GLYCATION OF HUMAN SERUM ALBUMIN BY DL-GLYCERALDEHYDE: A FLUORESCENCE QUENCHING STUDY

Miroslav STEPANEK[1] and Zdenek PAVLICEK[2]

Department of Physical and Macromolecular Chemistry, Charles University, 128 40 Prague 2, Czech Republic; e-mail: [1] stepanek@prfdec.natur.cuni.cz, [2] pavlicek@prfdec.natur.cuni.cz

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Process of glycation of human serum albumin (HSA) by DL-glyceraldehyde was studied using steady-state and time-resolved fluorescence spectroscopy in the course of 100 h. During this period, measurements of steady-state tryptophan (Trp) and non-tryptophan (non-Trp) fluorescence of the glycated HSA were carried out, together with measurement of the non-Trp fluorescence decay and steady-state quenching using potassium iodide as a quencher. Observed changes in both Trp and non-Trp fluorescence intensity, as well as changes in non-Trp fluorescence lifetimes and quenching efficiency are explained with respect to a probable mechanism of glycation.
Key words: Human serum albumin; Glycation; Fluorescence.

Proteins are able to react nonenzymatically with reducing sugars. This reaction, commonly known as nonenzymatic glycosylation or glycation, was at first described by Maillard[1].

Glycation is initiated by the reversible formation of a Schiff base between a reducing sugar and the amino group of a protein. The relatively unstable Schiff base forms a more stable Amadori product, undergoing a series of further reactions through dicarbonyl intermediates to form advanced glycation end products (AGEs), which are also known as advanced Maillard products[2]. The AGEs are a heterogeneous group of structures formed as both crosslinking and non-crosslinking adducts on proteins. AGEs accumulate in long-lived tissue proteins like lens crystallins and collagen during aging[3]. In diabetes AGEs accumulation in general is accelerated and associated with atherosclerosis, nephropathy, neuropathy, retinopathy and cataract[4,5].

As the AGEs are highly fluorescent and protein crosslinking species, their formation in proteins during long-term glycations manifests itself in non-tryptophan fluorescence and presence of protein oligomers.

Possible mechanism of tissue damage exerted by AGEs is connected with the generation of radicals and reactive oxygen species (ROS). They can occur spontaneously[6] or as a result of AGEs molecule acting as ultraviolet sensitizer[7]. Oxidation reactions thus contribute to formation of more permanent, irreversible chemical modifications and crosslinks in proteins, such as the glycooxidation products *N*-(carboxymethyl)lysine

and pentosidine[8]. Although the role of oxidation reactions and radicals in the Maillard reaction is widely recognized, the sequence of the reactions, glycation *versus* oxidation, is still uncertain[9]. Also the mechanisms of radicals and ROS forming are not known in detail.

To gain insight into the process of glycation and glycooxidation, we have studied the fluorescence phenomena during the glycation process using HSA as a model protein and DL-glyceraldehyde (GCA) as a model reducing sugar. The main aim of our study was to obtain information about distribution of lifetimes of non-Trp fluorophores and their behaviour during quenching and put it into context with the phenomena accompanying glycation.

EXPERIMENTAL

Materials. Human serum albumin (grade for medical purposes) was obtained from Imuna, Slovak Republic, GCA from Sigma, U.S.A. Other chemicals used were products of Lachema, Czech Republic (reagent grade purity).

A solution of 20 μM HSA and 10 mM GCA in 0.1 M sodium phosphate buffer pH 7.4, with 0.1% sodium azide as preservative, was incubated at 37 °C in a water thermostat, together with the same solution without GCA as a control. Samples taken from the solution were cooled to laboratory temperature before they were subjected to measurements in 1 cm quartz cuvettes.

