Nonenzymatic Glycation of Transferrin: Decrease of Iron-Binding Capacity and Increase of Oxygen Radical Production

Sadaki FUJIMOTO,* Naoko KAWAKAMI, and Akira OHARA

Laboratory of Biochemistry, Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607, Japan.
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The total iron-binding capacity (TIBC) and iron contents of diabetic rat serum, as well as the iron-binding capacity of glycated transferrin and oxygen radical production by the glycated proteins were examined. The TIBC and iron content of diabetic rat sera were found to be much lower than those of control rat sera. Incubation of human serum with glucose *in vitro* resulted in a significant fall of its unsaturated iron-binding capacity (UIBC) with time. When apotransferrin was incubated with glucose, its UIBC significantly decreased. The iron content of holotransferrin was markedly reduced by incubation with bathophenanthroline sulphonic acid (BPSA) in the presence of glucose, although the content was not altered by incubation with BPSA alone. The generation of superoxide radical (O_2^-) and hydroxyl radical (OH·) by the glycated holotransferrin was much greater than that by glycated apotransferrin. Glycated holotransferrin showed significantly accelerated hydroxyl radical production by the hypoxanthine-xanthine oxidase system, while intact holotransferrin did not. Treatment of holotransferrin with glucose caused the fragmentation of the protein, while the same treatment of apotransferrin did not. These results suggest that iron ions in the glycated transferrin molecule are bound loosely to the protein and are redox-active and the glycated holotransferrin produces oxygen radicals including O_2^- and OH· efficiently, and that the glycated transferrin does not function as an iron-binding protein.

Key words diabetes; glycation; transferrin; iron-binding capacity; oxygen radical

Nonenzymatic glycation of various proteins, such as hemoglobin,[1] albumin,[2,3] collagen,[4] erythrocyte membrane proteins,[5] lens crystallines,[6] and certain enzymes,[7,8] has been reported. A general mechanism for nonenzymatic glucosylation of protein is as follows: free amino groups in protein react with the acylic form of glucose to yield Shiff-base intermediate which can undergo Amadori rearrangement to stable ketoamine derivatives, which then cyclize to the hemiketal structure.[9] The Shiff base and/or Amadori products have been found to generate reactive oxygen species,[10-12] and divalent metals have also been implicated in the production of reactive oxygen species.[11,12]

Prolonged elevation of blood glucose in diabetes causes an increase in the levels of nonenzymatically glycated proteins such as glycated hemoglobin in erythrocytes[1] and glycated albumin in the serum.[13,14] The post-translational modification affects hemoglobin oxygen affinity[15,16] and sensitivity to the allosteric effector, 2,3-diphosphoglycerate,[16,17] and also affects the binding capacity of serum albumin with drugs such as sulfonylureas.[18] In addition, it has been found that Cu, Zn-superoxide dismutase (SOD) which can trap superoxide radical (O_2^-) was inactivated by glycation reaction,[19] and that glycation of Cu, Zn-SOD initially brings about a site-specific fragmentation of the molecule, and further incubation of the enzyme with glucose brings about complete fragmentation.[20] Therefore, it is presumed that nonenzymatic glycation of body proteins may also induce changes in their chemical, physical, and, ultimately, biological properties, leading eventually to the pathological sequelae of diabetes.

It has been recently reported that transferrin, an iron binding protein found in the vertebrate blood stream, and serum iron are both decreased in patients with insulin-dependent diabetes mellitus (IDDM).[21] This finding may suggest that the function of transferrin is affected by glycation reaction. The glycation of transferrin has, however, not yet been examined.

In this study, we examined the total iron binding capacity (TIBC) and iron contents of diabetic rat serum, as well as the iron binding capacity of glycated transferrin and oxygen radical production by the glycated proteins.

MATERIALS AND METHODS

Materials Apotransferrin (purity, above 98%; iron content, less than 0.0005%) and holotransferrin (purity, above 98%; iron content, above 0.1%), both from bovine plasma, streptozotocin (STZ), D-glucose, hypoxanthine (HX), and bathophenanthrolinedisulfonic acid (BPSA) were purchased from Wako Pure Chemicals, ferricytochrome c (Type III), L-phenylalanine, and standard proteins used in electrophoresis from Sigma Chemical Co., and xanthine oxidase (XO) from cow's milk from Boehringer Mannheim GmbH. XO activity was assayed by measuring the absorption at 290 nm of uric acid at 37 °C. One unit of activity was defined as the production of 1 nmol of uric acid per min at pH 7.4.

