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UV spectroscopy of entire proteins in the gas phase

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ABSTRACT

In-trap photoinduced electron detachment was performed on negatively charged proteins with molecular weights ranging from 8.6 to 77 kDa. The advantages of photodetachment over photodissociation are discussed in the context of action spectroscopy of very large ions. The resulting gas phase photodetachment yield spectra recorded in the UV range (220–310 nm) are presented and are compared with solution absorption spectra. The important question of the influence of the conformation and charge state of a protein on its optical gas phase spectrum is raised.

1. Introduction

In recent years, the concurrent development of versatile ion sources and ion trapping methods has contributed to the expansion of the field of biological ion spectroscopy. Nowadays, biological species ranging from the simplest molecular building blocks to massive protein complexes can be brought intact in the gas phase, manipulated, stored and analyzed in table-top mass spectrometry instruments. However, the ion density remains low and precludes the use of direct absorption spectroscopy. Therefore, action spectroscopy methods are mandatory in order to investigate the structural and optical properties of isolated biomolecular ions. In the field of trapped amino-acids, peptides and proteins, secondary structures can be identified by means of IRMPD spectroscopy [1–3] whereas fluorescence spectroscopy focuses on local conformational dynamics of dye-derivatized biomolecular ions [4]. Electronic spectra of trapped ions in the UV/vis range are also available via UV photodissociation [5–9] and more recently via measurements of electron photodetachment yields [10,11].

In this article, the limitations of single photon excitation dissociation for very large systems and the advantages of electron photodetachment measurements are discussed. Laser induced fragmentation of large molecular ions is indeed limited by the redistribution of the photon energy through the increasing number of vibrational modes of the molecule. In this context, the question of the possibility to perform linear (one photon excitation) action spectroscopy on very large systems remains open.

We present electron photodetachment measurements performed on a series of isolated entire proteins of molecular weights ranging from 8.6 to 77 kDa. The photodetachment yield spectra obtained in the 220–310 nm region for two proteins are compared with linear optical absorption in solution and the effects of various factors on the gas phase spectra are examined. The sensitivity of the method to the protein conformation is discussed.

2. Methods

2.1. UV/vis spectroscopy

The set-up for in-trap spectroscopy consists of a commercial LCQ Duo quadrupole ion trap mass spectrometer (ThermoFinnigan) coupled to a Panther OPO laser pumped by a 355 nm Nd: YAG PowerLite 8000 (5 ns pulse width, 20 Hz repetition rate). Frequency doubling allows scanning in the 220–310 nm range [8]. The vacuum chamber and the central ring electrode of the mass spectrometer were modified to allow the injection at the center of the trap of UV and visible lights. An electromechanical shutter triggered on the RF signal of the ion trap synchronizes the laser irradiation with the

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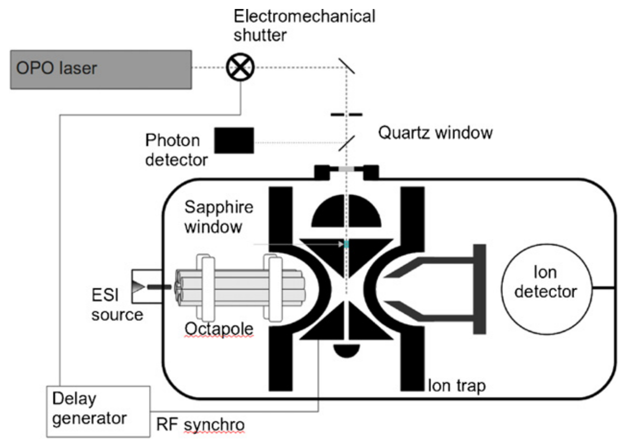

Fig. 1. Experimental set-up.

trapping of the ions (Fig. 1). The ions are produced with an electrospray ionization source, injected into the trap, mass-selected and laser irradiated. The product ions obtained after laser excitation are subsequently mass analyzed. In the case of polyanions, a species with one less charge than the parent ion is observed, as a result of electron loss. The electron photodetachment yield is then given by the relation: −ln (parent/(parent + product))/activation time/laser power/wavelength, where "parent" is the intensity of remaining parent ion after irradiation and "product" is the intensity of the photoinduced product. Gas phase UV spectra are obtained by recording the photodetachment yield as a function of the laser wavelength.

