CHEMILUMINESCENCE OF GLYCATED HUMAN SERUM ALBUMIN: COMPARISON WITH SYNTHETIC POLYMER

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The mechanism of protein glycation (nonenzymatic glycosylation) is complicated by existence of a variety of factors which affect both the kinetics and specificity of protein modification. In order to eliminate some of these factors, we have used a synthetic copolymer on the basis of polymethacrylic acid amide. Human serum albumin and the copolymer were incubated with glycolaldehyde for a period up to 168 h. The products of glycation were oxidized by atmospheric oxygen in the presence of horse-radish peroxidase leading to species in electronically excited states. The behaviour of human serum albumin and synthetic copolymer during glycation was compared on the basis of chemiluminescent data. In both cases, the chemiluminescence curves had biphasic character. On this experimental basis, a scheme was proposed for the glycation mechanism.

Key words: Protein glycation; Mechanism; Chemiluminescence; Human serum albumin.

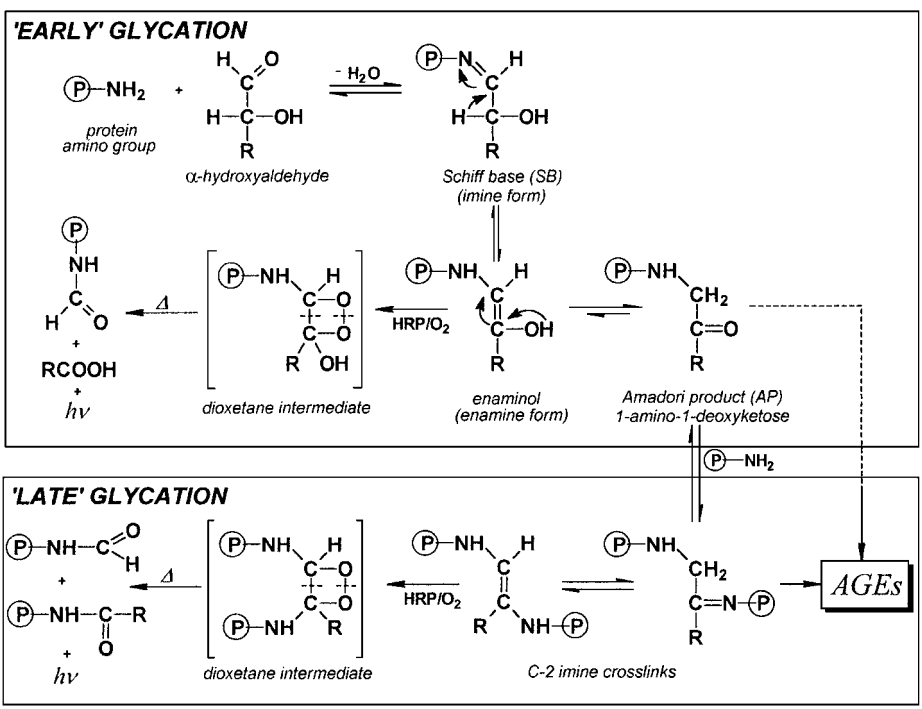
Glycation, an important post-translational modification of proteins, is a complex process which could be devided at least into two steps[1,2]. The first step involves condensation of a protein amino group with carbonyl group of a reducing sugar or low molecular weight aldehyde to form a Schiff base (SB)* followed by the consecutive shift of the double bond between carbons to form relatively stable ketoamine, an Amadori product (AP). In the second step, initially formed AP undergoes further complex reactions leading to the formation of so called advanced glycation end products (AGEs) which terminate the process of glycation (Scheme 1). Accumulation of protein-bound AGEs was implicated in pathogenesis of a number of diabetes mellitus-dependent complications[3,4].

There is evidence that C2 and C3 α-hydroxyaldehydes such as glycolaldehyde and glyceraldehyde react with proteins at a significantly higher rate and cause extensive

* Abbreviations: AGEs, advanced glycation end products; HRP, horse-radish peroxidase; HSA, human serum albumin; SC, synthetic copolymer; GA, glycolaldehyde; CL, chemiluminescence; BR, bilirubin; SB, Schiff base; AP, Amadori product; ROS, reactive oxygen species.