Induction of Hyperglycemia Male Wistar rats weighing 200—250 g were used as the STZ-induced diabetes rat model. STZ (60 mg/kg) dissolved in cold 0.1 M citrate buffer (pH 4.5) was injected *via* the tail vein under light ether anesthesia. Control rats were treated with the buffer alone. After 25 d, rats were bled from the descending aorta. The serum fraction was obtained by centrifugation and was assayed for glucose concentration, total protein, iron content, and TIBC as described below. The serum glucose concentration and total protein were determined with a Rapid Blood Analyzer (RaBA-Σ, Kyoto Daiichi Chemical Co.).

Preparation of Glycated Proteins Apotransferrin and

* To whom correspondence should be addressed.

holotransferrin (10 mg/ml) were incubated with and without glucose (1 M) in 50 mM phosphate buffer (pH 7.4) under sterilization at 37 °C. After 10 d of incubation, the reaction mixtures were passed through a Sephadex G-25 column equilibrated with 50 mM phosphate buffer (pH 7.4), to remove free glucose and released iron. The protein fractions thus obtained were used as glycated and nonglycated proteins. The protein concentration was determined by the method of Lowry *et al.* using bovine serum albumin as a standard protein.[22] The extent of glucosylation was assessed by the thiobarbituric acid reaction using 5-hydroxymethyl-2-furfural (HMF) as a standard compound.[23]

Measurements of TIBC, Unsaturated Iron Binding Capacity (UIBC) and Iron Content TIBC was determined by means of the Fe-Test Wako Kit using BPSA as a color reagent. UIBC and iron content were determined by means of the UIPC-Test Wako Kit and Fe C-Test Wako Kit, respectively, using 2-nitroso-5-*N*-(propyl-*N*-(sulfopropyl)amino phenol as a color reagent. The iron determination kits were obtained from Wako Pure Chemicals.

Determination of Superoxide (O_2^-) and Hydroxyl (OH·) Radicals O_2^- production by glycated proteins was assayed using cytochrome c reduction.[24] OH· was detected based on hydroxylation of phenylalanine by OH· as described previously.[25-27] Reaction mixtures containing phenylalanine (1 mM) and protein fractions (1 mg/ml) in 1.0 ml of 50 mM phosphate buffer (pH 7.4) were incubated at 37 °C with shaking. In the presence or absence of protein fractions (1 mg/ml), reaction mixtures containing phenylalanine (1 mM), HX (1 mM) and XO in 1.0 ml of 50 mM phosphate buffer (pH 7.4) were incubated at 37 °C with shaking. After incubation for the duration indicated, 100 μl of catalase (1 mg/ml) and then 100 μl of trichloroacetic acid (6 M) were added to stop the reaction. After centrifugation, 100 μl of each supernatant was injected directly into a HPLC column. *p*-, *m*- and *o*-Tyrosines derived from phenylalanine were evaluated by HPLC as described previously.[28]

Polyacrylamide Disc Gel Electrophoresis (PADGE) PADGE in the presence of sodium dodecyl sulfate (SDS) was carried out as described by Weber and Osborn.[29]

RESULTS

TIBC and Iron Content in the Serum of Diabetic Rats Rats were maintained for 25 d after injection of STZ, and hyperglycemia (mean blood glucose level, 34.0±1.9 mmol/l) was induced. As shown in Table I, the serum TIBC and iron content were significantly decreased in the diabetic group with hyperglycemia as compared with those in the control group (mean blood glucose level, 8.7±0.9 mmol/l). No significant difference was observed between the diabetes and control rats in the serum total protein concentration.

Effect of Glucose Treatment of Serum on Its UIBC In the examination of the effect of glycation on the iron-binding capacity of serum, we determined the UIBC during incubation of human serum with glucose *in vitro*. As shown in Fig. 1, incubation of human serum with glucose resulted in a time-dependent decrease of UIBC. This result suggests that the iron-binding capacity of transferrin (an iron-binding serum protein) may be lowered by glycation reaction. Therefore, we further examined the effect of the glycation reaction of transferrin on its iron-binding capacity and iron content.