UV–vis spectra in solution were recorded using an AvaSpec-2048 fiber optic spectrometer and an AvaLight-DH-S deuterium halogen light source.

2.2. Sample preparation

Three candidate proteins were examined: bovin ubiquitin, bovin alpha-lactalbumin and serotransferrin human (TRFE) (purchased from Sigma–Aldrich), referred to as P1, P2 and P3, respectively. They cover a broad mass range (8.6–77 kDa), contain a various number of aromatic chromophores in the form of tryptophan, tyrosine and phenylalanine (3–62) and a various number of cystein bridges (0–19). Their properties are summarized in Table 1 and their amino-acid sequences are given in Supplementary information.

Protein P1 was prepared in a 60:40 (v:v) methanol/water mixture with ammonium acetate buffer (pH 6.5). Native solutions of P2 and P3 were prepared in distilled water. 1–5% ammonia was added to the solution in order to optimize the formation of negatively charges proteins by deprotonation of their acidic residues. Proteins concentrations were approximately 100 μM.

Samples for solution spectra were prepared in distilled water with a concentration of 50 μM.

In case of protein P2, additional photodetachment measurements were performed on the reduced form after chemical cleavage of the cystein bridges. The S–S bonds were reduced by DTT treatment (6 mM) in the presence of 3 mM Guanidine and then heated at 70 °C for 1 h. The reagents were removed by filtration using ultracentrifugation (membrane <3 kDa) and the solutions were washed with water. The reduction of disulfide bridges was systematically verified by mass spectrometry and the UVPD spectra were recorded on fully reduced proteins (the rupture of the 4 disulfide bridges results in a mass difference of 8 Da).

3. Results and discussion

3.1. In-trap electron photodetachment of entire proteins

The full mass spectra of the three proteins recorded in the negative mode are presented in Fig. 2a. Maximum ion intensity is observed for charge states 5^-, 8^- and 39^- for P1, P2 and P3, respectively. Individual charges states were isolated in the trap and irradiated for 50 ms (for P2 and P3, one laser shot) and 500 ms (for P1, 10 laser shots) at 275 nm. At this wavelength, electronic excitation of the aromatic amino-acid residues is expected. The resulting mass spectra (MS^2 spectra) are shown in Fig. 2b. For all anionic proteins, the detachment of one electron is observed and results in a less charged species, which appears at a higher value of m/z in the mass spectrum. No additional fragment is observed. The photodetachment efficiency ranges from 0.2 to 7%. For comparison, photofragmentation spectra of the positively charged proteins obtained in the same laser conditions are displayed in Fig. 2c. No photofragment is observed under UV irradiation. Indeed, the conversion of the photon energy by IVR occurring through the tremendous number of vibrational modes available in an entire protein, leads to no observable photofragmentation in our time window. Hence, photoproduct can be observed for negatively charged proteins, whereas no photofragmentation is observed for positively charged species.

Electronic spectra of protonated amino-acids, along with small and medium size peptides are conveniently obtained by means of ion trap laser induced dissociation [5–8]. The method relies on the production of a photofragment after excitation by a single UV photon. After excitation, direct dissociation in the excited state competes with internal conversion to the electronic ground state and with radiative de-excitation. Around 266 nm (4.66 eV), electrons from the aromatic residues can be excited and a model that involves a charge transfer to a dissociative excited state has been proposed [12]. Specific fragmentation channels are detected, namely formation of radical cations following a H atom loss and Cα–Cβ bond cleavage fragments [13–15]. As observed in Fig. 2, when aiming toward larger molecules, IVR quickly reduces the probability of photodissociation and UV photodissociation method becomes inapplicable if dissociation in excited states is not the dominant channel. Note that increasing the laser power to perform UV multiphoton dissociation leads to non-linear absorption and spectra that does not reflect the linear absorption of the ions.

More recently, the relation between optical absorption and electron photodetachment yield of polyanionic peptides was examined [16]. In this work, we demonstrate that electron photodetachment spectroscopy can be successfully applied to entire proteins.

Table 1
Comparison of some P1, P2 and P3 characteristics.