protein crosslinking and polymerization. Studies of glycation mechanism showed that AP of glycolaldehyde-derived Schiff base contains an aldehyde group which is capable of further reaction towards another amino group to form protein crosslinks[5]. Some of the protein-bound compounds formed during glycation with glycolaldehyde have been shown to be readily oxidized by horse-radish peroxidase (HRP). This oxidation involves enzymatic addition of molecular oxygen to a carbon double bond followed by the formation of a labile dioxetane intermediate whose thermal decomposition is accompanied by generation of electronically excited species and emission of photons. In the "early" stages of glycation, an enaminol (a Schiff base tautomer) was suggested to be the real substrate for dioxygenation reaction whereas in the "late" phases a C-2 imine crosslinks may be involved in the chemiexcitation step[6,7] (Scheme 1). Resulting electronic excitation energy could be transferred to oxygen transforming it into reactive oxygen species (ROS) (singlet oxygen, superoxide anion radical, hydroxyl radical, hydrogen peroxide), which are known to cause collateral damage to proteins, lipids, DNA and other cellular components[8,9].

An investigation of glycation processes in proteins is complicated by the fact that their amino groups differ in susceptibility towards reaction with sugars. This suscepti-



SCHEME 1

bility is determined by a variety of factors which largely reflect the effect of neighbouring functional groups to catalyze Amadori rearrangement[10]. To eliminate these factors we have recently synthesized a copolymer with side chains terminated with amino groups and used it as a model compound for the study of glycation mechanism and subsequent formation of electronically excited states[11]. Here, we report on peroxidase-catalyzed generation of CL from systems of human serum albumin (HSA) and of the synthetic copolymer (SC), both glycated with glycolaldehyde (GA).

EXPERIMENTAL

Chemicals

Human serum albumin (Imuna, Slovak Republic), glycolaldehyde (Sigma, U.S.A.), horse-radish peroxidase (592 U/mg, Serva, Germany), bilirubin (Merck, Germany) were used as supplied. Synthetic copolymer based on polymethacrylic acid amide with side-chain amino groups (Fig. 1) was synthesized and characterized according to[11]. All the chemicals used in experiments were of the highest reagent grade obtainable. All solutions were prepared in 0.1 M phosphate buffer pH 7.4 at 25 °C. Solutions of bilirubin were prepared in 0.05 M NaOH and were kept in dark.

Procedures

Incubations with glycolaldehyde. Concentrations of HSA and SC were adjusted in order to achieve equivalent molar concentrations of amino groups (11 mM) and kept constant throughout all experiments. Solutions were incubated at 25 °C with equimolar quantities of glycolaldehyde (11 mM). At given time intervals aliquots of incubated sample were removed and HRP was added to the concentration of 1 μM. The concentration of O_2 naturally dissolved in the aqueous solution at 25 °C was considered to be sufficient for HRP-catalyzed oxidation. Experiments in the absence of oxygen were carried out by purging nitrogen through the solution which had been incubated with glycolaldehyde, 10 min prior to HRP addition.

Chemiluminescence measurements. Chemiluminescence from incubated reaction mixtures was measured as an integrated emission intensity in counts per minute (cpm) using a Beckman LS 5801 liquid scintillation counter in a single-photon monitor operating mode (counter with the coincidence circuit turned off). CL reaction was started by adding micromolar amounts of HRP to 5.0 ml of sample solution.

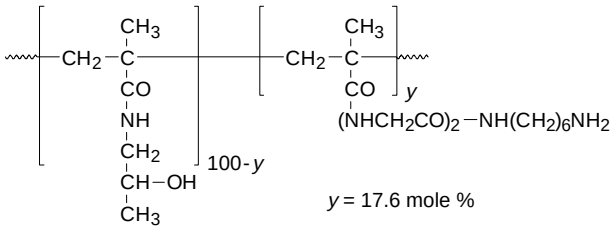


FIG. 1
Chemical structure of the synthetic copolymer

Spectral measurements. Difference absorption spectra were recorded on a Zeiss M40 Specord spectrophotometer. The rate of formation of electronically excited species during peroxidase-catalyzed oxidation was estimated according to the method that has been developed in our laboratory[12]. The samples were pipetted into quartz cuvettes and bilirubin (BR) was added to the concentration of 25 μM. After the reaction had been started by adding HRP (1 μM), absorbance at 450 nm was monitored against control. The controls were carried out by replacing HRP by the same volume of buffer. The rate of electronically excited species formation was estimated as the slope of linear time dependence of BR absorbance.

Oxygen uptake. The oxygen uptake during HRP-catalyzed oxidation was followed using a Clark type electrode which had been calibrated with the solution of Na_2SO_3.

