New fluorescence of nonenzymatically glucosylated human serum albumin

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Glucosylated human serum albumin (G-HSA) obtained under incubation with glucose at 37° C for 8 days showed a new fluorescence with a maximum at 430 nm, resulting in quenching of the fluorescence of only one tryptophan residue on HSA. The quantum yield of new fluorescence is 0.024 at 25° C. The analysis of the excitation spectra allowed us to conclude the absence of energy transfer. In G-HSA, non-disulfide cross-linking hexamer was confirmed by SDS–PAGE.

Nonenzymatic glucosylation *Human serum albumin* *Polylysine* *Fluorescence* *Energy transfer* *Aggregation*

1. INTRODUCTION

It has been reported that, in diabetic patients or in experimental animals with diabetes, glucose adducts of various proteins [1–3] as well as hemoglobin [4] and albumin [5] are increased. The reaction yields a stable ketoamine derivative of proteins through a Schiff-base between glucose and primary amine groups of protein, N-terminus or ϵ-amino of lysine residue. To investigate the pathological sequelae of this reaction, a characterization of changes in the structures and functions of glucosylated proteins attracts the researcher's interests. Serum albumin exerts a transport function for drugs through reversible binding [6]. The reduction of binding ability of glucosylated human serum albumin (G-HSA), which was obtained following the in vitro incubation with glucose, was observed for four kinds of sulfonylureas [7], bilirubin and *cis*-parinaric acid [8]. In this paper, we demonstrate the presence of a new fluorescence and this cannot be attributed to the energy transfer from tryptophan (Trp) residue. Glucosylated HSA is polymerized in the glucosylation process.

2. MATERIALS AND METHODS

All solutions were prepared with 1/15 M phosphate buffer, pH 7.4. HSA (Fujirebio Inc., Tokyo) solutions were incubated with glucose (200 mM) at 37°C for 8 days, followed by dialysis and lyophilization. The adsorbed fraction on the phenylboronate affinity column was referred to G-HSA, as in [7]. The glucose content of HSA was evaluated at 443 nm according to the thiobarbituric acid (TBA) method [9]. The glucosylated poly-lysine(G-p-Lys) was obtained by incubation of 0.1% solutions of poly-lysine hydrobromide (average molecular mass 55 000 Da, Sigma) with glucose (150 mM) for 1, 3 or 5 days, followed by dialysis against the buffer. SDS gel electrophoresis was carried out at room temperature using 5.0% polyacrylamide and Tris–HCl buffer, pH 7.4 [10]. The 0.1% solutions of protein were used for spectrum measurement. The corrected emission and excited fluorescence spectra were obtained at 25°C using a Hitachi MPF-4 spectrofluorometer following the correction by a rhodamine B quantum counter. The quantum yields of the fluorescence of G-HSA and G-p-Lys (25, 30, 35 and 40°C) were determined by

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comparison of the integrated corrected emission spectrum of a standard, quinine sulfate with a quantum yield of 0.54 in 0.1 N H_2SO_4 [11], with that of G-HSA and G-p-Lys obtained by excitation at 348 nm.

3. RESULTS AND DISCUSSION

As shown in the UV spectra (fig.1a), G-HSA and G-p-Lys show pigmentation at the wavelength ranging from 300 to 400 nm. At the same wavelength region, only one Trp residue on the HSA molecule shows fluorescence emission spectrum with the maximum at 340 nm by Ex. 290 nm (Ex. 290 nm or Em. 290 nm indicates that the excitation or emission wavelength is 290 nm) (fig.1b). However, this fluorescence was reduced by about a half of HSA as a result of glucosylation (fig.1b). Authors in [12] reported that an energy transfer occurs between two chromophores within 50 Å on a molecule if the emission spectrum of the energy donor overlaps with the absorption spectrum of the energy acceptor, and that the fluorescence of acceptor increases. Fluorescence energy transfer techniques have given useful information on the spatial relationships between active sites [13]. Therefore, we considered the possibility that the fluorescence energy of the Trp residue may be transferred to the pigment on G-HSA, i.e. a new chromophore, resulting in the quenching of Trp fluorescence. G-HSA showed a new emission spectrum with the maximum at 430 nm showing a small shoulder at 470 nm by Ex. 348 nm which is about the emission maximum of Trp fluorescence (fig. 1b). This fluorescence increased with incubation period, in contrast with the decrease of Trp fluorescence (fig.3). When the change of the emission spectra of Trp residue (Ex. 290 nm) was noted in detail, an isosbestic point at 430 nm was observed. This fact indicates the presence of a weak fluorescence at about 430 nm different from that of the Trp residue but dependent on the excitation energy at 290 nm. It is useful to investigate the excitation spectra, in order to estimate whether the Trp residue and the new chromophore on G-HSA could be assumed as donor and acceptor. For the complete efficient energy transfer, the excitation spectrum observed at the wavelength of emission maximum of the acceptor is identical with the sum