Methods. Absorbance measurements were performed with a Cecil 8020 spectrophotometer. Steady-state fluorescence was measured with a Perkin–Elmer LS-5 luminiscence spectrometer. The fluorescence of samples was quenched by adding solution of 4 M potassium iodide. The measured fluorescence intensities were corrected for sample dilution by the quencher. The quenching data were evaluated by means of the modified Stern–Volmer equation[10]. Derivation of this equation is based on the assumption that only a part of fluorophores in the described system is accessible to quencher. The equation has a form

$$\frac{I_0}{I_0 - I} = \frac{1}{f_a} + \frac{1}{f_a K_{SV}[Q]} \quad , \tag{1}$$

where I_0, I, f_a, K_{SV} and [Q] denote fluorescence intensity in the absence of quencher, fluorescence intensity in the presence of quencher, fraction of fluorophores accessible to quencher, Stern–Volmer quenching constant and quencher concentration, respectively.

Fluorescence decay measurements were carried out with a time-resolved Edinburgh Instruments 299/T fluorimeter. An excitation source was a thyratron-gated flashlamp filled with hydrogen at 45 kPa. The repetition frequency of excitation pulses was 40 kHz and average half-width of the pulses was *ca* 3 ns. All decay curves were collected at 1 024 channels of a multichannel analyzer (Ortec–Norland 5600) with the time resolution of 213 ps/channel. At least up $5 \cdot 10^3$ counts in maximum were collected in each measurement. The obtained curves were fitted by an iterative nonlinear least-squares method with a reconvolution procedure using the IBH DAS 4.2 software (IBH, Great Britain).

RESULTS AND DISCUSSION

Incubation of HSA with GCA leads to the production of a highly fluorescent moiety with an absorption maximum at 360 nm (Fig. 1). Similar observations have been made for HSA glycated by glucose[11].

GCA, together with glycolaldehyde, is one of the most reactive glycating agents, because of its inability to form hemiacetals. In contrary, hexoses are present in solution preferably in a cyclic hemiacetal form (99.997% for glucose at equilibrium[12]) which is not reactive. Therefore the formation of AGEs, indicated by violet fluorescence, is much faster for GCA than for hexoses.

Figure 2 shows that the fluorescence intensity of the HSA/GCA system has a maximum at about 1 day of incubation and later it begins to decrease, while the absorbance at 360 nm only increases towards an upper limit value (Fig. 1 inset). This behaviour is in contrast with results of a previous study[11] reporting both fluorescence and absorbance non-decreasing with time.

Since the absorbance at non-Trp fluorescence excitation wavelength, 360 nm, does not decrease, and reabsorption of the system about non-Trp fluorescence emission

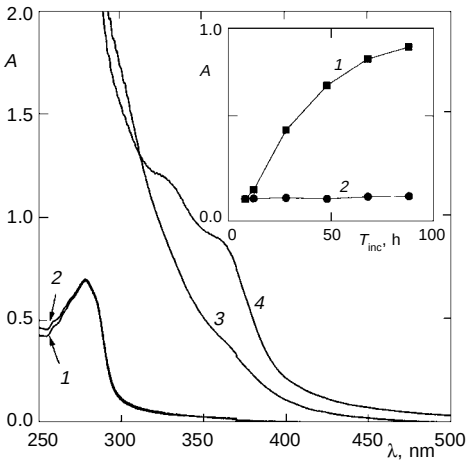

FIG. 1

Absorption spectra of the HSA/GCA system: *1* immediately after preparation, *2* after 1 h of incubation, *3* after 24 h of incubation, *4* after 96 h of incubation. Inset: Absorbance (*A*) at 360 nm *vs* time of incubation (T_{inc}): *1* HSA/GCA system, *2* control

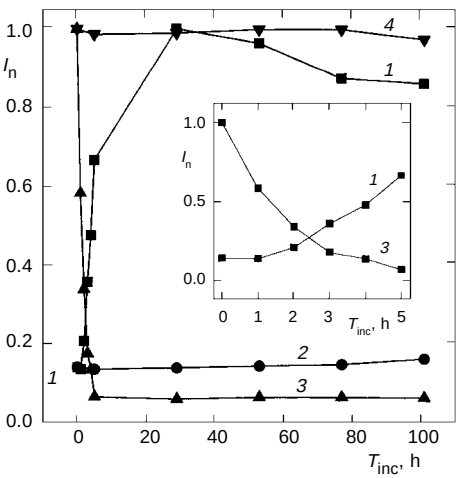

FIG. 2

Normalized non-Trp (excitation 360 nm, emission 440 nm) and Trp (exc. 295 nm, em. 345 nm) fluorescence intensity (I_n) *vs* time of incubation (T_{inc}): *1* non-Trp fluorescence of the HSA/GCA system, *2* non-Trp fluorescence of the control, *3* Trp fluorescence of the HSA/GCA system, *4* Trp fluorescence of the control. Inset: Detailed plot of the initial part of the curves *1* and *3*

maximum, 440 nm, is negligible, the decrease in fluorescence intensity must be caused by the decrease in the quantum yield of fluorescence, possibly either due to chemical modification of the fluorophores or due to quenching by side products of Maillard reaction.