Protein	P1: ubiquitin	P2: alpha-lactalbumin	P3: TRFE
Molecular weight (kDa)	8.6	14	77
Number of residues	76	125	698
S–S bonds	0	4	19
Aromatic amino-acid residues	1Y/2F	4W/4Y/4F	8W/26Y/28F
Acidic amino-acid residues	5E/6D	13E/7D	45E/42D

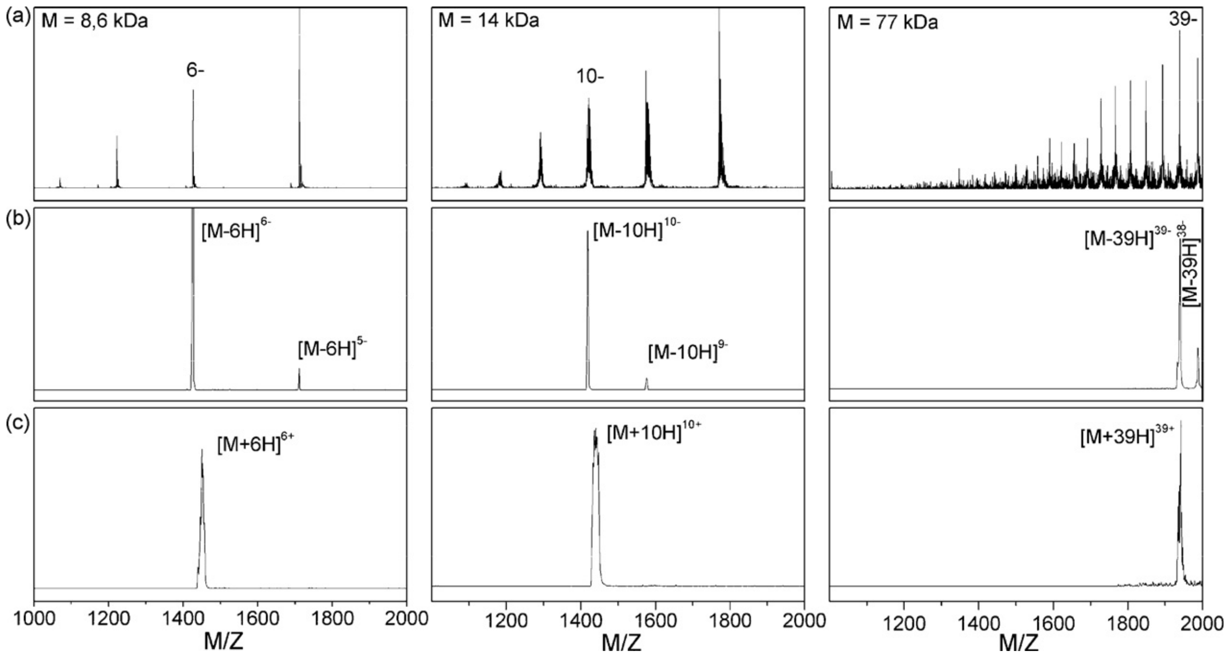

Fig. 2. (a) Mass spectra in the negative mode of P1, P2 and P3 proteins, (b) MS^2 spectra of P1, P2 and P3 after isolation and UV irradiation of charges states 6$^-$, 10$^-$ and 39$^-$, respectively and (c) MS^2 spectra of P1, P2 and P3 after isolation and UV irradiation of charges states 6$^+$, 10$^+$ and 39$^+$, respectively. The UV wavelength was 275 nm and the laser intensity was identical for measurements in the positive and the negative modes.

3.2. *UV spectroscopy of isolated deprotonated proteins*

The UV photodetachment yields of proteins P1 (charge state 6$^-$) and P2 (charge state 10$^-$) were recorded as a function of the wavelength from 220 to 310 nm. The resulting electron photodetachment spectra are shown in Fig. 3. Both proteins display two major features: a broad band centered around 280 nm and an intense response at high energy with an onset at 250 nm for P1 and at 240 nm for P2. Overall, the electron detachment yield obtained for protein P2 is two orders of magnitude higher than the one for protein P1. This is due to the number of chromophores in the two proteins: indeed P1 contains only a single tyrosine chromophore, while 4 tryptophans and 4 tyrosines are present in P2. The influence of the number of chromophores on the intensity of the photodetachment yield suggests that it is directly related to the optical absorption of the system.

