ELSEVIER

Biophysical Chemistry 105 (2003) 743–755

Biophysical Chemistry

www.elsevier.com/locate/bpc

Unusual susceptibility of heme proteins to damage by glucose during non-enzymatic glycation

Brian L. Cussimanio[a], A. Ashley Booth[a], Parvin Todd[b], Billy G. Hudson[b], Raja G. Khalifah[c,*]

[a]*University of Kansas Medical Center, Kansas City, KS 66160, USA*
[b]*Vanderbilt University Medical Center, Nashville, TN 37232, USA*
[c]*BioStratum Inc, 4620 Creekstone Drive, Durham, NC 27703, USA*

Received 2 January 2003; received in revised form 5 March 2003; accepted 5 March 2003

Abstract

Glucose modifies the amino groups of proteins by a process of non-enzymatic glycation, leading to potentially deleterious effects on structure and function that have been implicated in the pathogenesis of diabetic complications. These changes are extremely complex and occur very slowly. We demonstrate here that hemoglobin and myoglobin are extremely susceptible to damage by glucose in vitro through a process that leads to complete destruction of the essential heme group. This process appears in addition to the expected formation of so-called advanced glycation end products (AGEs) on lysine and other side-chains. AGE formation is enhanced by the iron released. In contrast, the heme group is not destroyed during glycation of cytochrome *c*, where the sixth coordination position of the heme iron is not accessible to solvent ligands. Glycation leads to reduction of ferricytochrome *c* in this case. Since hydrogen peroxide is known to destroy heme, and the destruction observed during glycation of hemoglobin and myoglobin is sensitive to catalase, we propose that the degradation process is initiated by hydrogen peroxide formation. Damage may then occur through reaction with superoxide generated (a reductant of ferricytochrome *c*), or hydroxyl radicals, or with both.

Keywords: Non-enzymatic glycation; Advanced glycation end products; Heme; Heme proteins

Abbreviations: AGE, advanced glycation (older term glycosylation) end products; CML, N^{ε}-carboxymethyl-L-lysine; metHb, methemoglobin; metMb, metmyoglobin; BSA, bovine serum albumin; RNase, bovine pancreatic ribonuclease A; DETAPAC, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; RBC, red blood cell.

*Corresponding author. Tel.: +1-919-433-1000; fax: +1-919-433-1010.
E-mail address: rkhalifah@biostratum.com (R.G. Khalifah).

0301-4622/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0301-4622(03)00100-5

1. Prologue

1.1. Personal introduction by RGK

My very first encounter with Walter Kauzmann was both memorable and consequential. We first met around November 1962, while I was a first year graduate student looking for possible thesis projects. As the subject drifted to protein folding, Kauzmann quickly opened a treasured Scientific American issue with an article by Kendrew [1] *describing the intricate three-dimensional structure of myoglobin. This was the very first protein to have its crystallographic structure determined to high resolution—and my first view of a protein. That historic stick-model illustration by Irving Geis was displayed as a stunning and colorful centerfold of myoglobin* (*perhaps it was only a two-page spread*)*, with its* α*-helices surrounding the luscious red heme group containing the space-filled ball of iron. This was as seductive as anything I had ever seen, and a lifetime of studying protein structure and function began then and there. Some 35 years later, chance observations that we made during studies on protein glycation would have gone entirely unappreciated, except for my familiarity with myoglobin that was developed during my thesis studies on its urea unfolding kinetics. It thus seems appropriate, on this special occasion honoring Walter Kauzmann, to report our novel observations on the glycation of heme proteins and the surprisingly complex reactions that distinguish them.*

2. Introduction

2.1. Statement of the problem

The direct chemical modification of protein amino groups by glucose, an aldehyde, has been implicated in the development of serious and life-threatening diabetic complications. Most of the damage occurs in the vasculature of tissues, e.g. the glomerulus of the kidney or the retina of the eye, where there is no regulation of glucose entry. The exposure in such cases directly follows the fluctuations of glucose blood levels, in contrast to cellular sites where entry is regulated by insulin. During our studies on the kinetics and mechanism of this extremely slow glycation process, we have observed greatly enhanced modification of metalloproteins containing redox metal ions. Furthermore, we made the surprising observation, which is the subject of this report, that certain heme proteins, but not others, are extremely vulnerable to damage by glucose. This damage is dramatically manifest by the complete destruction of the heme group, with obvious catastrophic consequences to structure and function. The broader implications of these findings for diabetic complications remain to be established. However, the results highlight new avenues to pursue in unraveling the complex 'glycoxidation' mechanisms by which glucose damages proteins. As our previous studies demonstrate [2,3], this is an essential prerequisite to the design of effective therapeutic inhibitors of hyperglycemic damage in diabetes [4–6]. Indeed, currently one such mechanism-based inhibitor is successfully advancing in phase 2 clinical trials for diabetic nephropathy [7].

2.2. Non-enzymatic glycation

It has been known for nearly a century that the exposure of amino acids to glucose results in a very slow 'browning' reaction, the so-called Maillard reaction [8,9]. This reaction has long interested food chemists [10], but only in the last two decades has it become of significant interest to biochemists studying diabetic complications [11]. The reaction is initiated by reversible Schiff base condensation of glucose, in its acyclic aldehyde form, with free amino groups (α or ε). The Schiff base (an aldimine) then undergoes an Amadori rearrangement, essentially irreversible, to form 1-deoxyfructosyllysine. This 'early glycation' product is the so-called Amadori intermediate, a ketoamine (Fig. 1). Half a century after Maillard's observations, the obscure hemoglobin variant HbA1c was shown to be an Amadori attachment of glucose to the terminal α-amino group of Val-1 of the β-chains [11]. While HbA1c is now a powerful clinical marker of glucose exposure in diabetics, its landmark identification also propelled the investigation of the biochemical and medical importance of non-enzymatic glycation [12,13]. Its

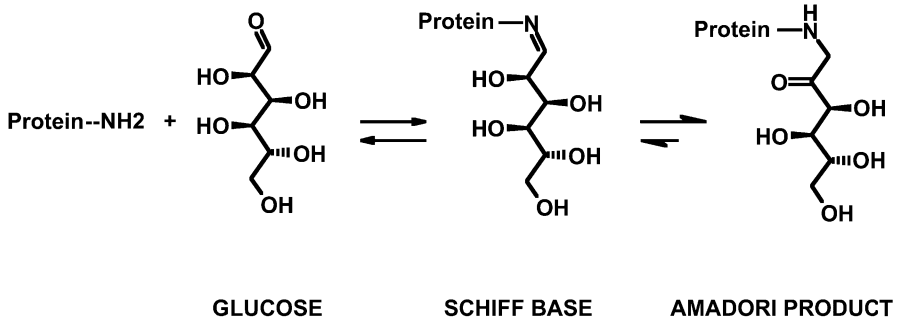

Fig. 1. Condensation of the acyclic glucose form with amino groups followed by the Amadori rearrangement.

presence in both diabetic and normal subjects proved that the aldehyde glucose, existing at only 0.002% in the reactive acyclic form, does indeed modify proteins.