RESULTS

Human serum albumin, as a model protein, and synthetic copolymer were incubated with glycolaldehyde. At certain time intervals the aliquots of incubated samples were removed, HRP was added and CL intensity was monitored. Both glycolaldehyde-modified HSA and SC samples exhibited intense CL whose kinetics was followed during the first 800 min after the addition of peroxidase (Figs 2 and 3). In general, the kinetics of CL generation in systems studied was dependent on the period for which the protein and/or copolymer were preincubated with GA. Although the kinetics of CL development in both systems is very complicated, there are two general differences between the two systems: (i) despite the fact that the molar concentration of amino groups was

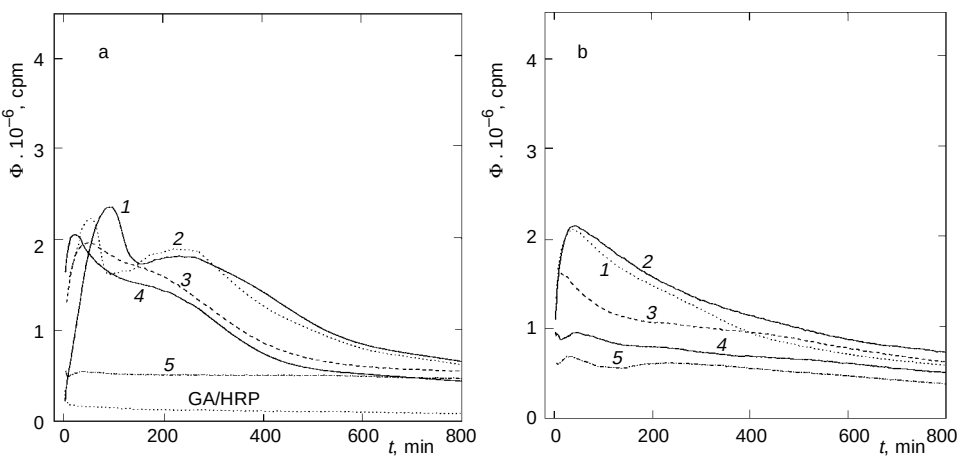


FIG. 2

Time dependence of chemiluminescence intensity Φ (counts per min) of HSA (0.2 mmol dm^{-3}, concentration of amino groups 11 mmol dm^{-3}) incubated with GA (11 mmol dm^{-3}) for 0–24 (a) and 48–168 h (b) at pH 7.4 and temperature 25 °C, after addition (t = 0) of HRP (1 μmol dm^{-3}) to mixture. Time of incubation (in h): *1* 0, *2* 2, *3* 8, *4* 14, *5* 24 (a); *1* 48, *2* 96, *3* 120, *4* 144, *5* 168 (b). The luminescence intensity of GA alone after addition of HRP is also shown for comparison

kept constant throughout experiments, the overall CL intensity is higher for glycated SC than for HSA (Fig. 3); (ii) the kinetics of peroxidase-induced CL of glycated HSA shows the presence of at least two distinct components (Fig. 2a), whereas in the samples of glycated SC the two components are not well resolved (Fig. 3). This biphasic character of CL is also completely lost in the samples of HSA that have been glycated for more than 24 h (Fig. 2b). As could be seen in Fig. 2a, the longer is the time of HSA glycation, the closer is the maximum of the initial burst of CL to the point at which HRP was added (for glycation period of 0–14 h).

Control experiments were performed to rule out a possibility that glycolaldehyde and/or its autooxidation products are responsible for the generation of CL. Glycolaldehyde incubated with HRP in the absence of both SC and HSA showed that the generation of CL is significantly inhibited (Fig. 2a). Furthermore, the samples of both glycated SC and HSA, in the absence of HRP, exhibited no measurable CL (data not shown), indicating that peroxidase-catalyzed oxidation of adducts between GA and SC or HSA is the sole source of CL.

