International Journal of Mass Spectrometry 277 (2008) 309–313

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

LILBID-mass spectrometry applied to the mass analysis of RNA polymerase II and an F_1F_o-ATP synthase

N. Morgner[a], J. Hoffmann[a], H.-D. Barth[a], T. Meier[b,*,1], B. Brutschy[a,*]

[a] Institute of Physical and Theoretical Chemistry, Goethe-University, Max-von-Laue-Str. 7, 60438 Frankfurt am Main, Germany
[b] Max-Planck-Institute of Biophysics, Max-von-Laue-Str. 3, 60438 Frankfurt am Main, Germany

ARTICLE INFO

Article history:
Received 26 March 2008
Received in revised form 30 July 2008
Accepted 6 August 2008
Available online 14 August 2008

Keywords:
Laser induced liquid bead ion desorption-mass spectrometry (LILBID-MS)
F_1F_o-ATP synthase
RNA polymerase
Membrane protein complexes
Proteomics

ABSTRACT

Mass spectrometry of large macromolecules is still a methodological challenge. We here report on the application of the recently developed LILBID (laser induced liquid bead ion desorption) mass spectrometry by which the biomolecules dissolved in microdroplets are desorbed/ablated by a mid-IR laser into vacuum. Two modes of desorption are possible: an ultrasoft mode at low laser intensity in which a macromolecule is desorbed as integral complex into vacuum and a harsher mode at higher intensity, by which it is dissociated into its covalent subunits. With this method we studied the soluble core polymerase II and a membrane-embedded F_1F_o-ATP synthase, solubilized by detergent. For both complexes the complete complex in different charge state is observed at ultrasoft conditions. At elevated laser intensities all 10 subunits could be assigned for the core Pol II. In the case of the ATP synthase under equal conditions all eight subunits appear in the mass spectrum, assigned by a correspondence of the expected theoretical masses of the subunits and the observed ones. In addition the method requires only sample volumes of microliter at micromolar concentration and is tolerant to detergents. Therefore it is a low consumptive method well adapted for the mass analysis of biomolecules of low availability such as membrane molecules.

1. Introduction

We recently developed a laser desorption mass spectrometry method [1] called LILBID (laser induced liquid bead ion desorption), in which biomolecules are laser desorbed/ablated from liquid microdroplets of solution. The method proved to be of nearly arbitrary softness, allowing to detect both specific weakly bound complexes at low laser intensity and also their covalent subunits at elevated intensity level. One of its major advantages is the low analyte consumption amounting to only few microliters of solution at micromolar concentration per analysis, making it an ideal tool for analysing biomolecules of low availability. This has been demonstrated recently for both large membrane-embedded molecules [2,3] and specific complexes of DNA and RNA with ligands [4]. Another important advantage is the tolerance of the method to various buffers in solution, which often play a crucial role in determining binding selectivity and strength as e.g., in the case of DNA/RNA–ligand complexes [5]. Here we demonstrate the power of LILBID to analyze two large protein complexes: the water-soluble RNA polymerase II (m/z = 443 kDa) and a large membrane-inserted molecular machinery, the ATP synthase (m/z = 542 kDa) from *Bacillus* sp. strain TA2.A1. In the latter case, not only the mass of the integral complex but also mass fingerprints of the individual subunits are of great interest and play an important role in the characterization and structural analysis of this enzyme as recently demonstrated [3]. Both enzymes described here play a leading role in life processes: polymerase II is the transcriptional machinery of eukaryotic cells, transcribing DNA into mRNA, while the ATP synthase converts ADP and phosphate into ATP, the universal energy currency of all cells.