The measurement of Trp fluorescence at 345 nm with excitation at 295 nm revealed its rapid decrease: After 1 h of incubation the Trp fluorescence intensity falls to half of its initial value, and after 5 h it completely disappears (Fig. 2 inset). Sakurai and coworkers[11] who reported much slower decrease in Trp fluorescence intensity during glycation of HSA by glucose, explained it as a consequence of internal filter effect because both excitation radiation and radiation emitted by Trp are absorbed by AGEs. This explanation seems to be unlikely because nearly no alternation in absorption spectrum of the system was observed after 1 h of incubation (Fig. 1). The observed quenching of Trp fluorescence could be more probably caused by a change of Trp-214 environment (the only Trp residue in HSA) *via* glycation of proximate Lys-212, nevertheless, we have no experimental evidence for that.

Decays of the non-Trp fluorescence are strongly nonexponential (Figs 3 and 4). At least three-exponential model decay function is required for an adequate fit. Models with one or two exponential components do not provide acceptable values of χ^2 and

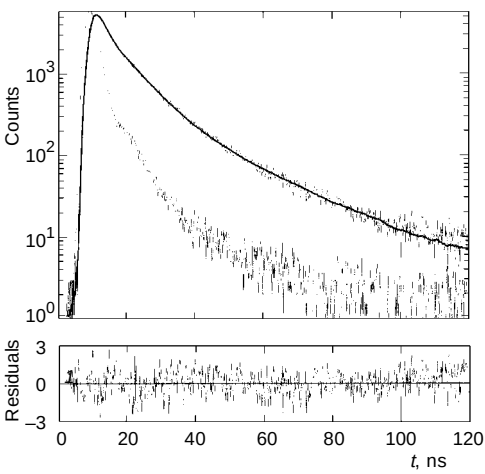

FIG. 3

Non-Trp fluorescence decay (exc. at 360 nm, em. at 440 nm) of the HSA/GCA system after 96 h of incubation, together with a flashlamp profile, a three-exponential fit of the decay (after convolution with the flashlamp profile) and residuals of the fit (χ^2 = 1.057)

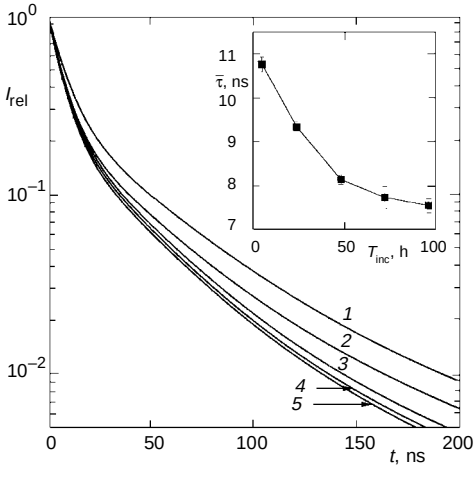

FIG. 4

Decay functions corresponding to the parameters in Table I, for different times of incubation (T_{inc} in h) of the HSA/GCA system: *1* 5, *2* 24, *3* 48, *4* 72, *5* 96. Parameters of the functions are given in Table I. Inset: Weighted average fluorescence lifetime of the HSA/GCA system, $\bar{\tau} = \Sigma_{i=1}^{3} F_i \tau_i$, calculated from the parameters

random distributions of weighted residuals. Since the real distribution of fluorescence lifetimes in the system is probably much more complex, parameters obtained by the three-exponential fit (Table I) cannot be interpreted as lifetimes and amounts of three fluorescent species. They only represent quantitative description of an experimental decay curve.

