Effects of Aminoguanidine and Pyridoxal Phosphate on Glycation Reaction of Aspartate Aminotransferase and Serum Albumin

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Summary Effects of aminoguanidine (AG) on binding of glucose and pyridoxal phosphate (PLP) to albumin, and on glycation reaction of cytosolic aspartate aminotransferase (cAST) were examined in an in vitro system. AG was found to inhibit not only glycation of albumin but binding of PLP to albumin, indicating that distribution of PLP into tissues is inhibited by AG. AG bound to PLP directly to produce a new compound, and in this manner AG inhibited cAST activity. AG could also inhibit glycation of cAST and the extent of inhibition was varied with sugars used. It appears that, although AG is a useful inhibitor of glycation proteins, it may be toxic from the viewpoints of vitamin B6 as an essential nutrient and also PLP-dependent enzymes.

Key Words aminoguanidine, aspartate aminotransferase, pyridoxal phosphate, glycation reaction

Non-enzymic glycosylation (glycation) was first described by Maillard (1), who observed browning of amino acids when they were heated with sugars. The first protein demonstrated to be glycated in vivo was hemoglobin. Rahbar (2) showed that there was a hexose molecule linked to the N-terminal valine of the \( \beta \)-chain of HbAlc which increases in diabetic patients. Several enzymes, such as Cu-Zn-superoxide dismutase (3) and horse liver alcohol dehydrogenase (4), have also been shown to be glycated in vivo. The glycation reaction begins with the non-enzymic formation of Schiff base resulted from the condensation of a sugar aldehyde or ketone with a free amino group of proteins. These Schiff base adducts then undergo rearrangement to the more stable Amadori product. The Amadori product is degraded into various highly reactive carbonyl compounds which react again with amino groups to form various intermediates and advanced glycation products (AGE) (5, 6). Accumulation of AGEs is considered to contribute to the pathology in diabetes and aging (7). Many pharmacological reagents have been developed that inhibit AGE formation. Among these reagents, aminoguanidine which was firstly demonstrated by Brownlee (8), is known to inhibit AGE forma-
tion effectively and has extensively been examined in animals and is planning to be used in humans. Previously, we reported (9, 10) that in the vitamin B6-deficient rat liver, the activity of cytosolic aspartate aminotransferase (cAST) was decreased and was not restored by the addition of pyridoxal phosphate (PLP) to the assay medium, but that antigenic activity of the enzyme was not decreased. We also observed (11) a similar phenomenon when the cytosolic fraction of rat liver was incubated in vitro. During studies of the factors responsible for inactivation of cAST, a glycation reaction was considered to be one of the possible mechanisms of inactivation. When purified cAST was incubated with various sugars, the enzymatic activity was lowered and the enzyme was shown to be glycated at the lysyl residue, which is the binding site of PLP (12, 13). We examined several inhibitors such as aspirin, diclofenac, ibuprofen etc. which have been reported to inhibit the glycation reaction of proteins and found that, among these drugs tested, aminoguanidine was the most effective inhibitor for albumin glycation under the conditions used.

The present study was conducted to examine the effect of aminoguanidine on glycation of cAST in vitro.

MATERIALS AND METHODS

Preparation of cAST from rat liver. Male Wistar rats, weighing about 200 g, were fed a 70% casein diet (9) for 1 week, then their livers were removed for purification of cAST. The enzyme was purified essentially by the method of Huynh et al. (14). The final preparation of cAST gave a single band on acrylamide gel electrophoresis with sodium dodecyl sulfate (SDS).

Chemicals. All reagents used were of the highest grade available and were purchased from Wako Pure Chemicals (Osaka, Japan), Boehringer (Mannheim, Germany), or Sigma Chemicals Co. (St. Louis, MO, U.S.A.). Ampholyte and pH markers were obtained from BioRad Laboratory (Richmond, CA, U.S.A.).

Preparation of samples. Glycation reaction of albumin and cAST was carried out at 37°C for the indicated periods. The incubation mixtures usually contained 0.1% NaN₃ in 0.1M sodium phosphate buffer.

Electrophoretic analyses. Isoelectric focusing (IEF) was carried out essentially as described by Reinhart and Malamud (15). Acrylamide gels (5%) 1 mm thick containing 2% ampholine were prepared on glass plates, and a current was applied in a horizontal IEF apparatus (Atto Co. Ltd., Tokyo) with 1 M NaOH as catholyte and 1 M H₃PO₄ as anolyte. Gels were chilled to 10°C and pretreated at 400 V for 1 h. Samples were then loaded onto the gels with a plastic applicator and focused to equilibrium (overnight, 400 V). The gels were washed with a 5% salicylic acid–10% TCA solution overnight to remove ampholyte and then stained for protein with a 0.25% Coomassie brilliant blue (CBB) G–10% acetic acid–30% methanol solution.

