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Investigation of the monomeric and oligomeric molecular forms of human growth hormone and poly-L-lysine using fluorescence anisotropy measurement method

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Summary

This work illustrates the usefulness of fluorescence anisotropy measurement using a long-lifetime fluorescence label bis-(bipyridine)-5-(isothiocyanatophenanthrolin)-$Ru(PF^{6-})_2$ for the investigation of large macromolecules - human growth hormone forms with different aggregation degree and poly-L-lysine hydrobromides with different molecular weights. Three molecular forms of this hormone – monomeric and two oligomeric (with polymerization degree 3 and 5) – and poly-L-lysine hydrobromides with different molecular weights were for the first time investigated using fluorescence anisotropy measurement by the constant viscosity. Fluorescence anisotropy and rotational correlation time are determined for each substance. Calculated rotational correlation times in investigated media are linear proportional to polymerization degree of molecular forms of the hormone. Relationship between rotational correlation time and molecular weight of random coil structure poly-L-lysines (with similar molecular weights) is nonlinear. It was shown what measured fluorescence anisotropy of the simple mixtures of two labeled molecular forms of the hormone well corresponds with theoretical anisotropy calculated with average values of the anisotropies of the separately investigated pure compounds.

Introduction

Human growth hormone (hGH) like many biological substances, is a heterogeneous polypeptide [1]. The main molecular form is so called monomeric hGH with molecular weight of 22 kDa, consisting of 191 amino acids. The second monomeric form is the 20-kD splice variant, in which amino acids 32 through hGH 46 are absent. Both macromonomers - monomeric 22 kDa or 20 kDa hGH - have two pairs of disulfide bonds and are polymerizable. Naturally occurs so called size variants of hGH, products of the homopolymerization of macromonomer – dimers and oligomers. There is also isoforms consisting of two or few non-covalently connected macromonomers (monomeric 22 kDa or 20 kDa hGH) [1]. Approximately 14% of the hGH molecules in the circulation are oligomeric forms. This value can vary from 0 % (in case of doping using athletes) up to 100 %. Specific assays in biological liquids

for each isoforms are currently unavailable, and every new method for the estimation of the monomeric and oligomeric molecular forms of hGH is welcome [1]. One of the possible ways for the estimation of the monomeric and oligomeric molecular forms of this hormone is fluorescence polarization measurement method with possibility of determination of molecular size of the hGH form [2]. Molecular size and rotational volume of fluorescent labeled molecules or bioconjugates are direct proportional to rotational correlation time and can be determined by the measurement of fluorescence polarization or anisotropy [3].

To characterize the polarized state of the emitted light, it was defined as a parameter called fluorescence anisotropy (r),

$$r = \frac{I_{II} - I_{\perp}}{I_T} = \frac{I_{II} - I_{\perp}}{I_{II} + 2 \cdot I_{\perp}} \qquad (\mathbf{1})$$

where I_{II} and $I_{\perp}$ are the components of the fluorescence intensity that are parallel and perpendicular to the electric vector of the excitation light, and I_T is the total fluorescence intensity [3]. In the absence of diffusion, the anisotropy is given by the relative orientation of the excitation and emission moments of the fluorophore, taking values between -0.2 and 0.4. In solution, the excited molecules can rotate, thereby changing their orientation. The angle θ between the average orientation of the molecules at a given time with respect to the original orientation depends on the time, the temperature, the solvent properties, and the size and shape of the molecule. This process, known as rotational diffusion, can be characterized by a parameter called rotational correlational time (φ), defined as the time after which $cos\ \theta = e^{-1}$ (θ is equal to c.a. 68,5 degrees) [4].

In 1926, Perrin proposed that rotational diffusion contributes to depolarizing the emitted light and postulated the following relationship to explain the dependence of the anisotropy with the rotational diffusion process [5],

$$r = \frac{r_o}{1 + \tau / \phi} \qquad (\mathbf{2})$$

where r_o is the anisotropy in the absence of diffusion, τ is the fluorescence lifetime (in this time the fluorescence anisotropy decreases up to $r_o \cdot e^{-1}$), and φ is the rotational correlation time of the fluorophore. Equation 2 predicts that, when $\tau << \varphi$, the particle would almost not rotate at all during the lifetime of the fluorophore, and r approaches the anisotropy measured in the absence of diffusion, *i.e.* r_o. When $\tau >> \varphi$, the emission dipoles of the molecule become randomly distributed as a consequence of the rotational diffusion, and r approaches zero. The anisotropy will be sensitive to factors affecting the rotational motions when $\tau \cong \varphi$ [4]. The rotational correlation time φ can be expressed by the Stokes-Einstein-Debye equation [5],

$$\phi = \frac{\eta \cdot V}{k \cdot T} \qquad (\mathbf{3})$$

where η is the solvent viscosity coefficient, k is the Boltzman constant, T is temperature, and V is the volume of the rotating particle. This approximation appears to be valid for proteins [4].