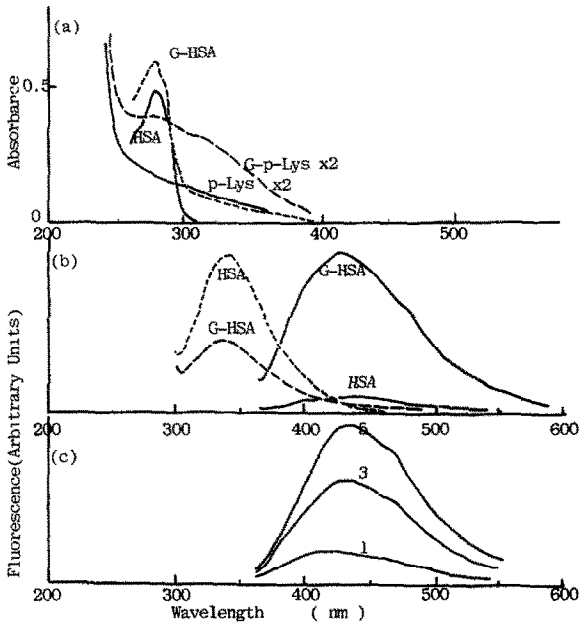

Fig. 1. Absorption spectra (a) and fluorescence spectra (corrected), (b,c), of human serum albumins and poly-lysines. (a) Absorbance scale of poly-lysines is one-half of that of HSAs. (b) The dashed lines with maximum at 340 nm were obtained while exciting at 290 nm. The solid lines with the maximum at 430 nm were obtained while exciting at 348 nm. (c) The spectra of poly-lysine glucose adduct were obtained while exciting at 350 nm. The 0.1% solutions of poly-lysine incubated with 150 mM glucose for 1, 3 and 5 days, followed by dialysis, were used.

of the excitation spectra of the donor and the acceptor [12]. In the absence of transfer, the excitation spectrum observed at the same wavelength is that of the acceptor itself. The latter criterion applies to G-p-Lys. The glucosylation of poly-lysine resulted in the fluorescence at the same wavelength region as G-HSA, increasing with incubation periods (fig.1c). The excitation spectra of G-p-Lys showed the maximum at 350 nm with a shoulder at 285 nm and a small band at 245 nm (fig.2c), indicating the presence of some chromophores contributing to fluorescence at about 420 nm. On the other hand, the excitation spectra of G-HSA observed at Em. 420 nm showed the maximum at 348 nm and 285 nm with a shoulder at 245 nm (fig.2b), showing a similar proportion of the sum of the excitation spectrum of the Trp residue of HSA (fig. 2a) and that of G-p-Lys (fig.2c), as if an