Other methods. Glucose linked to albumin was converted by 10 M acetic acid
for 16 h at 100°C to 5-hydroxymethyl furufuraldehyde (HMF) which was quan-
titated by reaction with 2-thiobarbituric acid (16). Glycation of albumin was
expressed as micromoles of HMF per gram of protein. cAST activity was measured
by a modified method of Karmen (17) as described previously (9). Absorption
spectra of complexes formed from PLP and AG, and from AG and sugars were
measured in a UVIDEC-660 (Japan Spectroscopic Co. Ltd., Tokyo). Measurement
of fluorescent spectra was carried out in a FP-770 (Japan Spectroscopic Co. Ltd.).

RESULTS AND DISCUSSION

1. Effects of AG on binding glucose and PLP to albumin

Effects of AG and PLP on the glycation reaction of albumin were observed in
vitro. Fifty milligrams of bovine albumin in 1 ml phosphate buffer (pH 7.0) was
incubated with 50 mM glucose at 37°C for 5 days. Glucose bound to albumin was
determined as HMF. As shown in Table 1, 252 μmol of HMF per gram of albumin
were found, and the addition of 10^{-3} M AG inhibited the glycation of albumin by
50%. As PLP is considered to be distributed into tissues through the bloodstream
by binding to albumin, the effects of AG and glucose on binding of PLP to albumin
were examined by determination of the isoelectric point (pI). Figure 1 shows IEF
pattern of albumin incubated with various substances. When PLP was added to
albumin, the pI was lowered, indicating that PLP was bound to albumin (lane 3).
Binding of PLP to albumin was not inhibited by glycation of albumin (lane 6). The
IEF pattern obtained by further addition of AG to the mixture of albumin, PLP,
and glucose, was very similar to that with albumin alone (lane 7). This means that
the addition of AG resulted in inhibition of binding PLP to albumin.

2. Reaction between PLP and AG

A direct reaction of AG with PLP was suggested, because PLP contains

| Table 1. Effects of AG and PLP on the glycation reaction of albumin. |
|-----------------|-----------------|-----------------|
| **Additions**   | **HMF formed**  | **%**           |
|                 | (μmol/g)        |                 |
| Albumin         | —               |                 |
| + Glucose       | 252             | 100             |
| + PLP           | 5.6             |                 |
| + Glucose, PLP  | 228             | 90.4            |
| + AG            | —               |                 |
| + Glucose, AG   | 134             | 53.1            |

—, negligible. Incubation was carried out at 37°C for 5 days. Reaction mixture
contained either one or two of albumin (50 mg), glucose (50 mM), PLP (10^{-3} M),
and AG (10^{-3} M), in 1 ml of 0.1 M phosphate buffer containing 0.1% NaN_{3}. Values
are means for 2 determinations.

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Fig. 1. Effects of glucose, PLP, and AG on IEF pattern of albumin. Albumin (50 mg/ml) was incubated at 37°C for 5 days in the absence or presence of 10⁻³ M AG, 10⁻³ M PLP, or 50 mM glucose. Additions are as follows: 1, albumin alone; 2, AG; 3, PLP; 4, glucose; 5, glucose + AG; 6, glucose + PLP; 7, glucose + AG + PLP; 8, pI markers (phycocyanin, 4.63; β-lactoglobulin B1 5.10; bovine carbonic anhydrase, 6.00).

Fig. 2. Effect of AG on absorption spectrum of PLP. a) 10⁻³ M PLP was mixed with an equimolar amount of AG in 0.1 M phosphate buffer (pH 7.4) and absorption spectra were measured. AG showed no absorption in the range of wavelength used. b) 10⁻² M NaBH₄ (at a final concentration of 10 mM) was added to the mixture of PLP and AG, and incubated at room temperature for 1 h. ---, PLP; ----, PLP + AG; -----, PLP + AG + NaBH₄; ------, PLP + NaBH₄.
3. Effects of AG on the enzymic activity and the glycation reaction of cAST

Effect of AG on PLP-dependent enzymes must be considered as the formation of a complex of PLP and AG has been shown. At first, an effect of AG on cAST activity was examined for short-term incubation. As shown in Table 2, the enzymic activity was inhibited by more than 70% upon the addition of $10^{-3} \text{M}$ AG, however, most activity was recovered by further incubation with excess PLP. It was indicated that PLP bound to the enzyme protein was released and as a result holoenzyme was converted into apoenzyme. As shown in the previous papers (12, 13), glycation of cAST occurred in the presence of various sugars, and so the effect
Table 2. Effect of AG on cAST activity.