2. Method

LILBID is a mass spectrometry method which allows an exact mass determination of single macromolecules dissolved in droplets of solution containing an adequate buffer, pH, ion strength, detergents etc. The details of the setup have been described elsewhere [1,5]. Briefly, droplets of solution of analyte are ejected by a piezo driven droplet generator. After their transfer into high vacuum they are detected droplet by droplet (ϕ = 50 μm, V = 65 pl, 10 Hz) by laser desorption/ablation. The laser is tuned to the stretching vibration of water at 2.9 μm. The total volume of solution required for a

* Corresponding author. Tel.: +49 69 79829587; fax: +49 69 79829560.
E-mail addresses: brutschy@chemie.uni-frankfurt.de (B. Brutschy), thomas.meier@mpibp-frankfurt.mpg.de (T. Meier).
[1] Tel.: +49 69 63033038; fax: +49 69 63033002.

1387-3806/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijms.2008.08.001

mass determination is only a few microliters in typically micromolar concentration. Thus amounts in the picomol or sometimes even femtomol range can be sufficient for an analysis. For these reasons, the method is ideal for studying biomolecules of low availability [1,2]. The amount of energy transferred into non-covalent complexes by the IR desorption/ablation process can be controlled in a wide range, starting from ultrasoft to quite harsh conditions, just by varying the laser intensity [2,3]. At ultrasoft conditions large macromolecules may be detected in their native stoichiometry. The complexes are detected in different charged states, preferentially as anions. The number of charge states observed increases with the size of the molecules, but is less than those observed in electrospray ionization (ESI) and considerably more than in matrix assisted laser desorption/ionization (MALDI). The mass measurements of ions up to an m/z of one million are based on a custom-built, novel Daly type ion detector applicable both for anions and cations and incorporating in addition helpful ion optics for the discrimination of metastable fragments. The 2.9 μm laser radiation is provided by a custom-built Nd-Yag pumped $LiNbO_3$-OPO with pulse energies of typically 30 mJ. The mass spectrometer is a home-built reflectron TOF-analyzer. Since the irradiation of the droplet at laser intensities of typically few MW/cm^2 induces an explosion of the solvent, the released ions have an initial velocity of about 800–1000 m/s. This hampers the resolution in a reflectron TOF and indeed for the present measurements the mass resolution $m/\Delta m$ of typically 100 is far from state of the art. It can be increased by orders of magnitudes by thermalizing the ions by collisions in an RF quadrupole followed by an orthogonal extraction into a TOF spectrometer, which is presently under construction.

3. Materials

The present species of polymerase II is a deletion version of the holo-polymerase II in which the two subunits Rpb4 and Rpb7 were removed. The polymerase was expressed and purified in the Lab of P. Cramer (LMU Munich) according to the purification procedure described [8]. The LILBID measurements were done with 1.6 μM polymerase II in 5 mM ammonium acetate at pH 7.5 or with 1.44 μM polymerase II with 100 mM ammonia at pH 10.5.

The F_1F_o-ATP synthase from *Bacillus* sp. strain TA2.A1 was heterologously expressed in *E. coli* cells and purified via immobilized metal affinity chromatography (IMAC) as described [10]. The ATP synthase-containing elution fractions of the Ni–NTA column procedure were pooled and precipitated by adding polyethyleneglycol (PEG) 6000 to 15% (w/v) for 14 h at 4 °C. The precipitated enzyme was collected and resuspended in 1 ml of 10 mM Tris–HCl (pH 8.0) and 0.05% DDM per 2 l of cell culture. The final purity of the enzyme was >95% with a specific activity between 30 and 40 U/mg. The LILBID spectra were recorded with a 5-μl aliquot at a protein concentration of 2.5 μM (approx. 0.5 mg/ml) in buffer containing 5 mM ammonium acetate (pH 8) and 0.05% dodecyl maltoside obtained by dialysis.