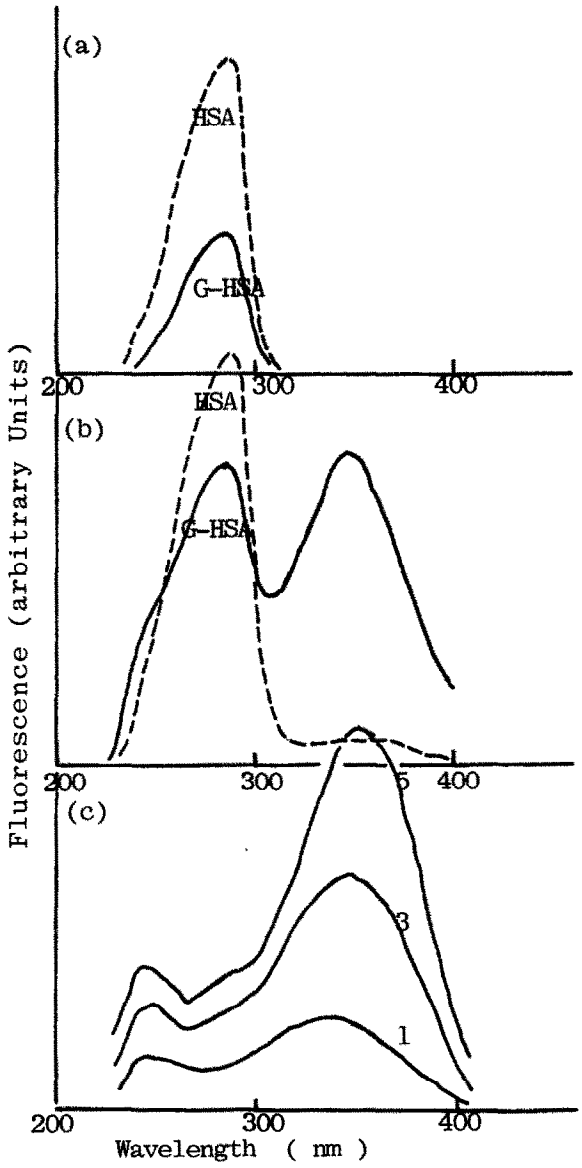

Fig. 2. Excitation spectra (corrected) of human serum albumins (a,b) and glucosylated poly-lysine (c). (a) Observed at emission wavelength of 340 nm. (b) Observed at emission wavelength of 420 nm. (c) Observed at emission wavelength of 420 nm. The incubation days are indicated above the lines.

efficient energy transfer were present. However, the emission band at 285 nm appeared more strongly in HSA than in G-HSA. This fact clearly indicates that the emission band at 285 nm does not originate in the new chromophore of G-HSA, but in the Trp residue. This phenomenon is understood by considering that the excitation spectrum of the Trp residue assumed as the donor overlaps on the spectrum of the new chromophore assumed as the acceptor, because of the wide emission spectrum of the former and the similar intensity of fluorescence in the Trp residue and the new chromophore even at 420 nm. Therefore, it is considered that a smaller decrease in the fluorescence of G-HSA at about 285 nm in the excitation spectra which was observed at Em. 420 nm (fig.2b) in comparison with that observed at Em. 340 nm (fig.2a) is attributed to the presence of a chromophore with an absorption band at about 285 nm in the new chromophore, as clearly shown in the excitation spectrum of G-p-Lys. The quantum yields of G-HSA and G-p-Lys at 25°C are 0.024 and 0.026, respectively, showing very similar values. This may reflect an absence of energy transfer. These values are one-third of the quantum yield of the Trp residue of HSA [13]. The quantum yield decreased as the temperature was increased (fig.4), but the shapes of the emission spectra remained unaltered. The change of the fluorescence and the glycosylation process is shown in fig.3. The absorb-

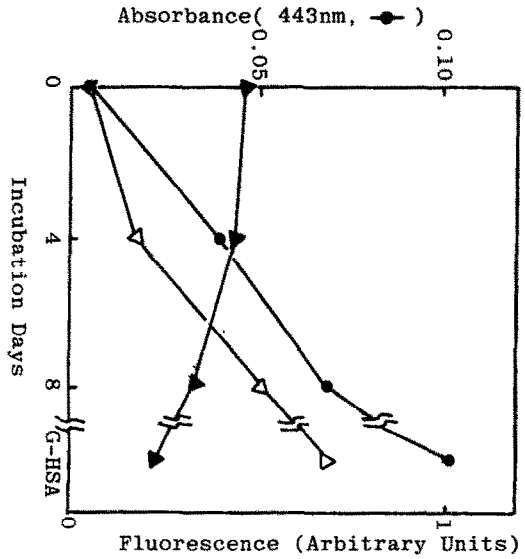

Fig. 3. Glucosylation and changes in fluorescence. Glucosylation was estimated at 443 nm by the TBA method. Trp fluorescence (—▲—) was monitored at 340 nm while exciting at 290 nm. For new chromophore fluorescence (—△—), the excitation and emission wavelengths were 348 nm and 430 nm, respectively.