TABLE I. TIBC and Iron Content of the Serum of Control and Diabetic Rats

	TIBC (μg/dl)	Iron content (μg/dl)
Control	496.8±35.4	239.7±27.7
Diabetes	293.4±43.3[a]	128.3±24.2[a]

The experimental animals, serum sample preparation methods, and TIBC and iron content assay are described in Materials and Methods. Each value represents the mean±S.D. for at least five diabetic or control rats. *a*) Significantly different from control value at $p<0.001$.

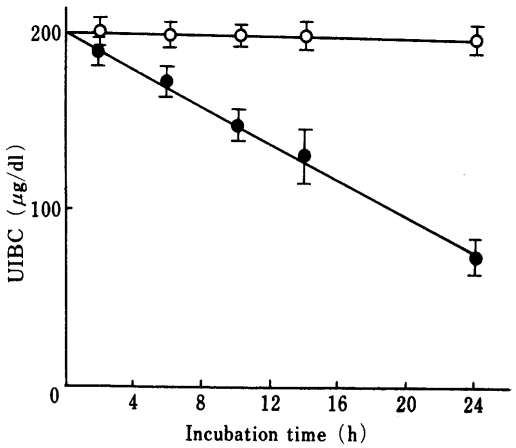


Fig. 1. Change in UIBC of the Human Serum by the Treatment with Glucose

Human serum was incubated with (●) and without (○) glucose (1 M) in 50 mM phosphate buffer (pH 7.4) at 37 °C for the indicated times, and then UIBC was assayed as described in Materials and Methods. Each value represents the mean±S.D. of three experiments.

Effect of Glycation Reaction on UIBC and Iron Content of Transferrin When apotransferrin and holotransferrin were incubated with glucose (1 M) for 10 d at 37 °C, a significant glycation was observed for each protein, although the extent of glycation of apotransferrin (20.4±3.42) was higher than that of holotransferrin (17.43 ±4.02; expressed as nmol HMF equivalent/mg protein). Holotransferrin was also incubated with glucose of 34 and 9 mM, which were observed in diabetic and control rat sera, respectively, for 10 d at 37 °C. The extent of glycation of the protein incubated with 34 mM glucose was significantly higher than that incubated with 9 mM glucose: 2.9±0.6* with 34 mM glucose ($n=3$) and 0.6±0.1 nmol HMF equivalent/mg protein (mean±S.D.) with 9 mM glucose ($n=3$) (*significantly different from that with 9 mM glucose at $p<0.01$). As shown in Table II, the UIBC of glycated apotransferrin was less than half that of intact apotransferrin, whereas the iron content of glycated holotransferrin was similar to that of intact holotransferrin. In the evaluation of the effect of glycation on the strength of iron binding to the protein, holotransferrin was incubated with glucose in the presence

TABLE II. UIBC and Iron Content of Glycated Transferrin

Protein fraction	UIBC (ng/mg)	Iron content (ng/mg)
Intact apotransferrin	1013±82.6	3.2± 0.4
Glycated apotransferrin	480±82.5[a)]	3.0± 0.3
Intact holotransferrin	25± 8.2	1275 ±100.2
Glycated holotransferrin	0	1285 ±110.4

Transferrin (10 mg/ml) was incubated with and without glucose (1 M) in 50 mM phosphate buffer (pH 7.4) at 37 °C and passed through a Sephadex G-25 column under the conditions described in Materials and Methods. The UIBC and iron content assays are described in Materials and Methods. Each value represents the mean±S.D. of four experiments. *a*) Significantly different from the value for intact apotransferrin at $p<0.001$.

TABLE III. O_2^- Production from Glycated Transferrins

Protein fraction	O_2^- produced (pmol/mg/min)
Intact apotransferrin	0
Glycated apotransferrin	14.4±0.4
Intact holotransferrin	0
Glycated holotransferrin	380 ±9.5[a)]

Ferricytochrome c (50 μM) was incubated with glycated or nonglycated transferrin (1 mg/ml) in 50 mM phosphate buffer (pH 7.4) at 37 °C. O_2^- was assayed as described in Materials and Methods. Each value represents the mean±S.D. of three experiments. *a*) Significantly different from the value for glycated apotransferrin at $p<0.0001$.