4. Results and discussion

4.1. RNA polymerase II

To demonstrate the power of LILBID to mass analyze large macromolecules we first studied RNA polymerase II, which is the central machinery in the transcription of DNA. Transcription is the process in which the information stored in a DNA is activated by synthesis of complementary mRNA by polymerases. RNA polymerase II (Pol II) is an enzyme found in eukaryotic cells. It is a 514-kDa complex and comprises 12 subunits with more than 28,000 atoms. Pol II is the best studied type of RNA polymerase and its structure was elucidated at a resolution of 2.8 Å by P. Cramer, D.A. Bushnell and R. Kronberg [6], for which the latter received the Nobel Prize in 2006. The polymerase II studied by them was from baker's yeast (*Saccharomyces cerevisiae*), which often serves as a model for eukaryotic organisms. According to the analysis of Pol II by these authors the complex has topological and functional regions, which are designated as cleft, saddle, foot, funnel etc. Here we studied Pol II Δ obtained from a deletion strain and consisting of 10 subunits (mass m/z = 470 kDa) instead of 12 comprised in Pol II. The difference to native Pol II is that the subunit Rpb7 was genetically deleted and with it also the subunit Rpb4 is missing, since Rpb4 is part of a subcomplex with Rpb7 that binds to a pocket formed by RPB1, RPB2 (see insert in Fig. 1). This fragment of the polymerase II is commonly called core polymerase (Pol II Δ).

By using ESI-mass spectrometry Heck and co-workers [7] were able to study holo-Pol II complexes in a charge distribution between 54 and 41 negative charges. By selecting a complex ion of defined charge state and by applying tandem mass spectrometry they were able to mass analyze 8 out of 12 subunits. However the largest subunits Rpb1, Rpb2, Rpb3 of the complex could not be detected by them. It should be noted that in their case they analyzed the full polymerase II and correlated the observed subunits of Pol II with its structure. In addition they analyzed Pol III containing 17 subunits.

The surface of Pol II is almost entirely negatively charged except for the uniformly positively charged lining of the cleft and active centre of Rpb1 (part of the shelf module) and the wall and saddle of

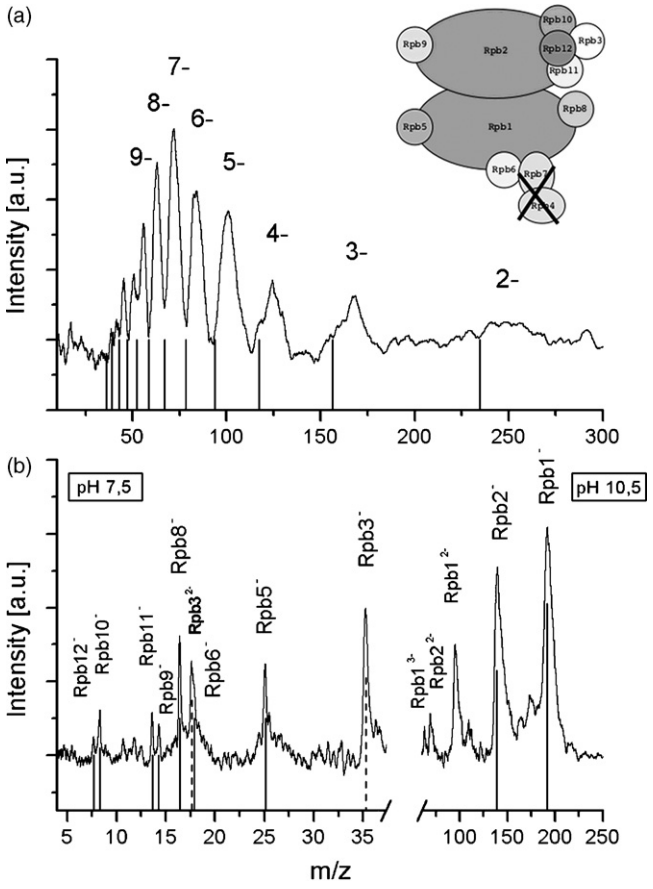

Fig. 1. (a) LILBID anion spectrum of the core polymerase Pol II Δ (see text). The spectrum has been recorded at ultrasoft conditions when the total mass is increased by solvent, buffer, divalent ions etc. (b) at elevated laser intensity the bare subunits appear. While Rpb1 and Rpb2 were only observed at a pH of about 10 the other 8 subunits are visible already at pH 7.5.