In order to estimate the immediate amount of a substrate susceptible to HRP-catalyzed oxidation, CL intensity in the first 30 min after the addition of HRP was integrated and plotted as a function of glycation period (Fig. 4). The amount of material susceptible to HRP-catalyzed oxidation in both HSA and SC sharply increases with time of glycation and then decreases to reach its minimum after 24 and 36 h, respec-

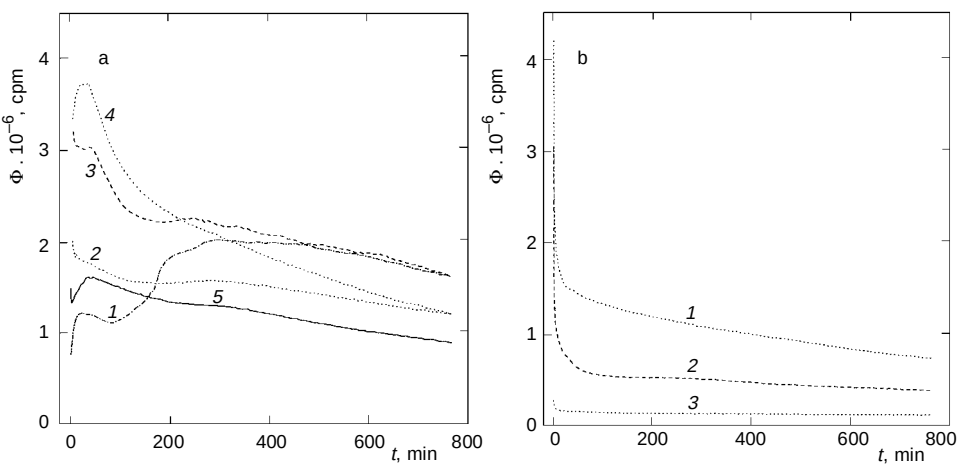


FIG. 3

Time dependence of chemiluminescence intensity Φ (counts per min) of copolymer (concentration of monomeric unit 63 mmol dm^{-3}, amino groups 11 mmol dm^{-3}) incubated with GA for 0–36 (a) and 48–168 h (b) after addition of HRP to mixture under the same conditions as in Fig. 2. Time of incubation (in h): *1* 0, *2* 8, *3* 14, *4* 24, *5* 36 (a); *1* 48, *2* 120, *3* 168 (b)

tively. Behind these points, the amount of substrate for HRP-catalyzed generation of CL again slowly increases with the time of glycation in both systems. In order to verify the acquired CL data, another comparative method was used to determine the amount of material available for HRP-catalyzed oxidation. Samples of HSA that had been glycated for various time intervals were supplemented by 25 μM of BR whose time-dependent photochemical degradation was monitored following the addition of HRP. Time dependence of the rate of BR degradation corresponded to that of integrated CL intensity (Fig. 4) and thus proved to be a useful comparative method for indirect measurement of CL intensity. In the case of SC, it was not possible to evaluate the changes in the rate of BR degradation because of nonlinearity of time-dependent decay of absorbance of BR at 450 nm.

To check whether oxidation processes are responsible for CL generation, concentration of oxygen was monitored after the addition of HRP. Inset in Fig. 4 depicts O_2 uptake during HRP-catalyzed oxidation in samples of HSA and/or SC with GA present only *in situ*, without any preincubation. Two hours after addition of peroxidase to the samples of glycated SC and HRP, nearly 77 and 53% of oxygen was consumed, respectively, indicating that the rate of O_2 uptake is directly proportional to the overall CL intensity. The control experiments carried out in the absence of O_2 showed that the development of CL species was completely impaired (data not shown).

DISCUSSION

The aim of this study was to compare the mechanisms of glycation in proteins with those in a model synthetic copolymer and to evaluate the possibility that some of the compounds formed during glycation are susceptible to HRP-catalyzed oxidation whose products may be responsible for light emission and consequent generation of free radi-

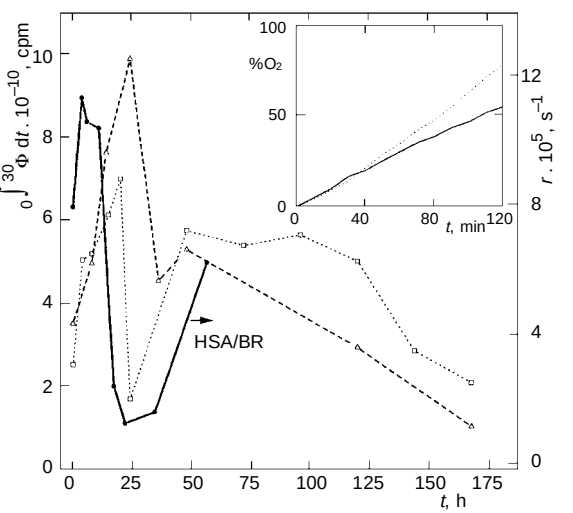


FIG. 4
Dependence of integral CL intensity Φ of HSA (dotted line) and SC (dashed line) on the time of its incubation with GA under the same conditions as in Fig. 2 and Fig. 3, respectively. Integral CL intensity is measured in the course of the first 30 min after addition of HRP. Solid curve shows the dependence of degradation rate of bilirubin (25 μmol dm^{-3}), *r*, on the time of incubation HSA with GA. Inset shows the kinetics of O_2 consumption (% O_2) in the solution of HSA (full line) and SC (dotted line) after addition of GA and HRP without any incubation