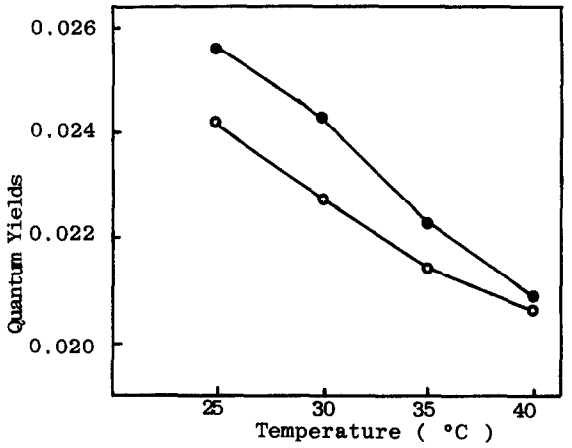

Fig. 4. The effect of temperature on the quantum yields of glucosylated human serum albumin (—○—) and glucosylated poly-lysine (—●—).

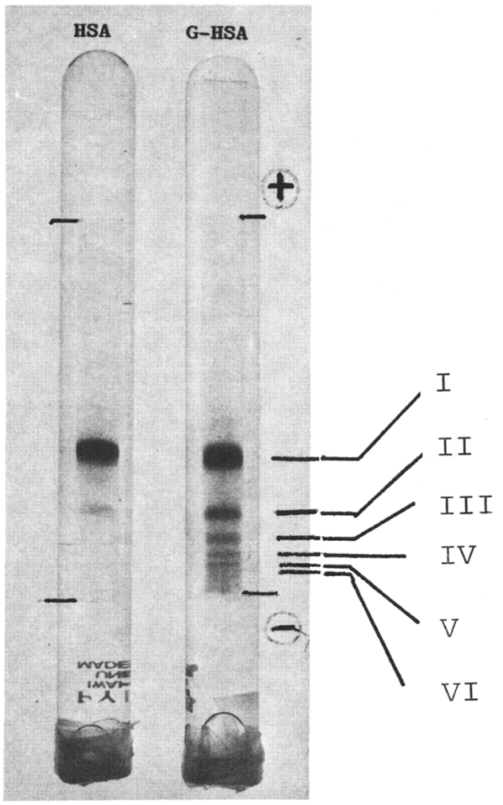

Fig. 5. SDS-PAGE of human serum albumin and glucosylated human serum albumin. Samples were heated for 3 min at 100°C in 1% SDS and 2% 2-mercaptoethanol before being placed on the gel.

ance at 443 nm increased linearly with incubation periods, but it seems that the increase in the fluorescence at 430 nm (Ex. 348 nm) and the quenching at 340 nm (Ex. 290 nm) appeared behind the increase in the TBA values. The fluorescence products may be the intermediates of a complicated Maillard reaction. The glucosylation of tendon collagen [14] has been reported to precede the appearance of fluorescence.

Maillard reaction has been studied in stored foods, though special interests have been taken in it in relation to aging and diabetes. Freeze-dried ovalbumin aggregated by storage with glucose at 50°C, and the content of lysine and α-helix decreased in proportion to the degree of aggregation [15]. In this experiment under physiological conditions, non-disulfide cross-linking was also confirmed by SDS-PAGE (fig.5). HSA contained monomer and dimer, but in G-HSA larger aggregates, from monomer to hexamer, were detected. Authors in [16] reported a very important fact that glucosylated ribonuclease A continued to generate its trimer through the reaction between glucosylated Lys residue and free Lys residues after the removal of free glucose. They suggest that a short term increase in blood glucose in vivo may also exert a long term physiological impact by subsequent cross-linking. Intermolecular cross-linking in HSA may be more easily formed than in ribonuclease A. The metabolism of the polymerized HSA may be extremely altered. Blue fluorescence and UV spectra obtained by this study are very similar to those obtained from cross-linked lens crystallin through photooxidation [17]. The relationship between new fluorescence and the aggregation of G-HSA remains to be investigated. The distance between Trp214 on domain II and Tyr411 on domain III of HSA as reported in [18] is approximately 25 Å, and the finding that the primary site of the glucosylation of HSA is Lys525 on domain III [8], suggest the possibility of the energy transfer, but spectroscopic analysis allows us to conclude the absence of energy transfer of Trp residue to the new fluorescence pigment. What contributes to Trp quenching may be the inter- or intra-molecular cross-linking.

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