2.3. Advanced glycation end products and diabetic complications

Glycation in normoglycemic subjects probably contributes to the aging processes [14], but more deleterious and more accelerated consequences for protein structure and function occur with the hyperglycemia of diabetes mellitus. A greater appreciation of the pathogenic consequences of glycation occurred later with the realization that the Amadori product is unstable. Its slow degradation can lead to a variety of drastic and irreversible changes, often producing fluorescent and poorly characterized products, including protein cross-links [15]. These modifications, in principle, affect protein structure, function, physical properties, enzymatic activity, cellular interactions and turnover. The reactions are extremely slow, making them difficult to study in vivo or in vitro. The modifications normally accrue on long-lived proteins, such as the collagen of the extracellular matrix, and have been implicated in the microangiopathies of diabetes such as nephropathy, neuropathy and retinopathy [13,16,17]. The Amadori product thus occupies a central role as the first committed intermediate on the road to this Maillard cascade of isomerizations, involving rearrangements, dehydrations, oxidations, and the formation of free radicals and reactive oxygen species [4]. Most of these 'glycoxidation' reactions usually require the presence of oxygen and trace redox metal ions [18,19], and the products are collectively referred to as advanced glycation end-products (AGEs). The most abundant AGE is probably carboxymethyllysine (CML), where the carboxymethyl radical can arise from fragmentation of the attached glucose between C2 and C3 through an ene-diol intermediate [20,21].

While this classical view ('Hodge pathway') involving a central role for Amadori intermediates has much support, it represents an incomplete description. Particularly as studied in vitro, where high concentrations of glucose near 1 M are utilized to speed the reaction, alternate pathways of AGE formation arise that can give similar AGE products [4]. For example, glucose itself autoxidizes ('Wolff pathway') to yield glyoxal and the pentose arabinose [22], both of which can modify lysine side-chains to produce AGEs such as CML. Similarly, the Schiff base is very susceptible to redox metal catalyzed oxidation ('Namiki pathway') to yield glycoaldehyde, which can also modify lysines to yield AGEs such as CML [23]. The Amadori intermediate itself is also subject to degradation to form very reactive dicarbonyls (1- or 3-dexoyglucosones). In vivo, other sources of AGEs have been identified that may contribute to diabetic complications. Oxidation of polyunsaturated lipids has been shown, at least in vitro, to even produce CML in the absence of sugar; the products of this pathway are referred to as advanced lipoxidation end-products (or ALEs) [24]. Others have suggested that increased pentose

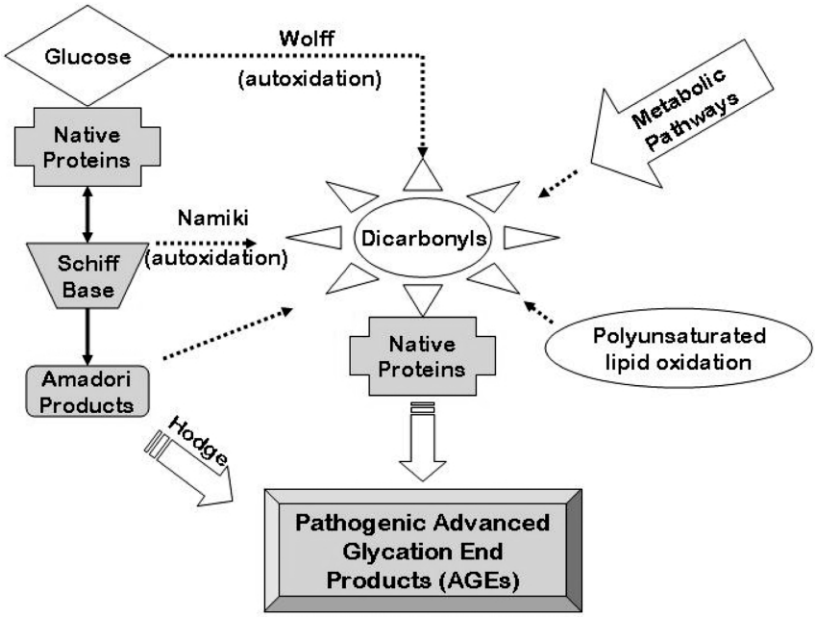

Fig. 2. Pathways of formation of AGEs in vitro and in vivo via a generalized increase in carbonyl stress. Protein products are shown as shaded. The classic Hodge pathway via Amadori is shown on the left.

phosphate metabolism in diabetes can produce intracellular precursor carbonyls such as methylglyoxal (pyruvaldehyde). These are also capable of modifying proteins to produce AGEs and can lead to mitochondrial or other damage [25]. These pathways are summarized schematically in Fig. 2. It is now clear that different pathways of AGE formation may predominate under different conditions, both in vivo and in vitro. The central challenge is to determine their relative contributions, if any, to pathogenic complications, and to find inhibitors that target them [4].

3. Experimental methods

3.1. Materials and chemicals

The following proteins and chemicals were purchased from Sigma: bovine heart cytochrome *c*; D-(+)-glucose; diethylenetriaminepentaacetic acid (DETAPAC); horse heart metmyoglobin; human methemoglobin; and bovine serum albumin. Pancreatic ribonuclease A was purchased from Worthington Enzymes. N^{ε}-Carboxymethyl-L-lysine (CML) and N^{α}-acetyl-N^{ε}-carboxymethyl-L-lysine were a gift from Drs Valeri Mossine and Milton Feather (University of Missouri–Columbia), and hippuryl-N^{ε}-carboxymethyl-L-lysine was a gift from Dr John W. Baynes (University of South Carolina).