For depolarization to occur, the macromolecule must display a rotational correlation time shorter than the lifetime of the fluorescent label. Some long-lifetime

fluorophores, such as chelates of Eu^{3+} and Tb^{3+}, have been used in time-resolved immunoassays [4], but they do not display polarized emission and are not useful for anisotropy measurements. Fortunately, ruthenium complexes display long lifetime τ (400-2700 ns) and high polarization (and anisotropy in the absence of diffusion r_o) [6], which expand the use of fluorescence anisotropy to detect molecules of up to 1000 kDa [7, 8]. These fluorescent labels can be used to label amine groups on biomolecules or macromolecules under mild conditions.
The first aim of this work was to determine fluorescence anisotropy and rotational correlation time of some such fluorescent labeled hGH forms – monomer and two oligomers, representing covalently connected monomeric hGH form with polymerization degree 3 and 5 and poly-L-lysine bromides with different molecular weights. The second aim was to investigate a possibility of quantitative analysis of these fluorescent labeled samples with different molecular weights in simple mixtures using fluorescence anisotropy measurement.

Experimental

Materials

Human growth hormone and its molecular forms were prepared and characterized as described earlier [2]. Mass-spectroscopy, HPLC and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of monomeric hGH show sample to be greater than 98% pure and molecular weight of 22 kDa. Oligomeric hGH forms were separated on Sephadex G-200 column. SDS-PAGE of two sample-fractions revealed that samples molecular weights are respectively 66 kDa and 110 kDa (approximately). Mass-spectroscopy and SDS-PAGE performed under reducing conditions revealed that oligomeric hGH forms consists of 22 kDa macromonomer units and are greater than 98% pure.
Bis-(bipyridine)-5-(isothiocyanatophenanthrolin)-Ru(PF^{6-})$_2$ ($[Ru(bpy)_2(phen\text{-}ITC)]^{2+}$) (Fluka) and glycerol (Aldrich) was used as received. Poly-L-lysine hydrobromide samples with different molecular weights (Sigma) were separated on Sephadex G-200 column. The molecular weights of poly-L-lysines were determined by the sedimentation equilibrium method in aqueous solutions of 1.0 M NaCl or 0.1 M Na_2CO_3 [9].

Instruments

Fluorescence measurements were performed using a Perkin Elmer luminescence spectrometer LS-5B equipped with excitation and emission polarization filters.

Preparation of the samples and measurements

To obtain constant viscosity for all samples special mixtures of aqueous glycerol solution (25%) and sample substances with viscosity of 2 cP (twofold higher than viscosity of H_2O) [10] were prepared. Because the polypeptides and buffer concentrations are very low, sample viscosities can be calculated from the glycerol concentration [10]. Every sample was prepared in duplicate.
Fluorescence anisotropy was measured in the mixture of sample in phosphate buffer (pH 7,2) and glycerol solution at 20 °C, setting the excitation at 467 nm and the

emission at 612 nm with bandwidths of 10 nm. Every measurement was repeated three times. Anisotropy was calculated using equation 4, including the instrument *G* factor defined as the ratio of sensitivities of the detection system for the vertically and horizontally polarized light [3].

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2 \cdot G \cdot I_{VH}}, with\ G = \frac{I_{HV}}{I_{HH}} \qquad (4)$$

where *H* and *V* refer to the horizontal and vertical positions, respectively, of the excitation (first subscript) and emission (second subscript) polarization filters.
Samples of hormone and poly-L-lysine hydrobromide were labeled with $[Ru(bpy)_2(phen\text{-}ITC)]^{2+}$ as described Youn and co-workers [11]. The labeling degrees of the samples were: 1,056 ± 0,09 (hGH monomer), 1,051 ± 0,11(hGH trimer) , 1,046 ± 0,09 (hGH pentamer), 1,054 ± 0,14 (poly-L-lysine hydrobromides). Only one label on each molecule can be excited by these labeling degrees (because of polarized excitation light).