| Addition     | Activity (U/ml) | (%)  |
|--------------|----------------|------|
| cAST         | 11.7±0.4       | 100  |
| + PLP        | 13.9±0.2       | 119  |
| + AG         | 3.18±0.07      | 27   |
| + PLP, AG    | 12.8±1.1       | 109  |

Purified cAST (40 μg/ml) was incubated at 37°C for 1 h with 10^{-3} M AG when added. Further incubation was carried out for 30 min after addition of 10^{-3} M PLP. Values are means±SD.

Table 3. Effects of AG on cAST activity and glycation reaction of cAST with various sugars for a long term incubation.

| Addition             | cAST activity | (%)  | cAST activity | (%)  |
|----------------------|---------------|------|---------------|------|
|                      | ACTIVITY (U/ml) | (%) | + PLP ACTIVITY (U/ml) | (%) |
| Experiment 1         |               |      |               |      |
| None                 | 56.4          | 100  | 135           | 100  |
| AG                   | 29.5          | 52.3 | 94.3          | 69.6 |
| d-Ribose             | 22.8          | 40.4 | 150           | 111  |
| d-Ribose, AG         | 10.8          | 19.1 | 101           | 74.8 |
| Glyceraldehyde       | 1.1           | 1.9  | 2.4           | 1.8  |
| Glyceraldehyde, AG   | 8.4           | 14.9 | 10.2          | 1.5  |
| Experiment 2         |               |      |               |      |
| None                 | 131           | 100  | 148           | 100  |
| AG                   | 62.7          | 47.8 | 118           | 79.7 |
| d-Fructose           | 25.3          | 19.3 | 110           | 74.3 |
| d-Fructose, AG       | 55.4          | 42.2 | 113           | 76.3 |

Incubation mixture contained approximately 400 μg/ml of purified cAST, 50 mM sugars, 10^{-3} M AG, and 10^{-3} PLP when added in 0.1 M phosphate buffer (pH 7.4). Incubation was carried out at 37°C for 3 days and for 24 h. Different batches of enzyme, stored for more than 3 months in Experiment 1 and freshly prepared in Experiment 2 were used. Values are means for 2 determinations in duplicate experiments.

of AG on the glycation of cAST was determined (Table 3). Extent of the glycation of cAST varied with the kind of sugars added. Ribose largely inhibited cAST activity, however, the inhibition was completely restored by the addition of PLP. On the other hand, when both ribose and AG were added, the inhibition of both reagents enzymatic activity was additive. This means that AG had no effect on the inhibition of the glycation reaction of cAST by ribose. Glyceraldehyde, which is also one of the intermediates of glucose metabolism, was found to greatly inhibit cAST activity and the inhibition was partly removed by the addition of AG, indicating that AG can inhibit glycation of cAST with glyceraldehyde. AG was
also effective to inhibit the glycation reaction of cAST with fructose.

4. Reaction between AG and sugars

Effect of AG as an inhibitor for the glycation reaction of cAST varied with the sugars used. Figure 4 shows absorption spectra of complexes produced by incubation of AG with various sugars. It is clear that AG shows different reactivity to various sugars and also shows very high reactivity to glyceraldehyde.

The principal findings of this study are as follows. At first, AG inhibited not only the glycation of albumin but the binding of PLP to albumin and therefore, the distribution of PLP absorbed from intestine into tissues will be inhibited by AG. Secondly, AG removes PLP from PLP-dependent enzymes and B6 deficiency will occur as their enzymatic activities are decreased. Thirdly, AG can bind to various compounds with aldehyde or ketone moieties, and consequently a shortage of some essential aldehyde and/or ketone compounds may result.

Glycation of proteins causes crucial damage in living organisms especially in diabetic complications and in the aging process. Although AG has been found to inhibit glycation of various proteins, the usefulness of AG must be reexamined from the viewpoint of safety for living organisms using an in vivo system. There are many problems which must be clarified, such as doses of AG given to humans, the possibility for combination therapy of AG and PLP, and so on. Development of some other pharmacological reagents for prevention of glycation reaction also will be desired in the near future.

Fig. 4. Absorption spectra of AG and sugars. Sugars and AG mixed in phosphate buffer (0.1 M, pH 7.4) were incubated at 37°C for 18 h in a), c), and d), and for 1 h in b). a) and b) glyceraldehyde, c) ribose, d) fructose.
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