3.2. Preparation of apomyoglobin

Apomyoglobin (apoMb) was prepared by removing heme from metmyoglobin using acid–butanone extraction [26]. A 1 mM solution of metMb was prepared in 5 mM phosphate buffer, pH 6.0 and was then cooled and acidified to pH 2.5 with additions of HCl. An equal volume of cold butanone was added to the solution and vortexed for 20 s. The freed protohemin was in the upper (butanone) layer and was siphoned off and discarded. This was repeated until the aqueous layer was colorless. The apoMb was then dialyzed against a series of buffers starting with 1 mM bicarbonate, 1 mM phosphate buffer, pH 6.3, 50 mM phosphate buffer, pH 7.0, and finally 0.2 M phosphate buffer, pH 7.5. The solution was finally

centrifuged to remove any precipitated denatured globin.

3.3. Glycation reactions

Typically, a 1 mg ml^{-1} solution of protein to be glycated was incubated at 37 °C with glucose (1.0 M) at pH 7.5 in 0.4 M phosphate containing 0.02% azide. When required, other compounds such as glycation inhibitors or facilitators were added at the initial time the solutions were prepared. Throughout the assay, the sample tubes were kept in the dark and were periodically checked for pH drift, and when necessary adjusted to pH 7.5 by the addition of either NaOH or HCl. The kinetics of formation of AGEs was monitored by taking aliquots at various time points, which were immediately frozen for later analysis at the end of the experiment.

3.4. UV-Vis spectroscopy

UV-Vis spectra were recorded using a computer-interfaced Hewlett Packard 8452A diode-array spectrophotometer. When necessary, second-derivative spectra were utilized in determining protein concentrations or to resolve band overlaps in order to minimize contributions from the 'browning' that accompanies glycation.

3.5. Amino acid and carboxymethyllysine analysis

Amino acid analysis was carried out at the KUMC Biotechnology Support Facility. PITC-derivatized amino acids were analyzed on a Perkin Elmer Applied Biosystems 420A derivatizer coupled with a 130A separation system and a 900A data analysis module. All glycated samples were dialyzed against deionized water and reduced with sodium borohydride prior to hydrolysis in order to avoid spurious generation of CML [27]. Hydrolysis was carried out under argon at 165 °C for 60 min. Modified gradient elution conditions were necessary in order to resolve CML from the normally overlapping methionine peak. Characterization and calibration of the CML peak was achieved by hydrolysis of a sample of authentic hippuryl-N^{ε}-carboxymethyllysine, which yields equal amounts of glycine and N^{ε}-carboxymethyllysine. Separate runs under standard elution conditions were carried out to observe and quantitate Ile, Leu, Phe and Lys. For CML content determinations, protein concentration was internally determined by a weighted average of Arg, Ala, Pro and Val, while for Lys content the internal reference was the weighted average of these four residues plus Ile, Leu and Phe. The latter three were not observed under the chromatography conditions for CML separation. Two or three separate analyses were typically carried out for each sample, with excellent inter-analysis agreement for Lys or for CML. Average values are reported.

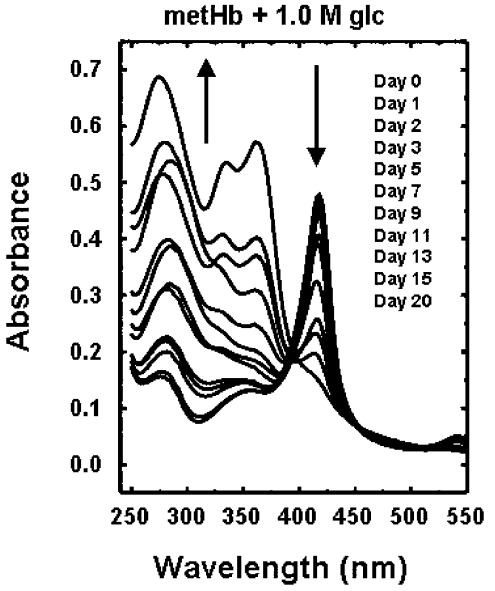

Fig. 3. Effect of 1 M glucose on the Soret band and UV absorbance of methemoglobin. Spectra were observed over 20 days of glycation.

4. Results

4.1. Spectral changes during glycation of hemoglobin

Glucose produces slow but profound effects on the UV-Vis spectrum of methemoglobin (metHb), as shown in Fig. 3 during the 20 days of incubation of 1 mg ml^{-1} metHb with 1 M glucose. In contrast,

this heme protein shows a stable spectrum in buffer alone (data not shown). First, there is a striking time-dependent decrease in the heme Soret band near 420 nm, paralleled in the visible bands, leading to complete bleaching (*downward arrow*). Second, there is an independent large intensification in absorbance in the UV region below the Soret band (*upward arrow*) with the appearance of new peaks, such as the 330–370-nm doublet. Careful examination reveals that this increase in the lower UV region appears somewhat delayed relative to the loss of the Soret band, and is mostly produced after most of Soret band bleaching has occurred. The UV bands generated are observed, although to a lesser extent, in non-heme protein glycation and are usually presumed to be part of the 'browning' that occurs. However, we have found that the UV chromophores are completely lost by dialysis or ultrafiltration.

4.2. Spectral changes during glycation of myoglobin and apomyoglobin

The spectral changes induced in methemoglobin are also observed to virtually the same extent with metmyoglobin (Fig. 4a). The distinct kinetics of the two processes at 416 nm (Soret band) and 360 nm can also be observed in time plots of the intensity changes (Fig. 5). When the heme group is absent or removed, as in apomyoglobin (apoMb), only the formation of non-Soret band spectral bands is observed, as expected (Fig. 4b). These non-heme changes are presumably due to the 'browning' that is typically observed when non-heme proteins are extensively glycated. However, closer inspection of the intensity changes reveals that, despite the qualitative similarity, the absolute changes in apoMb are far less than those observed in metHb and metMb. In other words, the presence of heme in metHb and metMb has led to far greater intensification in the non-heme spectral 'browning.' In separate experiments (data not shown), it was observed that the addition of hemin chloride at concentrations as low as 7 μM to non-heme proteins such as RNase and BSA greatly stimulated the glycation-induced 'browning' and also led to heme destruction.