cals and ROS. Peroxidase-catalyzed generation of exited triplet species by oxygen-consuming reactions has been reported for a variety of compounds (for review see ref.[13]), including simple aliphatic Schiff bases with amino acids[7] and covalent glycolaldehyde adducts with proteins[6]. Investigations of glycation process in proteins have shown that there is a variety of factors which affect the site-specificity of glycation[14]. To eliminate some of these factors we have used a synthetic copolymer with side-chain amino groups as a model system. Glycolaldehyde was used as the glycating agent because (i) glycation with GA is mechanistically simpler than that with glucose and (ii) leads to extensive crosslinking of the protein rather than to the generation of AGEs, such as pentosidine and pyrraline. Although GA is not direct product of glycolysis, its physiological significance stems from the fact that glycolaldehyde may be formed *in vivo* by retro-aldol cleavage of AP derived from glucose and lysine[15]. As a model peroxidase, HRP was used.

Copolymer and albumin were first glycated with glycolaldehyde and the resulting adducts were then subjected to HRP-catalyzed oxidation. Chemiluminescence intensity was monitored to determine the susceptibility of glycation adducts to oxidation reaction. As seen in Fig. 2 and 3, the kinetics of CL development is very complicated and the data acquired does not allow us to make any definite conclusions as to the precise molecular mechanism of the observed processes. However, a closer examination of the data shows that both systems (protein and the model copolymer) exhibit different susceptibilities to the formation of glycation adducts. Although the number of free NH_2 groups was the same in both systems, CL intensities (Fig. 3a) and rates of O_2 uptake (inset in Fig. 4) of the initially glycated SC (0–24 h) were generally higher than those of HSA, indicating that glycolaldehyde adducts with SC are formed at a significantly higher rate than with HSA. To explain the above observation we first have to make an approximation and to consider glycation as a series of two consecutive processes. In the first phase, a Schiff base between GA and a free protein or copolymer amino group is formed and undergoes subsequent slow rearrangement to the more stable AP. Amadori products and their precursors, such as SB, will therefore be collectively termed as "early" glycation products. In the second stage, APs undergo a series of complex reactions that lead to the formation of so called "late" glycation products such as covalent crosslinks and AGE products (Scheme 1).

In the initial period of glycation, the enamine-based compounds formed by imine–enamine tautomerization of a Schiff base were suggested to be real substrates for peroxidase-catalyzed oxidation and consequent generation of CL in initially glycated proteins[16]. As the positively charged nitrogen of a Schiff base (an imine) attracts electrons and thus facilitates the loss of a carbon-bound proton, formation of enamine compounds from Schiff bases is energetically more favourable than the keto–enol tautomerization of corresponding α-hydroxyaldehydes[17]. The rate of Schiff base tautomerization is very high so that small amounts of corresponding enamine will also be

present *in situ*; the rate of enamine formation will be directly proportional to the immediate concentration of protein- or polymer-bound Schiff base. Hence, if we accept the enaminol as a putative substrate of HRP-catalyzed oxidation of "early" glycation products, then we should consider the factors which influence SB formation in studied systems. Studies on the chemistry of SB have shown that low pK_a amino groups are the most reactive with the carbonyl groups. However, pK_a of the amino group is not only determined by its type (α-terminal or ε-side chain) but is also substantially influenced by its local environment. Thus, the covalent and three-dimensional structure of a protein may shift the pK_a of each amino group by as much as several pH units from its original value in the free amino acid as a result of electrostatic influence of nearby charged groups, medium effects arising from the proximity of groups of low dielectric constant, and the effect of hydrogen bonding associations[18]. Therefore each ε-amino group of lysine in HSA will have a different pK_a value and thus will exhibit different susceptibility to SB formation. Furthermore, as some of the amino groups in HSA are buried, they are inaccessible for modification and the rate of SB formation will be further decreased. Such effects are often observed in proteins and contribute undoubtedly to the site-specificity of glycation.