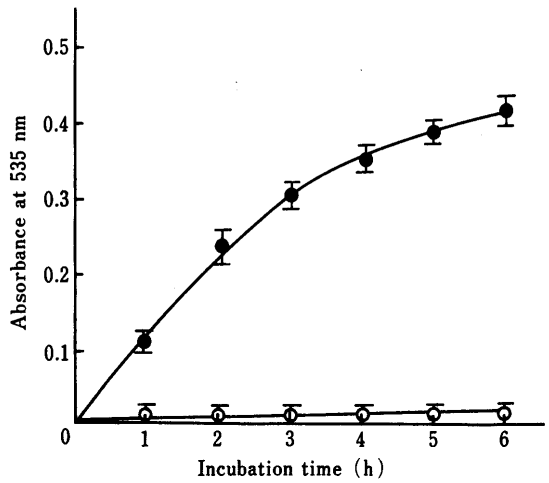


Fig. 2. Time Courses of BPSA–Iron Complex Formation by the Treatment of Holotransferrin with BPSA in the Presence of Glucose

Holotransferrin (1 mg/ml) was incubated with (●) and without (○) glucose (1 M) in the presence of BPSA (0.05%) in 50 mM phosphate buffer (pH 7.4) at 37 °C. Samples were incubated for indicated times, and then measured the absorbance at 535 nm. Each value represents the mean±S.D. of three experiments.

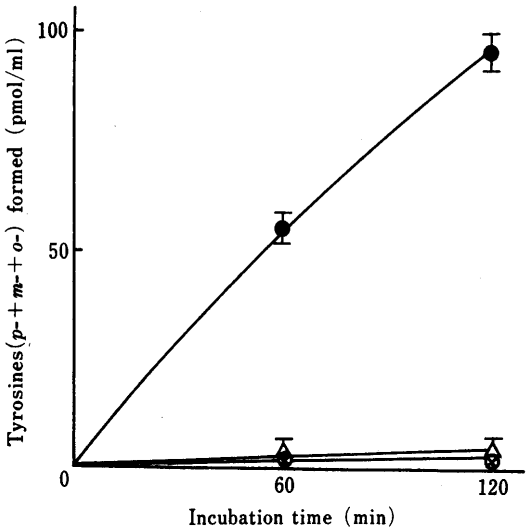


Fig. 3. Hydroxylation of Phenylalanine by Glycated Transferrin

Phenylalanine (1 mM) was incubated with glycated holotransferrin (●), or with glycated apotransferrin (△), or with nonglycated holotransferrin (○), or with nonglycated apotransferrin (×) (1 mg/ml) in 50 mM phosphate buffer (pH 7.4) under the conditions described in Materials and Methods. Samples were incubated for the indicated times, and the assayed as described. Each value represents the mean±S.D. of three experiments.

of BPSA, a chelating agent of ferrous ion. As shown in Fig. 2, absorption at 535 nm, based on the formation of BPSA–Fe^{2+} complex, increased with time, indicating that iron in holotransferrin was reductively released by the treatment with glucose in the presence of BPSA. After further incubation of the mixture containing holotransferrin, glucose and BPSA for 3 d at 37 °C, the reaction mixture was passed through a Sephadex G-25 column. Protein fractions were collected and assayed for iron content. The iron content of holotransferrin incubated with both glucose and BPSA was decreased to about one-tenth of that of holotransferrin incubated with BPSA alone (data not shown), although the iron content of holotransferrin was not altered by incubation with glucose alone. This result suggests that iron in glycated transferrin may be more easily released as compared with that of intact transferrin. Therefore, the reactivity of the iron bound to glycated transferrin may be higher than that of the iron bound to intact transferrin.