4.3. Effects of chelators on the spectral changes in metmyoglobin

It is known that the formation of most AGEs requires oxygen and redox metal ion catalysis. The reaction is inhibited by strong chelators such as EDTA and DETAPAC, which are also effective in general oxidative chemistries involving redox metal ion catalysis [28]. We thus examined the effects of the chelators EDTA and DETAPAC on the glucose-induced changes in metmyoglobin. Both of these strong chelators completely abolish the spectral changes at 1 mM in both the Soret band and the lower UV region, as shown for DETAPAC by the intensity plots of Fig. 5.

4.4. Effects of hydrogen peroxide on the spectral changes of metHb

Hydrogen peroxide interacts with many heme proteins and enzymes and at low concentrations has been shown to form complexes with various oxidation states of the heme iron. There have been increasing reports, however, that at higher concentrations it can destroy protein-associated heme groups through superoxide radical formation [29,30]. For comparison with glucose, we have examined the effects of hydrogen peroxide on the UV spectrum of the metHb. Under similar buffer conditions to the glycation reaction, addition of hydrogen peroxide, at a nominal concentration of 8 mM, to 1 mg ml^{-1} MetHb causes a rapid bleaching of the heme Soret band near 410 nm (Fig. 6). Complete destruction of the heme group was observed under these conditions with a half-life of approximately 10 min. This, of course, in itself does not prove that the destruction during glycation arises from peroxide. It is also important to note that, unlike glycation, these spectral changes were not accompanied by the UV increases that were noted above in the 250–375-nm range that were attributed to glycation-induced 'browning' of the protein. Two separate processes are thus involved (cf. Fig. 5).

4.5. Spectral changes of cytochrome c during glycation

While studying the effects on glucose on a variety of other heme proteins, we have observed

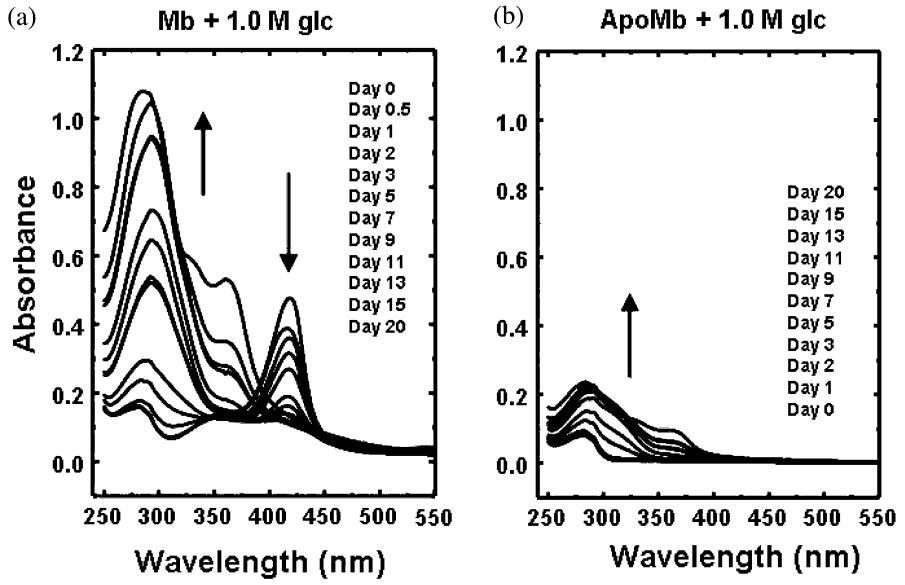

Fig. 4. Effect of 1 M glucose on the UV absorbance of (a) metmyoglobin and (b) apomyoglobin. Spectra were observed over 20 days of glycation and the arrows indicate the direction of the time course of changes in the different bands.

that cytochrome *c* behaves rather unusually, as shown in Fig. 7. Instead of bleaching of the Soret band, there is actually an intensification, with a slight red-shift of the maximum and the striking appearance of a doublet in the visible range. These changes are consistent with reduction of the ferricytochrome *c* to ferrocytochrome *c*. Furthermore, the changes in the lower UV region that are characteristic of 'browning' do occur, but are substantially attenuated when compared to hemoglobin and myoglobin.

4.6. Formation of CML during glycation of heme proteins

Since the identities of the UV-absorbing chromophores during 'browning' are not known, we have directly analyzed for the most abundant known AGE, carboxymethyllysine (CML) [20,21]. Fig. 8 demonstrates the extent of conversion of the lysines in metMb and metHb to CML during glycation at lower glucose concentrations (1–100 mM) for a longer reaction of 65 days. These concentrations include normal (4 mM) and diabetic (20 mM) glucose ranges. This duration is still shorter than the lifetime of red blood cells, often estimated at 120 days. The extent of formation of CML at glucose concentrations as low as 100 mM is quite remarkable, being in the 50–80% range, i.e. far greater than observed with non-heme proteins such as BSA and RNase (approx. 10% in 1 M glucose). The measurable levels at the diabetic glucose concentration of 20 mM are also equally remarkable.

5. Discussion

5.1. Destruction of the heme group by glucose

The impetus to this study was the accidental discovery that the UV spectrum of glycated hemoglobin bore no traces of the characteristic heme absorbance that is distinguished by the Soret band. We are unaware of any previous descriptions of such catastrophic heme damage by in vitro glycation.[1] We are also unaware of any reports of

[1] Subsequent to the completion of our studies and submission of this manuscript, we have come across one mention of the possibility of heme destruction by glucose, but no data were provided [31].

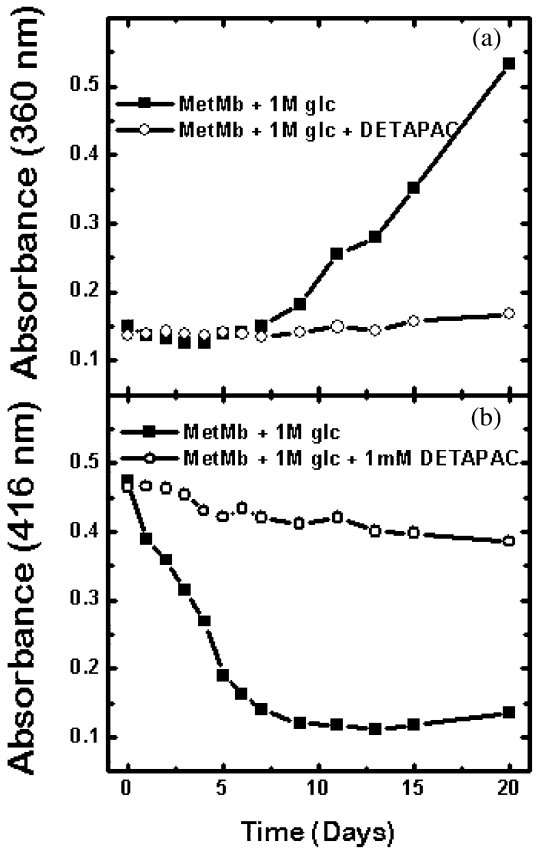

Fig. 5. Effect of 1 mM DETAPAC chelator on the 1 M glucose-induced spectral changes of metmyoglobin in the Soret band (416 nm) and in the lower UV range (360 nm). Spectra were observed over 20 days of glycation. For comparison, the changes in absence of DETAPAC (cf. Fig. 4) are also shown.

