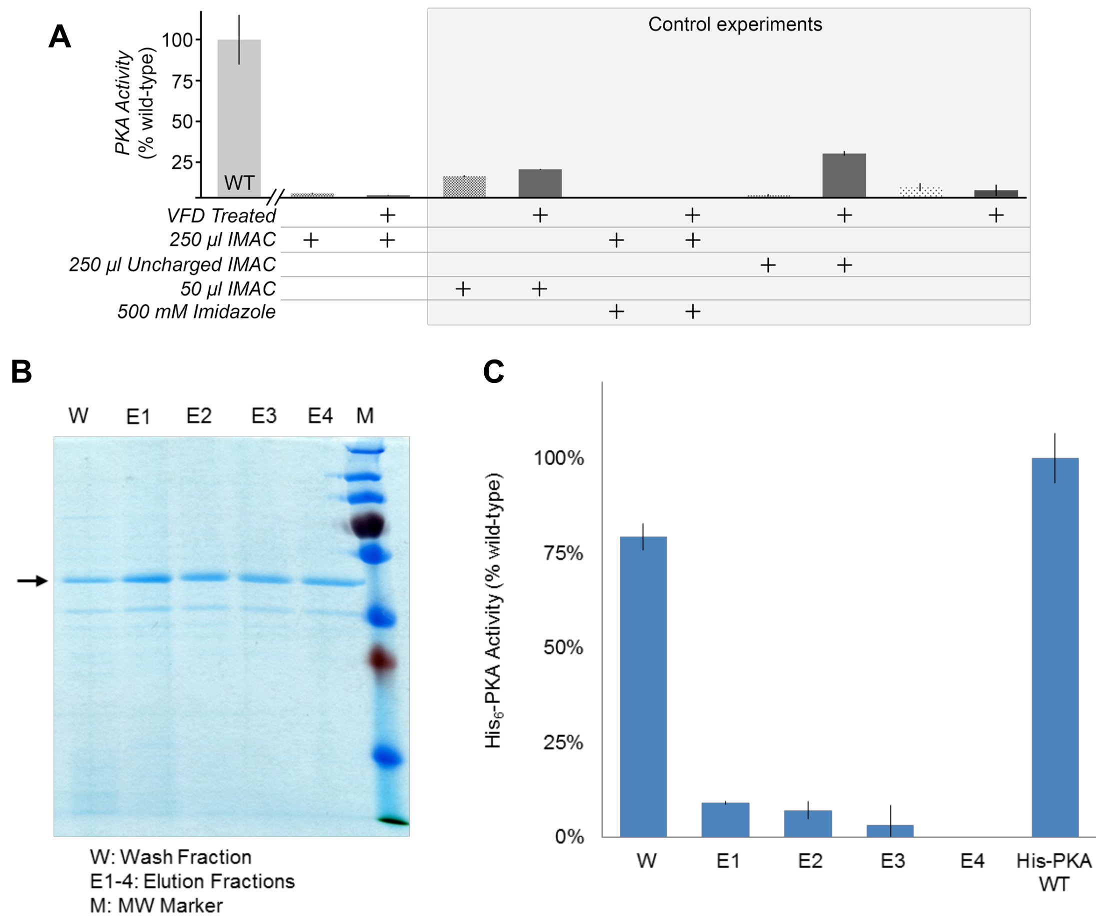


Figure S3. Kinase activity of PKA following VFD treatment. **A)** In this kinase assay, 1.7 mg PKA was pre-incubated with 250 or 50 µl IMAC resin in 6 M guanidine-HCl prior to dilution in 1 M guanidine-HCl and VFD treatment as described above. Excess PKA was removed by a low imidazole (1 mM) buffer (wash), and the remaining PKA was eluted by the addition of high imidazole (500 mM) buffer. Control experiments were performed with low resin, 500 mM imidazole, or uncharged IMAC resin during IMAC incubation, as described in the Figure 4 legend. **B)** PKA remaining bound to the IMAC resin during VFD treatment was then eluted from the resin (elution fractions E1 to E4) before dilution to decrease the imidazole concentration to 50 mM, and **C)** exhibited low PKA activity. Imidazole at concentrations up to 250 mM does not affect the kinase assay (data not shown).

References

[1] S. Majumdar, A. Hajduczki, R. Vithayathil, T. J. Olsen, R. M. Spitler, A. S. Mendez, T. D. Thompson, G. a Weiss, *J. Am. Chem. Soc.* **2011**, *133*, 9855–9862.

[2] R. S. Donovan, C. W. Robinson, B. R. Click, *J. Ind. Microbiol.* **1996**, *16*, 145–154.

[3] H. Block, B. Maertens, A. Spriestersbach, N. Brinker, J. Kubicek, R. Fabis, J. Labahn, F. Schäfer, in *Methods Enzymol.*, **2009**, pp. 439–473.

[4] N. Greenfield, *Nat. Protoc.* **2007**, *1*, 2876–2890.

[5] P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, D. C. Klenk, *Anal. Biochem.* **1985**, *150*, 76–85.

[6] K. Kiianitsa, J. A. Solinger, W. D. Heyer, *Anal. Biochem.* **2003**, *321*, 266–271.