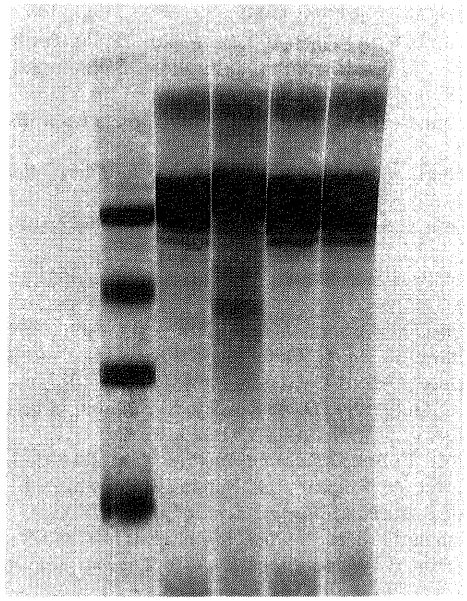
Production of Oxygen Radical by Glycated Transferrin It has been reported that glycated protein itself produces oxygen radicals in the presence of trace metal ions.[10-12] We examined the production of O_2^- and OH· of glycated apotransferrin and holotransferrin. As shown in Table III, the O_2^- production of glycated holotransferrin was much greater than that of glycated apotransferrin, even though the extent of glycation of holotransferrin was lower than that of apotransferrin, as described above. In addition, significant hydroxylation of phenylalanine was caused only by glycated holotransferrin, as shown in Fig. 3, indicating OH· generation by glycated holotransferrin. Furthermore, we examined the effect of these glycated protein fractions on OH· production by the HX–XO system well characterized as a generating system of O_2^-.[30] As shown in Table IV, the hydroxylation of phenylalanine by the HX–XO system was slightly decreased by the addition of apotransferrin, holotransferrin, or glycated apotransferrin. On the other hand, the addition of glycated holotransferrin to the HX–XO system resulted in a two-fold increase of phenylalanine hydroxylation. The tyrosines formation from phenylalanine by the HX–XO system containing glycated holotransferrin was much greater than the sum of the tyrosines formation by the HX–XO system alone and by glycated holotransferrin alone, indicating that the hydroxylation of phenyalanine by the HX–XO system is accelerated by glycated holotransferrin.

Fragmentation of Holotransferrin by Its Glycation Figure 4 shows SDS–PADGE bands of the protein

TABLE IV. Effect of Glycated Transferrins on the Hydroxylation of Phenylalanine by the HX–XO System

System	Tyrosines (*p*-+*m*-+*o*-) formed (pmol/ml/min)
HX–XO	92.5± 9.9
+intact apotransferrin	38.6± 6.9
+glycated apotransferrin	40.8± 8.8
+intact holotransferrin	64.2± 4.8
+glycated holotransferrin	207.5±30.0[a]
Intact apotransferrin alone	0
Glycated apotransferrin alone	0
Intact holotransferrin alone	0
Glycated holotransferrin alone	0.9± 0.1

Phenylalanine (1 mM) was incubated with glycated and nonglycated transferrins (1 mg/ml), HX (1 mM) and XO (12.5 units/ml), or with glycated and nonglycated transferrin alone in 50 mM phosphate buffer (pH 7.4) under the conditions described in Materials and Methods. Tyrosines formed were assayed as described in Materials and Methods. Each value represents the mean±S.D. of four experiments. *a*) Significantly different from that with HX–XO alone at $p<0.0012$.



A B C D E

Fig. 4. SDS–PADGE

(A) standard proteins, (B) holotransferrin alone, (C) holotransferrin+glucose, (D) apotransferrin alone, (E) apotransferrin + glucose, Each protein (10 mg/ml) was incubated with or without glucose (1 M) at 37 °C for 10 d, and then the protein solution was passed through a Sephadex G-25 column to remove excess glucose as described in Materials and Methods. Each protein fraction (50 μg) thus obtained was subjected to SDS–PAGDE according to the method described in Materials and Methods. Standards were bovine serum albumin (68000), ovalbumin (45000), carbonic anhydrolase (29000) and lysozyme (14300), from top to bottom.

fractions obtained by passage through a Sephadex G-25 column after incubation of transferrin (10 mg/ml) with or without glucose (1 M) for 10 d at 37 °C. Only glycated holotransferrin exhibited new protein bands, ranging from approximate 40 to 60 kDa, indicating that the glycation reaction of holotransferrin causes the fragmentation of the protein.