Some information can be, however, obtained from the time course of a weighted average fluorescence lifetime of the system (Fig. 4 inset). The decrease in the lifetime verifies the decrease in the quantum yield during the glycation. Moreover, if the decrease is caused by quenching, this quenching must be dynamic. We tentatively propose collisional quenching by free radicals as a possible mechanism. Free radicals are well known as side products of Maillard reactions, and radicals, in general, can quench fluorescence because of their non-zero spin multiplicity.

TABLE I

Parameters of a three-exponential fit of a fluorescence decay of the HSA/GCA system after different times of incubation, T_{inc}. Fitted function has a form $I(t) = \Sigma_{i=1}^{3}(F_i/\tau_i) \exp(-t/\tau_i)$, sum of F_i is normalized to one. Values given in the table are means and standard deviations from 5 measurements

T_{inc}, h	τ_1, ns	τ_2, ns	τ_3, ns	F_1	F_2	F_3
5	27.4 ± 1.5	8.5 ± 0.3	1.7 ± 0.1	21.9 ± 1.4	51.5 ± 0.7	26.6 ± 0.7
24	23.6 ± 0.7	7.4 ± 0.3	1.4 ± 0.0	22.7 ± 1.1	48.3 ± 0.5	29.0 ± 0.6
48	23.3 ± 0.9	7.0 ± 0.2	1.4 ± 0.1	18.0 ± 1.4	50.0 ± 1.5	32.0 ± 0.6
72	22.3 ± 1.4	6.8 ± 0.5	1.4 ± 0.1	17.4 ± 1.5	50.6 ± 1.5	32.0 ± 2.3
96	22.0 ± 1.5	6.8 ± 0.4	1.4 ± 0.1	17.1 ± 1.9	49.6 ± 1.0	33.3 ± 2.7

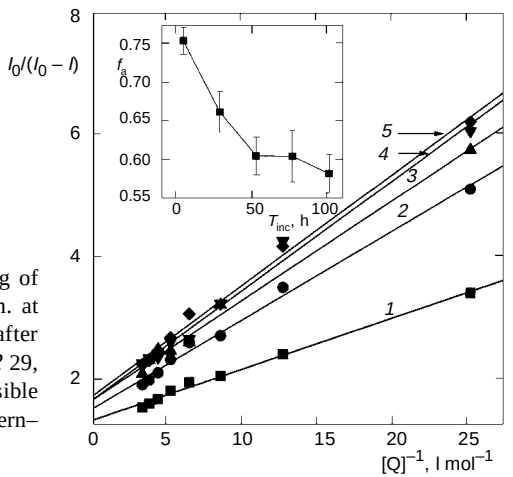

FIG. 5

Modified Stern–Volmer plots of the quenching of the non-Trp fluorescence (exc. at 360 nm, em. at 440 nm) of the HSA/GCA system by iodide after different times of incubation (T_{inc} in h): *1* 5, *2* 29, *3* 53, *4* 77, *5* 101. Inset: Fraction of accessible fluorofores, f_a, obtained from the modified Stern–Volmer plots, *vs* time of incubation

As evident from Fig. 5, the quenching of the non-Trp fluorescence in the system satisfied the modified Stern–Volmer equation. This is rather surprising because more complex behaviour of the system during quenching can be supposed. Although it can be a similar situation as in the case of evaluation of the fluorescence decays, and parameters of the modified Stern–Volmer equation obtained from fits have questionable physical meaning, efficiency of the quenching is definitely changed during glycation and values of f_a and K_{SV} represent good quantitative description of it.

Figure 5 inset shows the time course of f_a. If we interpret its value as a measure of the fluorescent moiety accessibility, its decrease can be caused by formation of crosslinked oligomers of HSA which was verified in HSA/glucose system by Sakurai *et al.*[11] by means of SDS–PAGE electrophoresis. The crosslinking is able to decrease iodide accessibility to the fluorescent moiety because a part of it is buried inside the oligomer. Also the time course of K_{SV} has similar character (data not shown). This result is probably associated with the time course of the lifetime distribution because quenching by iodide is dynamic and its efficiency is therefore proportional to lifetime of quenched fluorophore.

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