Results and discussion

Determination of the rotational correlation time of hGH forms and using fluorescence anisotropy measurement method

The molecular volume of the proteins and macromolecules is related to the molecular weight and rotational correlation time [4]. As shows SDS-PAGE without and under reducing conditions (and in regard of mass-spectroscopy), oligomeric hGH forms with molecular weight 66 kDa and 110 kDa consist respectively of three and five 22 kDa units. For the estimation of different 20-150 kDa macromolecules fluorescent label with fluorescence lifetime of 350-500 ns can be used [4]. It was interesting to study the possibility of the estimation of these molecular forms using fluorescence anisotropy measurement method with $[Ru(bpy)_2(phen\text{-}ITC)]^{2+}$ as fluorescent label with known fluorescence lifetime τ of 400 ns and anisotropy in the absence of diffusion r_o of 0,3 [11].
Results of the anisotropy measurements of $[Ru(bpy)_2(phen\text{-}ITC)]^{2+}$ labeled hGH forms and calculated (in regard to equations 2 and 3) rotational correlation times φ are shown in table 1.

Table 1. Fluorescence anisotropy values of different molecular forms of hGH and calculated rotational correlation times φ

Molecular forms of hGH	Polymerization degree	*r*	φ , ns
22 kDa	1	0,0224±0.0008	32,3±1,2
66 kDa	3	0,0608±0.0008	101,7±1,7
110 kDa	5	0,0892±0.0009	169,3±2,4

It can be seen from table 1, that the fluorescence anisotropy *r* values for all molecular forms are quite low in comparison to anisotropy of the fluorescent label in the absence of diffusion ($r_o = 0,3$). It means that all of the investigated forms are suitable to use as labeled antigen in the hGH fluorescence polarization immunoassay [4].

Calculated rotational correlation time φ in the investigated media was in good correlation with polymerization degree of the molecular forms of hGH (Table 1). As can be seen from figure 1, φ is linear proportional to polymerization degree. Rotational correlation time φ depends direct on the volume of the rotating particle V (see equation 3).

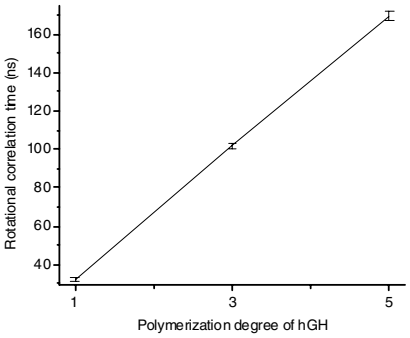

Figure 1. Correlation between rotational correlation time and polymerization degree of different molecular forms of hGH

One reason that can explain good correlation between calculated φ and polymerization degree of hGH – it must follow the condition, that the volume of the rotating particles in case of such big macromonomer is direct and linear proportional to aggregation degree [12].
Results of the anisotropy measurements of $[Ru(bpy)_2(phen\text{-}ITC)]^{2+}$ labeled poly-L-lysine hydrobromides with different molecular weights and calculated rotational correlation times φ are shown in table 2. The fluorescence anisotropy values for all samples in this case are also quite low in comparison to r_o. As can be seen from figure 2, φ is nonlinear proportional to molecular weights of poly-L-lysine hydrobromides, what can be explained with random coil structure of the macromolecules [12].

Table 2. Fluorescence anisotropy values of poly-L-lysine hydrobromides with different molecular weights and calculated rotational correlation times φ

M_W	r	φ , ns
20100	0,0153±0.002	21,5±3,2
29800	0,0310±0.002	46,1±3,2
68800	0,0563±0.003	92,3±4,8
135200	0,0739±0.003	130,7±6,5

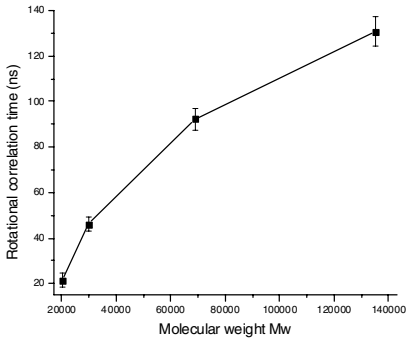

Figure 2. Relationship between rotational correlation time and molecular weights of poly-L-lysine hydrobromides

Estimation of quantitative proportions of the investigated forms of hGH in mixtures using anisotropy measurement of labeled hGH forms