DISCUSSION

Asayama *et al.* have reported that the iron content of the serum of patients with IDDM is significantly lower than that of normal subjects.[21] In the present study, we also observed that both the iron content and TIBC of serum were significantly reduced in diabetes rats (Table I), and that the iron-binding capacity of human serum was markedly reduced with time by the treatment of the serum *in vitro* with glucose. In addition, when holotransferrin was incubated with glucose, the UIBC of the protein was significantly reduced. The above results may suggest that glycated transferrin does not function as an iron-binding protein in the bloodstream.

Transferrin binds to iron (Fe^{3+}) tightly, and the iron is redox-inactive.[31] The iron content of holotransferrin was not altered by the treatment with glucose alone or with BPSA alone. However, when holotransferrin was incubated with glucose in the presence of BPSA, BPSA–Fe^{2+} complex was formed and the concentration increased with time, and iron content of the glucose-treated holotransferrin in the presence of BPSA was found to be greatly reduced. In addition, the glycated holotransferrin significantly accelerated the hydroxylation of phenylalanine by the HX–XO system, while intact holotransferrin did not. It has been described that reduction of transferrin-bound ferric ion (Fe^{3+}) to ferrous iron (Fe^{2+}), which binds only weakly to transferrin, is one mechanism by which iron is released.[32-34] Therefore, the above findings suggest that the iron ions in the glycated transferrin molecule are bound loosely to the protein and are redox-active.

Shiff-base and/or Amadori products formed during glycation reaction have been found to produce reactive oxygen,[10-12] such as O_2^- and OH·, and the role of divalent metal ions in production of oxygen radicals has been suggested.[11,12] In our experiments, only glycated holotransferrin was found to significantly generate O_2^-, but the extent of glycation of glucose-treated holotransferrin was lower than that of glucose-treated apotransferrin. In addition, significant hydroxylation of phenylalanine was produced by glycated holotransferrin, though neither nonglycated holotransferrin nor glycated apotransferrin produced hydroxylation. These results suggest that glycated holotransferrin produces oxygen radicals such as O_2^- and OH· more efficiently than does glycated noniron-protein.

Cu, Zn-SOD, which is the trapping enzyme of O_2^-, is inactivated by glycation reaction,[19] and at the same time the formation of oxygen radicals and the fragmentation of this enzyme itself occur.[20] These phenomena in OH· formation from O_2^- produced by glycated Cu,Zn-SOD are considered to occur as a result of the Haber–Weiss reaction[35] catalyzed by copper which is released from the glycated enzyme. The OH· thus produced causes the site-specific fragmentation of this enzyme. In the present study, fragmentation of protein was observed only in holotransferrin treated with glucose. The mechanism of the fragmentation may be as described above for Cu, Zn-SOD,[20] *i.e.*, fragmentation of the protein is caused by the OH· produced efficiently by Harber–Weiss reaction[35] catalyzed by iron ions, which bind weakly to the glycated transferrin molecule. The greater transferrin excretion in the urine because of fragmentation of transferrin by glycation reaction may be one of the causes

of the decreased serum transferrin level in IDDM.

Extent of glycation of holotransferrin by incubation with glucose at concentration observed in diabetic rat serum was significantly higher than that by the normal glucose concentration. The results may suggest that the glycated transferrin level in bloodstream of hyperglycemia is higher than that of normal subject. Oxidative stress is postulated to be increased in patients with diabetes mellitus.[36] Some reports suggest that oxidative cell injury caused by free radicals contributes to the development of angiopathy in the form of complications of diabetes mellitus.[37,38] Hydroxylation of phenylalanine by glycated transferrin was inhibited significantly by antioxidants such as ascorbic acid and reduced form of glutathione, nonspecific radical scavengers, in addition to hydroxyl radical scavengers including benzoate, mannitol, and formate (results not shown). Therefore, it is thought that oxygen radicals including O_2^- and OH· produced by glycated transferrin may be diminished by endogeneous radical scavengers such as ascorbic acid and sulfhydryl compounds in bloodstream. However, it is possible that the redox-active iron ions in glycated transferrin may contribute to the oxidative cell injury of endothelial cells. Endothelial cells have previously been shown to have cell surface receptors for transferrin.[39] Theoretically, transferrin-derived iron ions could potentiate free radical-mediated endothelial cell toxicity. From this point of view, it is important to examine the affinity of glycated transferrin for transferrin receptor. These experiments are now in progress.

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