accelerated loss of red blood cell hemoglobin, muscle myoglobin, or other heme proteins in diabetics. The spectral changes in the Soret band reported here leave little doubt that glucose is capable of destroying the heme cofactor in these proteins, even at diabetic glucose concentrations. The precise chemical mechanisms of the destruction are probably very complex. The most plausible hypothesis is that hydrogen peroxide produced during glycation is the initiating agent in the degradative mechanism [32,33]. Recent studies on hydrogen peroxide-mediated heme degradation have implicated the hydrogen peroxide complex of ferryl (Fe^{4+}) heme, the ferryl heme itself being produced from a peroxide complex of ferri- and ferrohemoglobin. The peroxide complex of the ferryl heme readily autoxidizes to form the highly reactive superoxide radical [29,30]. Fenton chemistry, producing damaging hydroxyl radicals, is also likely to occur in the presence of adventitious redox metal ions from the phosphate buffer or from iron released through destruction of the heme [34–36].

The proposed mechanism via initially coordinated peroxide is consistent with the observations of a lack of reactivity of the heme group of cytochrome *c*. The remarkable resistance of cytochrome *c* to glucose-mediated destruction is most easily ascribed to the inaccessibility of the heme iron to hydrogen peroxide due to the presence of the methionine sulfur ligand at the sixth coordination position of the iron. In contrast, this ligand site is open to the solvent in myoglobin and hemoglobin (Fig. 9) and, of course, binds water, oxygen, carbon monoxide, hydrogen peroxide and many other ligands.

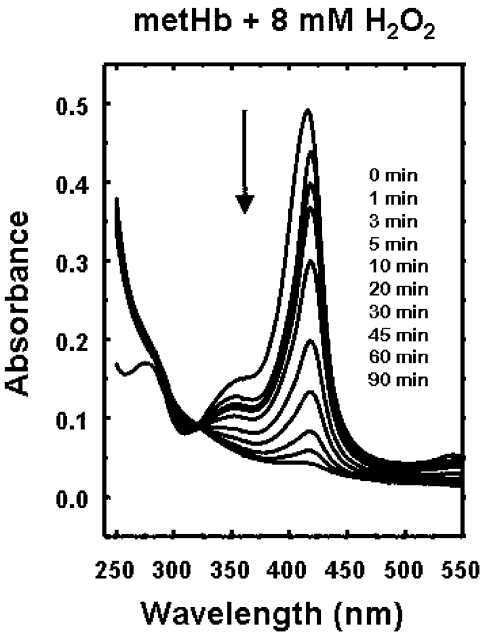

Fig. 6. Effect of hydrogen peroxide on the UV absorbance of metmyoglobin in the Soret band and UV regions.

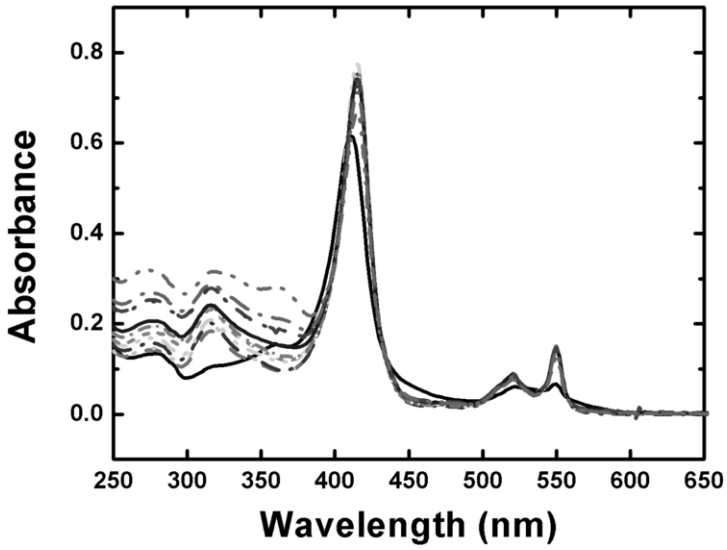

Fig. 7. Effect of 1 M glucose on the UV-Vis spectrum of cytochrome *c* during a 20-day incubation. Changes include intensification and shift of the Soret band, appearance of the doublet in the visible spectrum, and increased UV absorbance below the Soret band.

An alternative hypothesis to account for the difference in behavior of these heme proteins is that the heme destruction requires prior dissociation of the heme group from the globin. Support for this hypothesis comes from the fact that the heme group of cytochrome *c* is covalently attached (via thioether linkages to cysteines), while the heme groups of myoglobin and hemoglobin are reversibly bound and are well known to dissociate and exchange among globins [37]. Additional support comes from our observations, noted above, that added hemin is destroyed during glycation of

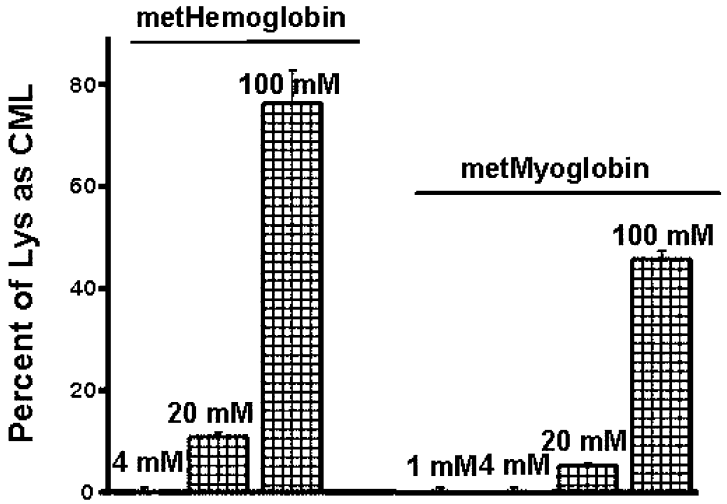

Fig. 8. Extent of formation of the AGE CML on lysines of methemoglobin and metmyoglobin during a 65-day glycation at the glucose concentrations indicated.