The advance of the investigation of systems with known fluorescence anisotropies of two compounds r_1 and r_2 is described by simple equation for the total anisotropy of the mixture r, containing part a_1 (calculated in %/100) of first compound and part a_2 of the second compound [12].

$$r = r_1 \cdot a_1 + r_2 \cdot a_2 \text{ , or } r = r_1 \cdot a_1 + r_2 \cdot (1 - a_1) \text{ , where } (a_1 + a_2 = 1) \quad (5)$$

As we see from equation 5 (and in regard that $a_2 = 1 - a_1$), we can estimate the parts of two compounds in the mixture from the value of the measured anisotropy r of the mixture. It has practical importance if this approximation agrees with practical results for investigated system (the two compounds in the mixture must rotate independent one from another [12]). Mixtures of three (or more) compounds also give total anisotropy proportional the part and anisotropy of all compounds in the mixture [12]:

$$r = r_1 \cdot a_1 + r_2 \cdot a_2 + r_3 \cdot a_3 \qquad (6)$$

It is often impossible to estimate the part of every compound in the mixture without time-resolved anisotropy technique [12].

It was interesting to investigate how measured fluorescence anisotropy of the simple mixtures of two or three labeled molecular forms agrees with theoretical anisotropy calculated with average values of the anisotropies from table 1.

Measured anisotropy r and calculated theoretical anisotropy r_t of specially prepared mixtures of known proportions of two or three molecular forms are summarized in table 3.

Table 3. Fluorescence anisotropy values of different mixtures of molecular forms of hGH

No.	Sample, containing two molecular forms in %			Measured anisotropy r	Theoretical anisotropy r_t
	22 kDa	*66 kDa*	*110 kDa*		
1.	50	50	-	0,0415±0.0004	0,0416
2.	50	-	50	0,0557±0.0006	0,0558
3.	-	50	50	0,0751±0.0007	0,0750
4.	20	80	-	0,0531±0.0005	0,0531
5.	20	-	80	0,0757±0.0007	0,0758
6.	-	20	80	0,0835±0.0004	0,0835
7.	-	80	20	0,0663±0.0007	0,0665
8.	80	20	-	0,0299±0.0004	0,0301
9.	80	-	20	0,0355±0.0004	0,0358
10.	25	50	25	0,0583±0.0007	0,0583
11.	40	15	45	0,0583±0.0006	0,0582

How we can see from table 3, the measured anisotropies of all mixtures are in good agreement with calculated theoretical values. The good agreement between measured and theoretical anisotropy can confirm the statements that the fluorescence lifetime of the label is constant and that the fluorescence quantum yield of the used label does not change for all samples [7].These conditions [12] talk about good suitability of steady state fluorescence anisotropy measurement method for investigated compounds with $[Ru(bpy)_2(phen\text{-}ITC)]^{2+}$ as fluorescent label. All measured r values for systems of two molecular hGH forms correspond to one proportion of two investigated molecular forms. Samples of three molecular forms (for example, No.10 and No.11 in table 3) have very different ratios of hGH forms and, unfortunately, very similar r and r_t values. It means, that for the estimation of the mixtures of all of this three hGH forms time-resolved technique is required. The high enough difference in rotational correlation time φ of all three hGH forms (see table 1) and long fluorescence lifetime

of label speak about suitability of such labeled macromolecules for the quantitative estimation with time-resolved technique [12].

Conclusions

It was found that calculated rotational correlation time in the investigated media is linear proportional to the polymerization degree of the molecular forms of hGH that can be explained the condition, that in the investigated media the volume of the rotating particles in case of such big macromonomer is linear proportional to aggregation degree. Relationship between rotational correlation time and molecular weight of random coil structure poly-L-lysines (with similar molecular weights) is nonlinear. The high enough difference in rotational correlation time of all three hGH forms and long fluorescence lifetime of label show suitability of such labeled macromolecules for the quantitative estimation with time-resolved technique. The fluorescence anisotropy values for all polymer samples and molecular forms of the hormone labeled with bis-(bipyridine)-5-(isothiocyanatophenanthrolin)-Ru(PF^{6-})$_2$ is low enough in comparison to anisotropy of the fluorescent label in the absence of diffusion. The anisotropy of the mixtures of two labeled molecular forms of the hormone well corresponds with theoretical anisotropy of the mixture calculated with values of the anisotropies of the separately investigated compounds that talks about suitability of such anisotropy measurement for the quantitative estimation of the investigated monomeric and oligomeric molecular forms of the hormone.

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