In the synthetic copolymer, the situation is markedly different. As the only charged groups present in SC are the side-chain amino groups, each of them will have the same pK_a comparable to that of ε-NH_2 of Lys. In addition, the random-coil structure of SC along with the extended side chains will also contribute to higher accessibility of their amino groups towards modification. Hence the "early" glycation products of SC will be formed with most of its amino groups, whereas in HSA their formation will be restricted to a limited number of sites, thus providing lower amounts of a substrate for HRP-catalyzed oxidation.

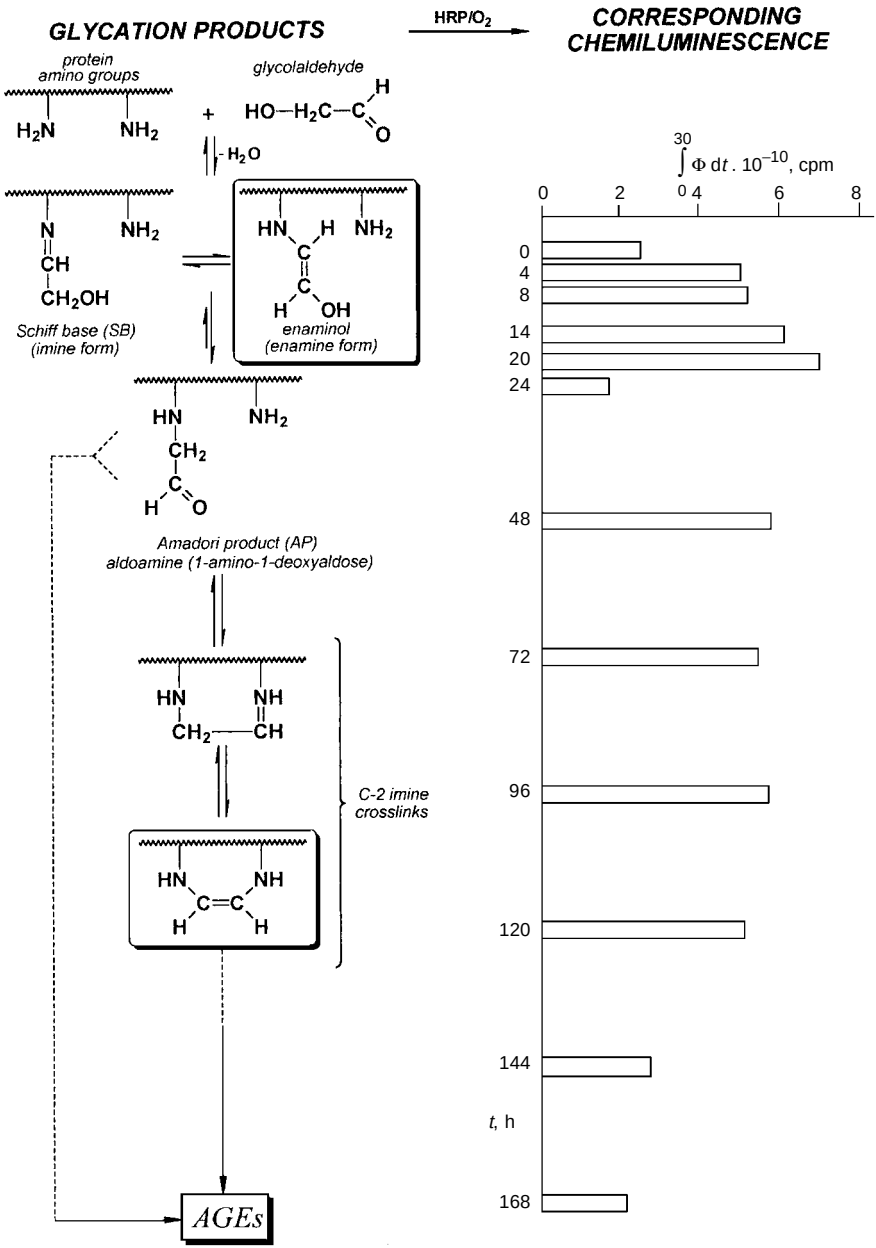
Generation of light from "early" glycation products in the presence of HRP is likely to proceed through the formation of a dioxetane intermediate which is known to emit photons upon thermal decomposition (Scheme 1). Dioxetane ring is formed by HRP-catalyzed addition of molecular oxygen to the carbon double bond of enamine (dioxygenation reaction). Although the peroxidase activity of HRP is well documented in literature, less is known about its dioxygenase activity which is responsible for the formation of dioxetanes and generation of CL. Apart from the enamine, HRP and O_2, traces of organic peroxides are also known to be required for the initiation of enzymatic reaction. Since the reaction mixtures were not supplemented by H_2O_2 or other peroxides, the initiation of oxidation was dependent upon its trace presence or their formation during glycation. As could be seen from the kinetics of CL in glycated HSA (Fig. 2a), the time of initial burst of CL was dependent upon the time for which the protein was glycated with GA, so that, for example, an initial lag phase of almost 100 min was required for the maximal CL intensity in HSA which has not been previously glycated. In contrast, the samples that have been glycated for 14 h developed CL almost immedi-