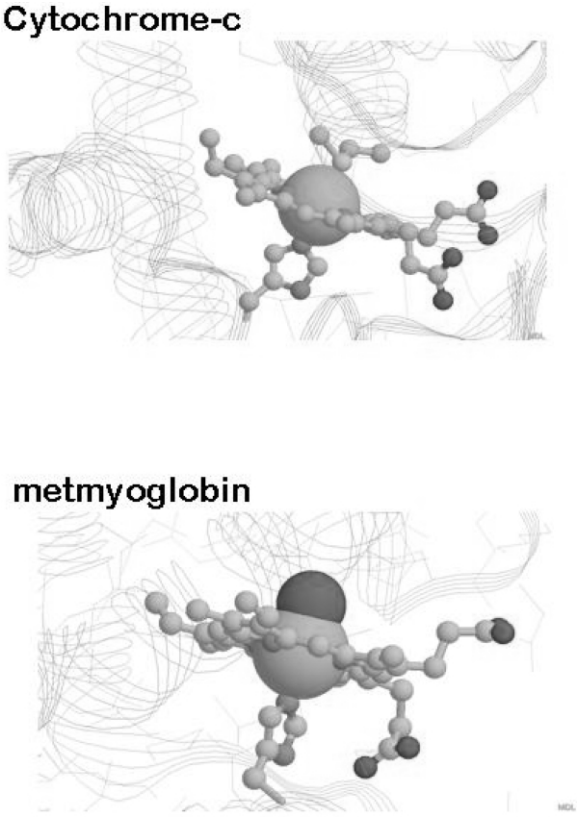

Fig. 9. Comparison of the heme environments at the sixth coordination position of the iron in cytochrome *c* (top) and metmyoglobin. The former has a sulfur ligand from methionine, and the latter coordinates a solvent ligand.

non-heme proteins. Nevertheless, we consider this plausible explanation much less likely. This is due to a combination of very low equilibrium concentration of free heme in hemoglobin or myoglobin (affinity of the order of 10^{13} M^{-1}) [37] and a very low steady-state concentration (nM range) of hydrogen peroxide generated by the Maillard reaction [38]. Even completely free hemin displays a very slow rate of destruction in protein–glucose solutions of the order of days (data not shown).

Experimental support for a hydrogen peroxide mechanism of destruction comes from several sources. For example, glycated proteins are known to autoxidize in the presence, and sometimes absence [38,39], of redox metal ions, thus generating hydrogen peroxide, free radicals and other reactive oxygen species [34,40,41]. The obligatory requirement for hydrogen peroxide can also be tested by examining the potential protective effect of catalase or glutathione peroxidase. We have carried out such studies and have found protection in the case of catalase, as will be reported elsewhere.

Evidence for an accompanying generation of superoxide can also be observed in the spectral changes in cytochrome *c*. Not only is the heme group protected during glycation, but there is also very rapid reduction to ferrocytochrome *c*. Indeed, reduction of ferricytochrome *c* has become a sensitive method for detecting superoxide generation in a variety of systems [42,43]. Glycated proteins have frequently been reported to contain 'reductones' that are capable of such reductions. Dual sources of superoxide production are thus possible in hemoglobin and myoglobin, i.e. from glycation on lysines via the Maillard cascade and from autoxidation of ferryl–peroxide transient intermediates. These must conspire to accelerate the destruction of the protein. The resistance of the heme group itself to degradation observed in cytochrome *c* suggests, as discussed above, that Maillard-generated superoxide may not be as effective as the superoxide generated in the heme pocket via autoxidation of the ferryl heme. The diminished 'browning' in cytochrome *c* further attests to lack of release of iron from the bound heme.

5.2. Potential implications for diabetic complications

The sensitivity of the ubiquitous heme proteins myoglobin and hemoglobin to destruction by glucose that we observed in this study raises novel questions regarding the toxicity of glucose in diabetic hyperglycemia. For example, it has been reported that ferryl heme is formed more readily by peroxide when the iron is in reduced form [29,30]. The red blood cell maintains such reduction for the functional deoxy and oxygenated states, but this is difficult to maintain in vitro due to well-known autoxidation to methemoglobin. This favors a greater tendency for the peroxide pathway of damage in vivo than under our present experimental conditions. It should also be noted

that there is a low flux of hydrogen peroxide in RBC, where it is estimated that 3% of hemoglobin is autoxidized in 24 h [30]. Why then do we not observe massive RBC damage in diabetics? Indeed, even AGEs appear to be present at extremely small concentrations in RBCs compared to plasma [44].

The AGE and browning results reported here make it even more surprising that we do not observe substantial evidence of AGE formation or damage in RBC. Our in vitro glycation experiments reveal that substantial CML formation occurs on heme proteins and probably all redox-active metalloproteins in general. The unusually large browning spectral changes we have observed are another reflection of enhanced AGE formation. The contribution of heme destruction to the copious AGE formation on metHb and metMb needs to be recognized, since this process releases the catalytic metal ion. This process leads to a prediction of an overall autocatalytic AGE formation, and this may be supported by the kinetics of browning observed (cf. Fig. 5a). We note that the absence of heme destruction in cytochrome *c* is accompanied by lesser 'browning.'

It thus becomes evident that the RBC anti-oxidative defenses powerfully protect against glycoxidative damage by glucose in normal and diabetic states. Catalase and glutathione peroxidase in RBCs may be crucial in this regard. In addition, the intracellular scavenging of free iron must also be involved in case damage occurs on bound heme prior to its removal. While we infer that the RBC has evolved excellent defenses against glucose-mediated pathways of heme protein degradation, little information is available to assess defenses in other extracellular and intracellular compartments, such as heme-rich mitochondria. Hypotheses have recently been advanced that diabetic complications result primarily from increased fluxes of highly reactive metabolic intermediates such as methylglyoxal [25,45]. However, as in RBCs, intracellular defensive enzymes such as glyoxalase exist that offer sufficient protection [46]. The site of greatest damage in diabetes remains the extracellular matrix, where no defensive anti-oxidant enzymes have been established. In view of the results presented here, it is perhaps no coincidence that few heme or redox-active metalloproteins are normally found in this highly functional but fragile biological matrix.

