Protein Yoctowell Nanoarchitectures: Assembly of Donut Shaped Protein Containers and Nanofibres

Andreas Schreiber,^{*a,b*} Ekaterina Zaitseva,^{*c*} Yi Thomann,^{*d*} Ralf Thomann,^{*b,d*} Jörn Djengel,,^{*e,f*} Ralf Hanselmann,^{*b*} and Stefan M. Schiller,*^{*a,b*}

^cBiophysics Section, Institute of Molecular Medicine and Cell Research, University of Freiburg, Hermann Herder Str. 9, D-79104 Freiburg, Germany.

^dFreiburger Materialforschungszentrum, University of Freiburg,

Stefan-Meier-Str. 21, 79104 Freiburg, Germany

Habsburgerstr. 49, 79104 Freiburg, Germany

Table of contents

1.	Protein expression and purificatio	n2
2.	MALDI-TOF measurements:	
3.	Circular dichroism spectroscopy	
4.	Variable temperature measurement	
5.	Intrinsic tryptophan fluorescence of Hcp1	
6.	Hcp1 layer on ultra flat gold	
7.	O ₂ Plasma	
8.	SDS-PAGE for monomer and hex	amer analysis
9.	Transmission electron microscopy	7 (TEM)
10.	Environmental scanning electron	microscopy (ESEM)3
11.	Atmoic force microscopy (AFM).	3
Figur	'es	Error! Bookmark not defined.
Figur	Figure 6 : ESEM-micrograph at 850:	
Figur	Figure 6 : ESEM-micrograph at 850: lyophilized Hcp1[6.11 μM]	Error! Bookmark not defined. x(A) and 9000x(B) magnification of Error! Bookmark not defined.
Figur	Figure 6: ESEM-micrograph at 850: lyophilized Hcp1[6.11 μM] Figure 7: SDS-Page of Hcp1	Error! Bookmark not defined. (A) and 9000x(B) magnification of Error! Bookmark not defined. Error! Bookmark not defined.
Figur	Figure 6: ESEM-micrograph at 850: lyophilized Hcp1[6.11 μM] Figure 7: SDS-Page of Hcp1 Figure 8: CD-spetroscopy of Hcp1 i	Error! Bookmark not defined. (A) and 9000x(B) magnification of Error! Bookmark not defined. Error! Bookmark not defined. n aqua dest
Figur	Figure 6: ESEM-micrograph at 850: lyophilized Hcp1[6.11 μM] Figure 7: SDS-Page of Hcp1 Figure 8: CD-spetroscopy of Hcp1 i Figure 9: CD-spectra of Hcp1 measure	Error! Bookmark not defined. (A) and 9000x(B) magnification of Error! Bookmark not defined. Error! Bookmark not defined. n aqua dest
Figur	Figure 6: ESEM-micrograph at 850: lyophilized Hcp1[6.11 μM] Figure 7: SDS-Page of Hcp1 Figure 8: CD-spetroscopy of Hcp1 i Figure 9: CD-spectra of Hcp1 measure Figure 10: Wavelength at maximal to	Error! Bookmark not defined. (A) and 9000x(B) magnification of Error! Bookmark not defined. Error! Bookmark not defined. n aqua dest
Figur	Figure 6: ESEM-micrograph at 850: lyophilized Hcp1[6.11 μM] Figure 7: SDS-Page of Hcp1 Figure 8: CD-spetroscopy of Hcp1 i Figure 9: CD-spectra of Hcp1 mease Figure 10: Wavelength at maximal to SDS and urea concentration	Error! Bookmark not defined. (A) and 9000x(B) magnification of Error! Bookmark not defined. Error! Bookmark not defined. n aqua dest
Figur	Figure 6: ESEM-micrograph at 850: lyophilized Hcp1[6.11 μ M] Figure 7: SDS-Page of Hcp1 Figure 8: CD-spetroscopy of Hcp1 i Figure 9: CD-spectra of Hcp1 measur Figure 10: Wavelength at maximal to SDS and urea concentration Figure 11-13: Atomic force micrographics	Error! Bookmark not defined. (A) and 9000x(B) magnification of Error! Bookmark not defined. Error! Bookmark not defined. n aqua dest
Figur	Figure 6: ESEM-micrograph at 850: lyophilized Hcp1[6.11 μM] Figure 7: SDS-Page of Hcp1 Figure 8: CD-spetroscopy of Hcp1 i Figure 9: CD-spectra of Hcp1 measure Figure 10: Wavelength at maximal to SDS and urea concentration. Figure 11-13: Atomic force micrograph Figure 14: TEM micrograph showing	Error! Bookmark not defined. (A) and 9000x(B) magnification of Error! Bookmark not defined. Error! Bookmark not defined. n aqua dest