Table 1
Subunits and the masses of Pol II and Pol II Δ

Subunit	Theoretical mass (kDa)	Experimental mass by ESI [7] (kDa)	LILBID mass (kDa)
Rpb1	191.6	–	191.8
Rpb2	138.8	–	139.7
Rpb3	35.3	–	35.3
Rpb4	25.4	25.46	–
Rpb5	25.1	25.127	25.1
Rpb6	17.9	17.902	17.9
Rbp7	19.1	19.062	–
Rpb8	16.5	16.425	16.5
Rpb9	14.3	14.265	14.3
Rpb10	8.3	8.28	8.3
Rpb11	13.6	13.66	13.6
Rpb12	7.7	7.692	7.65
ΣPol II Δ	469.1	–	470.2
ΣPol II	513.6	–	–

Rpb2 [6]. Hence these positive charges may be one of the reasons why ESI had problems with these subunits in the anion mode.

With LILBID, however, it is very easy to observe anions of polymerase. Fig. 1(a) shows the mass spectrum in the ultrasoft mode.

The ion distribution corresponds to the integral core Pol II Δ corresponding to an m/z of 470 kDa. The theoretical masses appear at the low mass side of each charge state peak because in this ultrasoft mode the masses are often shifted to higher values by solvent and buffer molecules and metal ions, which stay attached after the desorption process [2]. This adduct covering of the macromolecule has to be accepted in order to keep the weakly bound complexes intact. At harsher laser desorption conditions the mass spectrum obviously changes, showing now narrow single mass peaks, as depicted in Fig. 1(b). All peaks can be assigned to known masses, calculated for the polymerase and assigned in the publication of Heck and coworkers. Their masses correspond exactly to the values observed with LILBID. As evident from Table 1, Heck and co-workers [7] could not observe the subunit Rpb3 as well as the two largest subunits Rpb1, Rpb2 which form the active centre and exhibit large areas of positive charge. These three subunits form together with Rpb11 the evolutionary conserved core that is also found in bacterial RNA polymerase.

In case of the LILBID spectra recorded at pH 7.5 Rpb1, Rpb2 could also not be detected. When ammonia was added to the buffer, increasing the pH to about 10.5, strong signals for Rpb1, Rpb2 appeared as shown in the right part of Fig. 1(b). The sum of the theoretical masses of all subunits of the core Pol II Δ corresponds to a total mass of 470 kDa.

4.2. F_1F_o-ATP synthase

For the second LILBID case study presented in this work we have chosen the membrane-associated F_1F_o-ATP synthase, which has been under investigation for already more than 40 years. This protein complex is the main producer of the universal energy currency adenosine triphosphate (ATP) in all living cells and can be found in membranes of mitochondria, chloroplasts, and bacteria. It can be divided into two main subcomplexes, a water-soluble F_1 harboring the subunits $\alpha_3\beta_3\gamma\delta\varepsilon$ and the membrane-embedded F_o part, which in its simplest version has the composition ab_2c_n. The crystal structure of the bovine F_1 complex has a mushroom-like shape with a headpiece made of three alternating α and three β subunits [11]. It wraps around an extended α-helical γ subunit, which forms an asymmetric stalk together with the ε subunit. In accordance with the 'binding change mechanism' [12], the central stalk rotates within the $\alpha_3\beta_3$ cylinder which ultimately leads to the synthesis of three ATP molecules per full turn. The F_1 rotary machine is tightly coupled with the F_o complex, which is a rotary motor by itself. The synthesis of ATP requires energy. The F_o-ATP synthase is able to convert the electrochemical H^+ or Na^+ gradient across the membranes into rotation and thereby transports these ions across the membrane [13–15]. Two F_o subunits are directly involved in ion translocation: the stator-subunit a and the oligomeric assembly of c subunits [16] which together with the γ and ε subunits represent the rotor element of the enzyme [13]. It has turned out in the past, that the c-ring stoichiometries vary from 10 to 15 c subunits, depending on the species. This variance is meaningful; it has a direct impact on the 'ion-to-ATP-ratio' a parameter for the bioenergetic efficacy of cells. LILBID turned out to be a time-saving and accurate method for the determination of these rotor ring sizes. We were able to elucidate the so far unknown tridecameric stoichiometry of the proton-driven rotor ring from the thermoalkaliphilic *Bacillus* sp. strain TA2.A1 F_1F_o-ATP synthase [3]. In the present work, we have applied LILBID to measure the integral F_1F_o-ATP synthase from the same bacterial strain and we were also able to dissociate the complex into its different subunits at elevated laser intensities. This F_1F_o-ATP synthase comprises in total 25 subunits in the stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_{13}$ giving a total of 77,222 atoms ($C_{24,172}H_{39,159}N_{6,563}O_{7,162}S_{166}$) with a molecular mass of 541,635.4 Da. However, the theoretically calculated mass obtained from the single amino acid sequences of these different eight subunits derived from the raw DNA sequence of the *atp* operon [17] might deviate marginally from the real mass of the enzyme present in a living bacterial cell.