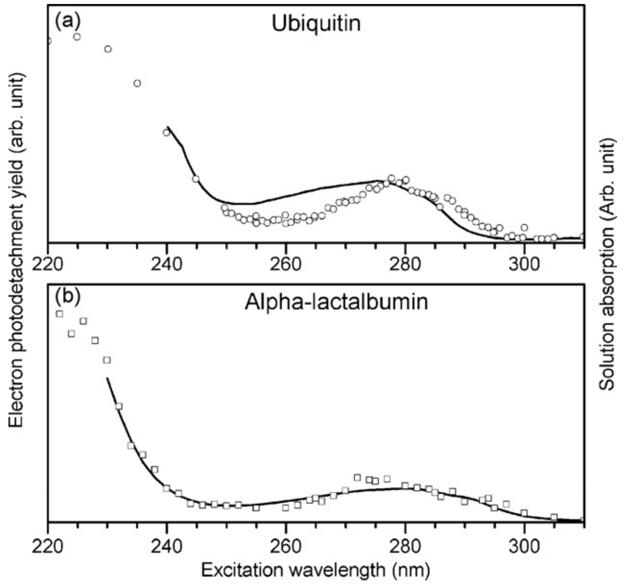

Fig. 3. Electron photodetachment yield measured as a function of the laser wavelength (open symboles) and solution absorption spectra (line) of proteins P1 (a) and P2 (b).

For comparison, the solution spectra of the two proteins are also displayed in Fig. 3. They display similar features around 280 and at higher energy, which correspond to the well-known resonant $\pi\pi^*$ excitation of the chromophores, and to excitations involving the backbone atoms, respectively. The consistency between the measurements of the photodetachment yields and solution spectra indicates that electron detachment measurements reflect the optical absorption of the system. In particular, the band centered around 280 nm in the gas phase photodetachment spectra can be assigned to $\pi\pi^*$ excitation of the aromatic residues.

As seen in Fig. 3b, a perfect match between the solution and gas phase spectra is observed for protein P2, whereas in the case of P1 (Fig. 3a), the $\pi\pi^*$ band in the gas phase is redshifted as compared to the absorption in solution. Protein P1 contains a single significant chromophore in the form of a tyrosine amino-acid (two phenylalanine are also present, but have a lower absorbance). The experimental redshift may be due to the presence of a negative charge in the vicinity of the tyrosine, as already observed in the case of gas phase negative tryptophan [11]. On the other hand, crystal structure [17] suggests that the chromophore residue is located at the surface of the protein, hence particularly sensitive to the outside environment. The addition of water is also expected to cause a redshift of the tyrosine absorption spectrum [18]. However, solvent molecules may shield the charges in neighboring of the chromophore, thus causing a screening of the charge effect mentioned above. The shift between solution and gas phase would then be due to the addition of several contributions with opposite effects. The resulting shift is difficult to predict. Besides, one cannot exclude that protein P1 in the gas phase, adopts a different conformation as compared to the solution, due to the fact that no disulfide bridge constrains the structure. On the opposite, P2 contains eight chromophores partly embedded inside the protein. This results in a scrambling of the individual contributions combined with a lesser sensitivity to the presence of solvent, in agreement with the observations.

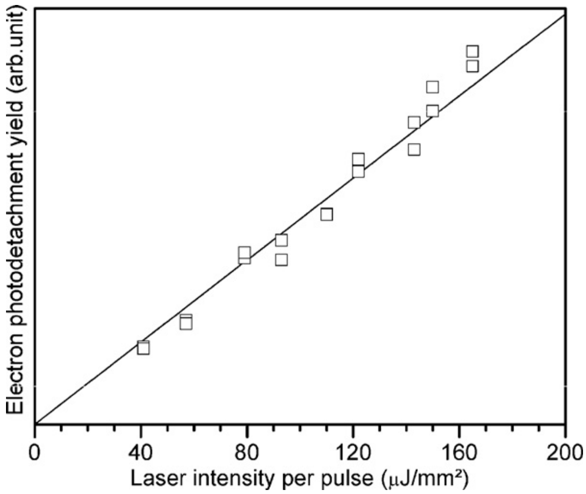

Fig. 4. Electron photodetachment yield of P2 (charge state 10^-) as a function of laser intensity at 280 nm (squares). Linear regression of experimental data (line).