^aFreiburg Institute for Advanced Studies (FRIAS), School of Soft Matter Research, University of Freiburg, Albertstr. 19, 79104 Freiburg, Germany

E-mail: Stefan.Schiller@FRIAS.Uni-Freiburg.de

Fax: +49 761 203 97451

^bInstitute for Macromolecular Chemistry, University of Freiburg, Stefan-Meier-Str. 31, 79104 Freiburg, Germany

^eFreiburg Institute for Advanced Studies, School of Life Science-LIFENET, University of Freiburg, Albertstr. 19, 79104 Freiburg, Germany. ^fCenter for Biological Systems Analysis, University of Freiburg,

1. <u>Protein expression and purification</u>

The plasmid pET 29b Hcp1Q54C (Mougous group) was transformed and amplified in E. coli Top10 and the sequence was confirmed by sequencing. E. coli strain BL21 (DE3) was used for expression of Hcp1Q54C. All bacteria where grown while shaking at 250 rpm on 2YT medium at 37°C until they reached OD₆₀₀ 0.7. At this point over expression was induced with 1 μ M IPTG and after 5 h at 37°C cells were harvested by centrifugation at 4000 x g for 20 min. After resuspension in lysis buffer (500 mM NaCl, 50 mM Tris 7.5, 10% Glycerol, 2 μ M β -mercaptoethanol and 10 μ g/ml lysozym per liter of culture volume) the bacteria where incubated on ice for 30 min, frozen and thawn. For further purification cells were sonified and loaded onto a Ni-NTA-column (*5 PRIME*) and eluted with 150-300 mM imidazol.

2. MALDI-TOF measurements:

All measurements were performed on *Bruker AutoflexIII TOF/TOF* and acquired in linear mode. Samples were transferred into 70% Acetonitril 30% aqua dest. 0.1% TFA and mixed with matrices either Sinapinic acid (SA) or dihydroxyacetophenone (DHAP). Preparations of the sample matrix was according to Bruker Daltonik, the dried droplet method: 50 μ mol 2,5 μ l DHAB in 375 μ l ethanol and 125 μ l (10 μ mol) aqueous diammonium hydrogen citrate solution. The protein solution (2 μ l), 2% TFA (2 μ l) and 2 μ l matrix solution were added to prepare the matrix sample mixture.

3. <u>Circular dichroism spectroscopy</u>

For CD-measurements the protein buffer was exchanged using *vivaspin* concentrators (10 kDa molecular cut off). The protein was measured on *Jasco 810 spectrometer* at 8°C in a 200 μ l solution at a protein concentration of 15.4 μ mol/l. All samples were measured at cell length, range, data pitch and band width of 10 mm, 300-180 nm, 0.1 nm and 1.00 nm, respectively. The scanning speed was 200 nm/min and 3 accumulations taken.

4. Variable temperature measurement

The same device and sample parameter were chosen as for CD-spectra. Heating and cooling occurred at 0.5° K/min while target temperature was kept +/-0.10°C for 5 seconds. Measurement started at 7.92°C temperature, heated up to 73.01°C and 45°C respectively and reversed back to 8°C and 131 data points were taken. Milli degree of the monitored wavelength 216.4 nm were recorded.

5. Intrinsic tryptophan fluorescence of Hcp1

As for CD-measurements protein buffer was exchanged and $100 \,\mu$ l of protein solution (1.54 μ mol/l) in MilliQ water was applied to a 10 mm quartz cuvette. The proteins were excited at 280 nm in a *Perkin Elmer LS45 luminescence spectrometer* and emission was recorded between 290-400 nm at a slit of 10 nm. Scanning speed was 300 nm/min and samples were kept at 8°C while 3 accumulative measurements were recorded.