ately after the addition of HRP. This observation suggests that progressive accumulation of an initiator (hydrogen peroxide or other peroxides) during glycation period may play a role in the kinetics of CL development. Other likely mechanism for the accumulation of peroxides would be the autooxidation of unreacted GA in the solution catalyzed by traces of transition metals as reported in ref.[19]. The lack of this effect in SC may be due to the fact that the sample of SC may have been already contamined by peroxides during its synthesis and isolation.

In the context of the proposed mechanism, one may speculate whether GA or its autooxidation products could also be sources of CL species. However, control experiments have shown that GA incubated with HRP exhibited CL slightly above the level of backround (Fig. 2a). As some low molecular weight aliphatic aldehydes (2-methylpropanal) are known to undergo HRP-catalyzed oxidation accompanied by CL generation[20], it is possible that GA alone (or its enol tautomer) may act as a substrate for HRP. However, because the formation of enol form of GA, substrate for HRP, is not as favourable as the formation of enamine from its corresponding SB, its contribution to the overall CL will be very limited (Fig. 2a). In addition to this possibility, glycated HSA and SC do not exhibit increased CL in the absence of HRP (data not shown). The proposed mechanism in which the formation of dioxetane intermediate is responsible for CL generation is further supported by the evidence that oxygen is consumed during HRP-catalyzed reaction (inset in Fig. 4) and that the development of CL is completely impaired in the absence of O_2 (data not shown).

One of the most striking features is that the intense emission of light form both systems lasts for more than 10 h after addition of HRP. Although the initial intensity of CL (0–30 min) could be taken as a good measure of the amount of substrate susceptible to HRP-catalyzed oxidation (Fig. 4), kinetics of further CL development is very complicated and may be influenced by a variety of factors. For example, the presence of the second CL component in samples of HSA (Fig. 2a) may be explained by the exhaustion of oxygen normally dissolved in solution. In later phases of enzymatic reaction, the concentration of oxygen may become rate-limiting and thus the generation of CL would be decreased. This hypothesis is supported by the fact that after 120 min of the enzymatic reaction the concentration of O_2 in the solution is substantially diminished (inset in Fig. 4). Another fact to mention is that the species produced upon decomposition of proposed dioxetane intermediate may contain reactive aldehyde groups (Scheme 1), which are capable of further reactions *in situ*, thus propagating the glycation process and promoting the duration of CL generation. Nevertheless, the above hypotheses are only speculative and therefore molecular characterization of reaction products will be necessary to elucidate the precise mechanism of observed changes.

As mentioned above, the integrated CL intensity in the first 30 min was taken as a measure of the amount of the substrate immediately susceptible to HRP-catalyzed oxidation. Integrated CL intensity was followed as a function of the period for which HSA


GLYCATION PRODUCTS
HRP/O2
CORRESPONDING CHEMILUMINESCENCE
protein amino groups
glycolaldehyde
- H2O
Schiff base (SB) (imine form)
enaminol (enamine form)
Amadori product (AP) aldoamine (1-amino-1-deoxyaldose)
C-2 imine crosslinks
AGEs
30 ∫ Φ dt . 10−10, cpm
0 2 0 4 6 8
0
4
8
14
20
24
48
72
96
120
144
t, h
168

SCHEME 2

or SC were incubated with GA (Fig. 4). The initial increase of CL intensity soon reaches a plateau which is likely to correspond to the highest concentration of enamine tautomer of a Schiff base whose role in the generation of CL was discussed in previous paragraphs (Scheme 2). Schiff base then undergoes a slow, nearly irreversible rearrangement to form more stable AP, so that after a given time all SB in the glycation mixture are converted to AP. Due to their chemical nature, AP are unable to act as substrates for oxidation by HRP and thus the decrease in the integrated CL intensity of glycated HSA and SC represents the moment at which all available SB were converted into AP. Observation that this decrease comes earlier during glycation of HSA (24 h) than of SC (36 h) indicates that the rate of Amadori rearrangement is higher in the protein than in the copolymer. Indeed, it is well established fact that in proteins the clusters of basic amino acid residues (Arg, His, Lys) may act as local acid-base catalyst of the Amadori rearrangement on neighbouring Lys residues[21]. In contrast to HSA, the rate of Amadori rearrangement at the amino groups of SC will be substantially attenuated due to the absence of such catalytic mechanisms.