In order to further discuss how photodetachment spectroscopy relates to linear optical absorption, the linearity of the UVPD process was examined. Fig. 4 shows the photodetachment yield measured for protein P2 with a single laser shot at 280 nm as a function of the laser fluence. The trend is linear, demonstrating that the detachment of an electron is a single photon process in our experimental conditions, even for a large protein.

Additionally, gas phase measurements – as compared to solution phase spectroscopy – benefit from the specific potential of mass spectrometry. Indeed, charge state resolved spectroscopy can be achieved. The electron detachment spectra of individual charge states 11^-, 10^- and 9^- of protein P2 are given in Supplementary information as an example. No perceptive effect of the charge is observed in this particular case, which indicates that the electronic states of the chromophores are not significantly perturbed by the change in charge state. However, it has already been shown that the UV spectra of peptides are sensitive to the ionization state [10] and radical state of their chromophore [19] thus making charge state resolved spectroscopy a relevant diagnostic of the charge localization in a protein.

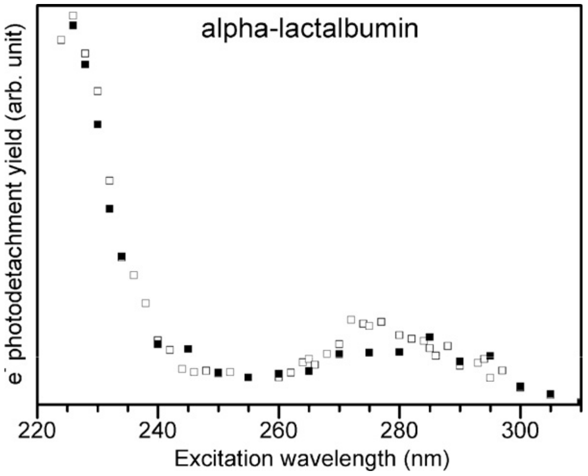

Fig. 5. Electron photodetachment yield as a function of the laser wavelength for P2 in the non-reduced form (open squares) and reduced form (black squares).

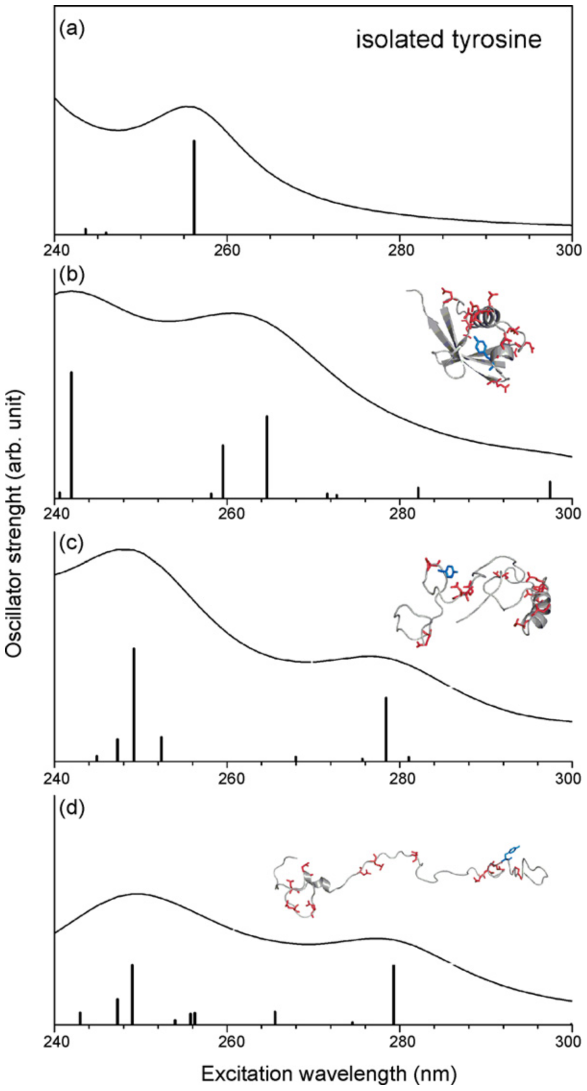

Fig. 6. Convolution (full line) of the simulated spectra of isolated tyrosine (a), P1 in compact form (b), P1 in extended form with helices preserved (c) and P1 in extended form (d). Transitions are shown by bars.