Table 2
Theoretical and measured masses of the *Bacillus* sp. strain TA2.A1 F_1F_o-ATP synthase and their single subunits (calculator: www.expasy.org/tools/protparam.html)

Subunit (s)	Theoretical mass (Da)	LILBID mass (kDa)
F_1F_o ($\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_{13}$)	541,635.4	542.0
α	54,955.8	55.0
β	51,035.0	51.2
γ	31,891.5	31.9
δ	20,284.6	20.3
ε	14,956.3	14.9
a	26,650.8	26.6
b	19,503.7	19.6
c	7,023.4	7.1

Fig. 2(a) shows a mass spectrum of the complete ATP synthase in a charge distribution of up to six charges. The peaks are comparably narrow, without the typical broad shape usually observed if a lot of detergent molecules stay attached. So most of the complexes remain intact even under conditions where most of the detergent and water molecules are already stripped off. Some fragmentation has occurred though, giving some signals in the low mass region. Under higher laser intensity all complexes are dissociated into their subunits as depicted in Fig. 2(b). The mass region between 0 and 60 kDa is spread and shown in the inset for better recognizability. The inset spectrum is combined out of three different mass spectra each measured under optimised conditions for the respective mass region. All eight subunits are visible (Table 2), out of which the single α and β subunits give the most intense signals and appear up to five times charged. This is not surprising since three copies each are present per enzyme and additionally both have low theoretical pI values (between 5 and 5.5) and therewith high net charges at pH 8 and represent the lion's share of the surface from the water-soluble F_1 subcomplex $(\alpha\beta)_3$. Therefore, at high laser intensity, a strong signal from these two disintegrated subunits seems feasible [2]. However, the other subunits can only be observed singly charged. Additional peaks appear which can be assigned to oligomeric c_n subcomplexes (n = 1–4), stemming from the incompletely disintegrated c-ring as documented earlier [3].