6. Postscript: a tribute to Kauzmann personal comments by RGK:

The intervening years since graduation have provided numerous opportunities to apply the scientific lessons assimilated from Kauzmann. Gratifyingly, the significant discoveries always seemed to arise from recognition (perhaps fruits of a 'prepared kinetics mind') of some anomalous kinetics, whether studying the diffusion-limited catalysis by one of the fastest known enzymes (carbonic anhydrase) [47] or the ultra slow (months) protein modifications by glucose (glycation) [2–4,48]. Despite the disparity in time scales, the problems always yielded to common-sense kinetic strategies designed to simplify the system and to model it. However, reflection suggests that Kauzmann's lasting influence as a mentor has been deeper and a lot more subtle. It really emanates from his unique mentoring style that is appropriately described from a student perspective, since its essence is that he leaves no student behind.

Kauzmann's unusual didactic approach uniquely complemented his great intellectual breadth and depth. His students quickly realize that he can admirably expound the merits of the opposite sides to almost any scientific question, including the weaknesses and limitations of his own notable theories and ideas (such as the hydrophobic bond concept). When praised in public, he would vigorously disavow taking sole credit for his achievements and would point out the many important contributions of others in the field. He was also quick to defend his scientific opponents against blanket dismissal or criticism, always pointing out the specific good things his rivals had done. He could equally be counted on to challenge, find holes in, or even dash, claims of discovery (that devastating 'Oh?') that were often prematurely advanced.

Kauzmann's approach was quite difficult on unaware ambitious students, but it opened wide the doors of learning to humble others. The turning point is reached when you begin to recognize that

his hallmark ability to synthesize theory and experimental knowledge was always based on understanding the elements of the problem all the way down to the most basic and elementary level. His exemplary practice was that you do not accept or apply theories if you do not know their derivations, including all the assumptions involved. Similarly, you do not build theories or hypotheses on data you have not analyzed yourself. Little in the literature is assumed proven or taken for granted, but criticism is accepted only if it is not personal in nature. If Kauzmann comprehended science at this elementary level of simplicity, then it seemed perfectly acceptable for the student to focus on understanding and interpreting simple results. Kauzmann genuinely conveyed that there was no experiment, successful or not, from which you cannot learn something interesting or useful. Indeed, he would often spend hours helping the discouraged student bring that out by a discourse on topics related to the data. Unashamedly, I took full advantage of that and enjoyed the process immensely. Perhaps my students and younger associates did the same too!

Acknowledgments

This work was supported by grant KS95GS45 from the American Heart Association, Kansas Affiliate Inc (R.G.K.) and by a grant from Bio-Stratum Inc (R.G.K.). Portions of this work were submitted as a thesis by B.L.C. in partial fulfillment for the requirements of the Graduate School at the University of Kansas Medical Center.

References

[1] J.C. Kendrew, The three-dimensional structure of a protein molecule, Sci. Am. 205 (1961) 96–111.
[2] R.G. Khalifah, P. Todd, A.A. Booth, S.X. Yang, J.D. Mott, B.G. Hudson, Kinetics of non-enzymatic glycation of ribonuclease A leading to advanced glycation end products. Paradoxical inhibition by ribose leads to facile isolation of protein intermediate for rapid post-Amadori studies, Biochemistry 35 (1996) 4645–4654.
[3] A.A. Booth, R.G. Khalifah, P. Todd, B.G. Hudson, In vitro kinetic studies of formation of antigenic advanced glycation end products (AGEs). Novel inhibition of post-Amadori glycation pathways, J. Biol. Chem. 272 (1997) 5430–5437.
[4] R.G. Khalifah, J.W. Baynes, B.G. Hudson, Amadorins: novel post-Amadori inhibitors of advanced glycation reactions, Biochem. Biophys. Res. Commun. 257 (1999) 251–258.
[5] T.P. Degenhardt, N.L. Alderson, D.D. Arrington, et al., Pyridoxamine inhibits early renal disease and dyslipidemia in the streptozotocin-diabetic rat, Kidney Int. 61 (2002) 939–950.
[6] A. Stitt, T.A. Gardiner, N.L. Alderson, et al., The AGE inhibitor pyridoxamine inhibits development of retinopathy in experimental diabetes, Diabetes 51 (2002) 2826–2832.
[7] T.P. Degenhardt, R.G. Khalifah, R.J. Schotzinger, Pharmacokinetics of oral Pyridorin™, a novel AGE inhibitor, in human subjects, Diabetes 51 (Suppl. 2) (2002) A185.
[8] J.W. Baynes, V.M. Monnier (Eds.), The Maillard Reaction in Aging, Diabetes, and Nutrition, Alan R. Liss, New York, 1989.
[9] P.A. Finot, H.U. Aeschbacher, R.F. Hurrell, R. Liardon (Eds.), The Maillard Reaction in Food Processing, Human Nutrition and Physiology, Birkhauser Verlag, Basel, 1990.
[10] J.E. Hodge, Chemistry of browning reactions in model systems, J. Agric. Food Chem. 1 (1953) 928–943.
[11] J.J. Harding, Non-enzymatic covalent posttranslational modification of proteins in vivo, Adv. Protein Chem. 37 (1985) 247–334.
[12] R.J. Koenig, A. Cerami, Hemoglobin A1c and diabetes mellitus, Annu. Rev. Med. 31 (1980) 29–34.
[13] M. Brownlee, Advanced protein glycosylation in diabetes and aging, Annu. Rev. Med. 46 (1995) 223–234.
[14] V.M. Monnier, Toward a Maillard reaction theory of aging, Prog. Clin. Biol. Res. 304 (1989) 1–22.
[15] K.J. Wells-Knecht, E. Brinkmann, M.C. Wells-Knecht, et al., New biomarkers of Maillard reaction damage to proteins, Nephrol. Dial. Transplant 11 (Suppl. 5) (1996) 41–47.
[16] D.R. McCance, D.G. Dyer, J.A. Dunn, et al., Maillard reaction products and their relation to complications in insulin-dependent diabetes mellitus, J. Clin. Invest. 91 (1993) 2470–2478.
[17] R. Singh, A. Barden, T. Mori, L. Beilin, Advanced glycation end-products: a review, Diabetologia 44 (2002) 129–146.
[18] M.X. Fu, K.J. Wells-Knecht, J.A. Blackledge, T.J. Lyons, S.R. Thorpe, J.W. Baynes, Glycation, glycoxidation, and cross-linking of collagen by glucose. Kinetics, mechanisms, and inhibition of late stages of the Maillard reaction, Diabetes 43 (1994) 676–683.
[19] J.W. Baynes, S.R. Thorpe, Glycoxidation and lipoxidation in atherogenesis, Free Radic. Biol. Med. 28 (2000) 1708–1716.
[20] M.U. Ahmed, S.R. Thorpe, J.W. Baynes, Identification of *N*-epsilon-carboxymethyllysine as a degradation product of fructoselysine in glycated protein, J. Biol. Chem. 261 (1986) 4889–4894.