6. <u>Hcp1 layer on ultra flat gold</u>

Reagents were used as purchased without further purification. N α , N α -bis(Carboxmethyl)-lysine hydrate (lysine NTA) was purchased from *FLUKA* and dithiobis-[succinimidylpropionate] (DSP) was bought from *Pierce*. For the protein-gold surface attachment a silicium wafer coated with titanium and an ultra flat gold layer was received from *PVD-coatings Georg Albert*. The polycristalline gold surface was of 30 nm thickness on a 100-4 inch wafer with rms-roughness of less then 1 nm. A 10 mM Lysin-NTA, 1 mM DSP 100 mM NaHCO₃ solution in dry DMSO was reacted for 2 h. Subsequently the reaction mixture and the clean gold substrates were incubated over night. The subtstrates were rinsed

with MilliQ water and exposed to a 50 mM NiSO₄-hydrate solution for 20 min. Before protein immobilisation the substrate was washed with MilliQ water and buffer.

Hcp1 (100 μ M) in buffer (250 mM NaCl, 25 mM Tris, 5% glycerol) was incubated with the Ni-NTA terminated monolayer for 2 h at 4°C and washed with milli Q water afterwards. For unspecific protein attachment Hcp1 [16.25 μ M] was incubated on the ultra flat gold surface for 2 hours.

7. <u>O₂ Plasma</u>

On the gold surface attached Hcp1 was plasma cleaned with *Plasma System Femto UHP* (*Diener electronics*) low pressure plasma cleaner. After O₂-purging the chamber for 5 min the sample was applied for 4 min at maximum level and subsequently an AFM image was recorded.

8. SDS-PAGE for monomer and hexamer analysis

Hcp1(45 μ g) on standard ten percent Tris/Glycine sodium dodecylsulphate SDS polyacrylamide gel electrophoresis (PAGE). In the stacking and resolving gel as well in running buffer SDS concentrations were adjusted to 0.025% and 0.1% depending on the analysis. Samples were applied without prior boiling nor DTT additive. Proteins were stained with coomasie blue.

9. Transmission electron microscopy (TEM)

TEM-measurements were performed on *Zeiss LEO 912 omega 120 kV*. Samples were prepared in 250 mM NaCl, 25 mM Tris, 5% glycerol at a protein concentration of 100 μ g/ml and negatively stained with 2% uranyl acetate. For imaging the protein was transferred on a carbon coated copper grid.

10. Environmental scanning electron microscopy (ESEM)

Hcp1 [6.11 μ M] in 5 ml MilliQ water was frozen in liquid nitrogen and lyophilized at 0.1 mbar over night. For ESEM images samples were sputtered with gold for 1 min and captured at 23 kV.

11. <u>Atmoic force microscopy (AFM)</u>

AFM Measurements are executed on a multi mode AFM with a *Nanoscope IIIa controller* (*Veeco DI Instruments*), where tapping mode and Si-cantilevers of super sharp type, with about 2-10 nm radius of curvature and 160 kHz resonance frequency, were used. Standard parameters were applied and varied to get optimized image quality.

Figures



Fig.6: ESEM-micrograph at 850x(A) and 9000x(B) magnification of lyophilized Hcp1[6.11 μ M] out of 5 ml MilliQ water sputterd with gold: Tube width of about 300-400 nm.



Fig.7: SDS-Page of Hcp1 (native) run in 0.025% SDS (A) and 0.1%SDS gel (B) and LMW-marker in kDa (C).



Fig.8: CD-spetroscopy of Hcp1 in aqua dest. milli degree at 216.4 nm at increasing tempertaure up to 45° C (A) and to 73° C (B) and reversed back at heating and cooling rate of 0.5° C/min.



Fig.9: CD-spectra of Hcp1 measured at different pH (5-10).



Fig.10: Wavelength at maximal tryptophane fluorescence depending on SDS and urea concentration.



Fig.11: Atomic force micrographs of a (A) Ni-NTA terminated monolayer and (B) Hcp1 bound to this self assembled monolayer. (SAM)



Fig.12: AFM micrographs A) O₂-plasma stripped Hcp1 layer B) Hcp1 bound to Ausurface via ionic interactions. C) Hcp1 bound to an ultrasmooth goldsurface.



Fig.13: AFM z- profil of Hcp1 bound to a gold surface.



Fig.14: TEM micrograph showing hexameric superstructures (hexamers of hexameric Hcp1 interacting in the x-y plane) and tubes of Hcp1 corresponding to a single hexameric donut shaped Hcp1 width (9 nm) eluted from a Ni-NTA column (after being loaded as monomers in solution of 1% SDS).