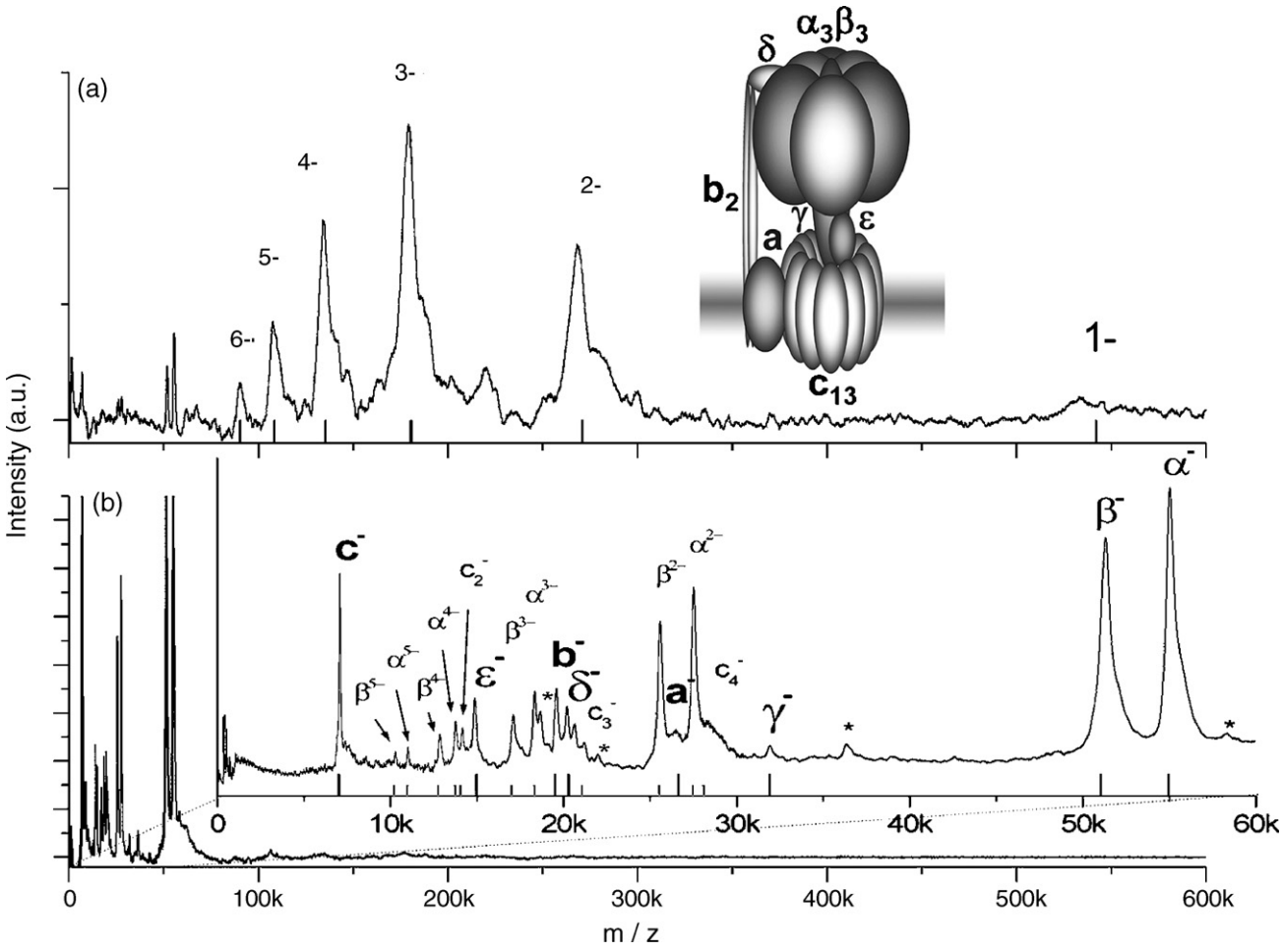

Fig. 2. (a) LILBID anion spectrum of the intact ATP synthase measured under soft conditions. (b) Using higher laser intensities all eight subunits are visible (indicated by black marks). Additionally the α and β subunits appear multiply charged and fragments consisting of oligomeric *c* subunits (c_n) are visible as well (shorter, gray marks). Peaks indicated by asterisks could not yet be accounted for and probably represent minor impurities from the enzyme preparation.

However, fully assembled *c*-rings (c_{13}) as well as other F_1F_o subcomplexes could not be detected at the lower laser intensity level.