[21] S. Reddy, J. Bichler, K.J. Wells-Knecht, S.R. Thorpe, J.W. Baynes, *N*-epsilon-(carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins, Biochemistry 34 (1995) 10872–10878.

[22] K.J. Wells-Knecht, D.V. Zyzak, J.E. Litchfield, S.R. Thorpe, J.W. Baynes, Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose, Biochemistry 34 (1995) 3702–3709.

[23] M.A. Glomb, V.M. Monnier, Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction, J. Biol. Chem. 270 (1995) 10017–10026.

[24] M.X. Fu, J.R. Requena, A.J. Jenkins, T.J. Lyons, J.W. Baynes, S.R. Thorpe, The advanced glycation end product, *N*-epsilon-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions, J. Biol. Chem. 271 (1996) 9982–9986.

[25] M. Brownlee, Biochemistry and molecular cell biology of diabetic complications, Nature 414 (2001) 813–820.

[26] F.W. Teale, Biochim. Biophys. Acta 35 (1959) 543.

[27] J. Hartkopf, H.F. Ebersdobler, Modelluntersuchungen zu bedingungen der bildung von *N*-epsilon carboxymethyllysin in lebensmitteln, Z. Lebensm. Unters Forsch. 198 (1994) 15–19.

[28] M.C. Wells-Knecht, S.R. Thorpe, J.W. Baynes, Pathways of formation of glycoxidation products during glycation of collagen, Biochemistry 34 (1995) 15134–15141.

[29] E. Nagababu, J.M. Rifkind, Reaction of hydrogen peroxide with ferrylhemoglobin: superoxide production and heme degradation, Biochemistry 39 (2000) 12503–12511.

[30] E. Nagababu, S. Ramasamy, J.M. Rifkind, Y. Jia, A.I. Alayash, Site-specific cross-linking of human and bovine hemoglobins differentially alters oxygen binding and redox side reactions producing rhombic heme and heme degradation, Biochemistry 41 (2002) 7407–7415.

[31] I.I. Stepuru, N.A. Chaikovskaya, V.P. Vodoevich, V.V. Vinogradov, Reduction of methemoglobin and ferricytochrome *c* by glycosylated amino acids and albumin, Biochemistry (Moscow) 62 (1997) 967–972.

[32] A.I. Alayash, R.P. Patel, R.E. Cashon, Redox reactions of hemoglobin and myoglobin: biological and toxicological implications, Antioxid. Redox Signal. 3 (2001) 313–327.

[33] E. Nagababu, J.M. Rifkind, Heme degradation during autoxidation of oxyhemoglobin, Biochem. Biophys. Res. Commun. 273 (2000) 839–845.

[34] P.J. Thornalley, S.P. Wolff, M.J.C. Crabbe, A. Stern, The oxidation of oxyhaemoglobin by glyceraldehydes and other simple monosaccharides, Biochem. J. 217 (1984) 615–622.

[35] B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, 3rd ed., Oxford University Press, Oxford, 1999.

[36] S.P. Wolff, Z.Y. Jiang, J.V. Hunt, Protein glycation and oxidative stress in diabetes mellitus and ageing, Free Radic. Biol. Med. 10 (1991) 339–352.

[37] A. Rossi Fanelli, E. Antonini, A. Caputo, Hemoglobin and myoglobin, Adv. Protein Chem. 19 (1964) 73–222.

[38] Z.Y. Jiang, A.C. Woollard, S.P. Wolff, Hydrogen peroxide production during experimental protein glycation, FEBS Lett. 268 (1990) 69–71.

[39] V.V. Mossine, M. Linetsky, G.V. Glinsky, B.J. Ortwerth, M.S. Feather, Superoxide free radical generation by Amadori compounds: the role of acyclic forms and metal ions, Chem. Res. Toxicol. 123 (1999) 230–236.

[40] J.V. Hunt, R.T. Dean, S.P. Wolff, Hydroxyl radical production and autoxidative glycosylation, Biochem. J. 256 (1988) 205–212.

[41] S.P. Wolff, Free radicals in glycation, in: B.P. Yu (Ed.), Free Radicals in Aging, CRC Press, Boca Raton, 1993, pp. 123–142.

[42] J.M. McCord, I. Fridovich, The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interaction of sulfite, dimethylsulfoxide, and oxygen, J. Biol. Chem. 244 (1969) 6056–6063.

[43] H. Ukeda, T. Shimamura, M. Tsubouchi, Y. Harada, Y. Nakai, M. Sawamura, Spectrophotometric assay of superoxide anion formed in Maillard reaction based on highly water-soluble tetrazolium salt, Anal. Sci. 18 (2002) 1151–1154.

[44] Z. Makita, H. Vlassara, E. Rayfield, et al., Hemoglobin AGE: a circulating marker of advanced glycosylation, Science 258 (1992) 651–653.

[45] E.A. Abordo, H.S. Minhas, P.J. Thornalley, Accumulation of alpha-oxoaldehydes during oxidative stress: a role in cytotoxicity, Biochem. Pharmacol. 58 (1999) 641–648.

[46] M. Shinohara, P.J. Thornalley, I. Giardino, et al., Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced increases in macromolecular endocytosis, J. Clin. Invest. 101 (1998) 1142–1147.

[47] R.G. Khalifah, Reflections on Edsall's carbonic anhydrase: paradoxes of an ultra fast enzyme, Biophys. Chem. 100 (2003) 159–170.

[48] A.A. Booth, R.G. Khalifah, B.G. Hudson, Thiamine pyrophosphate and pyridoxamine inhibit the formation of antigenic advanced glycation end-products: comparison with aminoguanidine, Biochem. Biophys Res. Commun. 220 (1996) 113–119.