Once Amadori products are formed on protein and/or copolymer they undergo further complex reactions to form "late" glycation products – brown and highly fluorescent compounds that are collectively called advance glycation end products (AGEs). The glycation reaction of GA is rather exceptional because the corresponding protein- or polymer-bound AP contains another reactive aldehyde group which is capable of intra and intermolecular crosslinking reaction with another free amino group. The crosslinking by GA is therefore mechanistically unique compared to that of bifunctional reagents such as dialdehydes, because the crosslinking potential of GA is exposed only after Amadori rearrangement of its SB has occurred. Recently, it has been shown that GA-derived protein crosslinks have a C-2 imine structure, *i.e.* they are formed by condensation of GA-derived AP with a free amino group[22] (Scheme 1). The C-2 imine crosslinks were also proposed to be involved in the HRP-catalyzed formation of electronically excited species from GA-lysozyme adduct[6] suggesting that the formation of imine crosslinks in HSA and/or SC may be responsible for CL generation in the "late" phases of glycation, *i.e.* in the samples that were glycated for more than 36 h (Fig. 4). Chemiluminescence intensity from "late" glycation products is decreased when compared to that of "early" glycation, suggesting lower concentration of imine crosslinks. As the probability of imine crosslink formation is not only influenced by the concentration of AP but also by the availability of a free and sterically accessible amino group, the amount of imine crosslinks formed will be reduced. Although the formation of AGEs, such as pyrazine, is responsible for increase in fluorescence and coloration observed during "late" phases of glycation, their susceptibility to HRP-catalyzed chemiluminescent oxidation cannot be evaluated on the basis of acquired data.

At this place we should also note that both "early" and "late" glycation was itself shown to be a source of light, free radicals and ROS (refs[23–25]). Proposed mechanisms

involve oxidative processes at multiple stages of glycation, such as autooxidative glycosylation and glycoxidation[26,27], which lead to the transition metal-catalyzed generation of ROS. Recently Namiki *et al.*[25] suggested that 1,2-dioxetane structures formed by the rearrangement of carbon-centered free radicals of α-diketone/enediol complex may be responsible for weak CL observed in the "early" stages of glycation. Although the role of transition metal-mediated oxidative processes during glycation is well established, their role *in vivo* might be attenuated by the fact that transition metals present in the cell are mostly chelated and therefore not able to catalyze oxidative processes[28]. Our results show that catalysis by peroxidase may substantially increase the rate of oxidative processes that lead to far more intense CL and consequent generation of ROS.

To conclude, comparison of acquired CL data for HSA and SC indicated a variety of factors which are responsible for their different behaviour during glycation by GA. Although the SB-type adducts are more readily formed with SC, formation of covalent crosslinks is quicker in HSA. This finding reflects the unique ability of proteins to self-catalyze the Amadori rearrangement. Although the initial concentration of SB in HSA is lower than that of SC, increased rate of Amadori rearrangement ensures higher concentrations of protein-bound AP whose presence is essential for imine crosslinking. The copolymer used thus proved itself to be a useful model for the investigation of factors that influence processes of glycation *in vitro*. On the basis of CL data, we have also proposed mechanism by which glycated proteins, in the presence of peroxidases, may be a source of chemiluminescent species whose electronic excitation energy could be transferred to oxygen transforming it into ROS. We have suggested that in the "early" stages of glycation an enaminol, a SB tautomer, could be a substrate of HRP-catalyzed oxidation whereas in the "late" stages a C-2 imine crosslinks can occur (Scheme 2). As the above mechanism is only hypothetical, the structures of corresponding reaction products should be investigated in order to establish the precise molecular mechanism of observed processes. Peroxidase-catalyzed oxidations of glycation products thus may provide a missing link between increased glycation and generation of ROS, both seen *in vivo* in diabetes and normal ageing.

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