In order to address the question of the influence of the 3D organization of the protein on its UV spectrum, photodetachment yield spectra of non-reduced and reduced forms of protein P2 were compared. The tertiary structure of protein P2 in its native form is constrained by 4 disulfide bonds formed between pairs of cystein residues. In the denaturated conditions, the four bridges are cleaved by reduction with DTT, which allow greater flexibility of the structure. The photodetachment spectrum of reduced P2 was measured between 220 and 310 nm and is compared to the one obtained for the non-reduced form in Fig. 5. The two spectra similarly show the two features at 230 and 280 nm, as discussed above. However, the $\pi\pi^*$ region is more active in the 270–280 nm region in the case of the native protein. This difference between the two spectra is partially interpreted as the optical signature of the presence of cysteine bridges in the native protein. Indeed, a disulfide bridge is optically active in the 240–280 nm region [20] whereas individual SH groups in cysteine residue are not. A crude evaluation of this contribution is made by adding the individual absorbances of each chromophore contained in the protein (see Table 1). Although the absorption of the four tryptophan residues remains dominant, the cysteine bridges would account for a 15% increase in the absorption at the left part of the $\pi\pi^*$ band, as compared to the reduced form. This is qualitatively consistent with the observations, although the strength of the effect is insufficient.

This result points out that a complete interpretation of the experimental spectra implies to take into account the conformational difference between the two forms of the protein, and that a detailed understanding of the influence of the structure on the optical spectrum of gas phase proteins is of considerable importance. However, the theoretical efforts that are necessary to predict the optical properties of entire proteins containing a number of chromophores are beyond the scope of this work.

Conformation effects were theoretically explored for P1 which contains a single tyrosine chromophore. Absorption spectra of P1 were predicted for three distinct forms of the protein: (i) native, (ii) unfolded with helical sections preserved and (iii) totally unfolded. In all three structures, the charges were placed as follow: carboxylic acids were deprotonated, lysine and arginine residues were protonated, and the tyrosine residue was in the neutral form. Two-layer ONIOM (QM-high:MM-low) [21] scheme was applied for this simulation. TD-DFT (B3LYP/aug-cc-pvdz) was used for the QM method and the Amber force field for the MM method. The chromophore of the protein (tyrosine) is the model system (QM). The partial charges from the MM region are included in the QM Hamiltonian [22].

The Gabedit [23] software was used to build and optimize the three starting structures at Amber level of calculation. The ONIOM calculations were carried out by use of computational package Gaussian03 [24]. The three absorption spectra are shown in Fig. 6 and are compared to the spectrum of isolated neutral tyrosine calculated with TD-DFT(B3LYP/aug-cc-pvdz). Depending on its environment, the $\pi\pi^*$ band corresponding to the excitation of the tyrosine residue ranges from 260 to 280 nm. The simulations show that the optical spectrum of the chromophore is modified when incorporated in a protein and that changes in protein conformation may induce optical shifts. This opens the way to further experimental and theoretical explorations.

4. Conclusion

It was shown that photoinduced electron detachment on anionic species allows overcoming the limitation of photodissociation arising for large ions, and that linear UV action spectroscopy of entire proteins is possible without size restrictions. Applied in the present work to proteins, the electron photodetachment spectroscopy has proved to be also an efficient tool for DNA polyanions [25] and could be used as a universal action spectroscopy for large biomolecular ions.

The gas phase electron photodetachment spectra of a series of proteins were reported and the influence of several parameters (size, charge state, denaturation, number of chromophores) was discussed. The sensitivity of the method to the protein conformation was established in the case of reduced/non-reduced alpha-lactalbumine. Although a complete interpretation of the spectra requires extensive theoretical efforts that are beyond the scope of this work, it appears clearly that the question of the influence of the protein conformation on the gas-phase UV spectrum is of considerable importance. Possible improvements of the techniques include grafting of artificial chromophore in order to improve the sensitivity to the conformation. The action spectroscopy described here could be completed by ion mobility measurements to assess the global shape of protein polyanions in the gas phase; we are currently developing this approach.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2010.05.015.

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