5. Conclusion

With LILBID-mass spectrometry we studied large macromolecules, which consist of many non-covalently bonded subunits. As example we first studied the water-soluble RNA polymerase II Δ, an enzyme involved in the transcription of DNA to mRNA and consisting of 10 subunits. The second example is an archetypical membrane-embedded protein complex. The ATP synthase, or complex V, we used in this study comprises in total 25 molecules forming a ATP producing nanomotor driven by the proton motive force which is usually produced by the previous four complexes of the respiratory chain. To demonstrate the variabitlity in softness of the LILBID process both macromolecules have been studied first under ultrasoft conditions, where the integral protein complexes appear in the mass spectrum in several charge states. Under these conditions adducts from buffer, detergent and solvent cannot be completely avoided. At harsher desorption conditions, i.e., at higher intensity of the IR laser, the complexes dissociate into their single subunits and therefore lose all the above mentioned adducts. Here LILBID demonstrates its particular advantage for the mass determination of highly hydrophobic subunits.

From these mass fingerprints, new stoichiometries of such complexes may be elucidated. This represents a particular need e.g., in the structural characterization of new protein complexes as well as for an efficient checking of different complex assemblies e.g., after the deletion of regulatory genome parts. Furthermore, this method also has a large potential for future approaches towards the characterization of proteomes in large scale.

Taken together the two examples presented in this work clearly demonstrate several striking advantages of our technology if compared with commonly used mass determination methods: LILBID allows the mass determination of non-covalently assembled, detergent solubilized proteins, consisting of complex stoichiometric compositions at least up to the 500 kDa range with a high accuracy and in the presence of structure-maintaining buffer solutions.

Acknowledgements

We gratefully acknowledge the lease of sample of polymerase II by Prof. J. Michaelis and Prof. P. Cramer (LMU Munich) and Prof. G. Cook (University of Otago, New Zealand) for critically reading the manuscript. TM was supported by the Cluster of Excellence "Macromolecular Complexes" at the Goethe University Frankfurt (DFG Project EXC 115).

References

[1] N. Morgner, H.-D. Barth, B. Brutschy, Austr. J. Chem. 59 (2006) 109.

[2] N. Morgner, T. Kleinschroth, H.-D. Barth, B. Ludwig, B. Brutschy, J. Am. Soc. Mass Spectrom. 18 (2007) 1429.
[3] T. Meier, N. Morgner, D. Matthies, D. Pogoryelov, S. Keis, G.M. Cook, P. Dimroth, B. Brutschy, Mol. Microbiol. 65 (2007) 1181.
[4] N. Morgner, H.D. Barth, T.L. Schmidt, A. Heckel, U. Scheffer, M. Göbel, B. Brutschy, Zeit. Phys. Chem. 221 (2007) 689.
[5] N. Morgner, H.D. Barth, B. Brutschy, U. Scheffer, S. Breitung, M. Göbel, J. Am. Soc. Mass Spectrom., doi:10.1016/j.jasms.2008.07.001.
[6] P.T. Cramer, D.A. Bushnell, R.D. Kornberg, Science 292 (2001) 1863.
[7] K. Lorenzen, A. Vannini, P. Cramer, A.J.R. Heck, Structure 15 (2007) 1237.
[8] F. Brueckner, U. Henneke, T. Carrell, P. Cramer, Science 315 (2007) 859.
[10] D.G. McMillan, S. Keis, P. Dimroth, G.M. Cook, J. Biol. Chem. 282 (2007) 17395.
[11] J.P. Abrahams, A.G. Leslie, R. Lutter, J.E. Walker, Nature 370 (1994) 621.
[12] P.D. Boyer, Biochim. Biophys. Acta 1140 (1993) 215.
[13] R.A. Capaldi, R. Aggeler, Trends. Biochem. Sci. 27 (2002) 154.
[14] P.D. Boyer, Annu. Rev. Biochem. 66 (1997) 717.
[15] P. Dimroth, C. von Ballmoos, T. Meier, EMBO Rep. 7 (2006) 276.
[16] T. Meier, P. Polzer, K. Diederichs, W. Welte, P. Dimroth, Science 308 (2005) 659.
[17] S. Keis, G. Kaim, P. Dimroth, G.M. Cook, Biochim. Biophys. Acta 1676 (2004) 112.