Aldehydes or dicarbonyls in non-enzymic glycosylation of proteins

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The non-enzymic post-translational glycosylation of certain proteins has been implicated in the production of diabetic sequelae. In the present paper the possibility that it is not the glucose aldehyde that binds to proteins but a dicarbonyl autoxidation product is investigated. Earlier experiments may not have distinguished between these two possibilities. The rate of binding of 2-deoxyglucose (a non-autoxidizable sugar) to lens α -crystallin is compared with that of glucose (an autoxidizable sugar). The stabilized Schiff-base adducts from reaction of glucose with glycine were isolated. The structure of the adducts was investigated by using proton n.m.r. and fast-atom-bombardment mass spectroscopy to distinguish whether they are the product of aldehyde or dicarbonyl addition. We conclude that it is the open-chain aldehyde of glucose that binds initially to amino groups and that there is no participation of dicarbonyl autoxidation products in the initial non-enzymic protein glycosylation reaction.

Non-enzymic, post-translational, glycosylation of proteins has been implicated in many diabetic complications. The modification may induce conformational changes that affect protein function (Harding, 1985).

The nature of the glucose-protein linkage was first investigated in relation to haemoglobin A_{1c}, a minor component of haemoglobin A. Haemoglobin A_{1c} was found to differ from haemoglobin A solely by the attachment of glucose to its *N*-terminus. The initial linkage was formed via a reversible Schiff base, which could be stabilized by sodium borohydride reduction for studies *in vitro* (Holmquist & Schroeder, 1966; Bookchin & Gallop, 1968). Later experiments suggested that the Schiff base undergoes an Amadori rearrangement *in vitro* to form a stable ketoamine (Bunn *et al.*, 1975), most of which is in the β -pyranose form (Roper *et al.*, 1983), this process probably being the rate-determining step of protein glycosylation (Flückiger & Winterhalter, 1976). Lysyl amino groups can also be glycosylated (Shapiro *et al.*, 1980), the rate of modification being site-dependent. These investigations have led to the accepted reaction mechanism of glucose with amino groups shown in the left-hand branch of Scheme 1.

Certain triose sugars undergo rapid autoxidation under physiological conditions, producing oxygen free radicals (Yamaguchi & Nakagawa, 1983).

Dicarbonyl compounds have been detected as one of the autoxidative by-products (Thornalley *et al.*, 1984), and it has been postulated that these could bind to protein amino groups via a Schiff base, stabilized by imine-carbonyl conjugation (Wolff, 1984). If glucose were to autoxidize at a significant rate, it could be the glucose dicarbonyl, not the open-chain aldehyde, that binds to proteins (right-hand branch of Scheme 1). Both aldehyde and dicarbonyl bind initially via Schiff bases. Reduction of these bases with sodium borohydride would yield identical products, hence studies such as that by Bookchin & Gallop (1968) would not distinguish whether the aldehyde or dicarbonyl bound initially to amino groups. Furthermore, experiments where incubations are sealed for purposes of sterility may minimize the formation of dicarbonyls that could bind to proteins. If autoxidation products are responsible for the protein glycosylation associated with many diabetic complications, then antioxidant drugs may be therapeutically useful in conjunction with good blood glucose control.

Materials and methods

Materials

D-[U-¹⁴C]Glucose (278 mCi/mmol) and D-2-deoxy[U-¹⁴C]glucose (310 mCi/mmol) were obtained from Amersham International. AnalalR D-

Scheme 1. Two mechanistic schemes for the binding of glucose to amino groups

The left-hand branch shows the accepted mechanism involving the open-chain aldehyde binding directly to amino groups. The right-hand branch shows a simplified scheme where the aldehyde autoxidizes to produce dicarbonyls, which then bind to amino groups.

glucose and chromatographically pure glycine were purchased from BDH Chemicals. 2-Deoxyglucose was obtained from Sigma Chemical Co. Sodium cyanoborohydride and deuterium oxide were obtained from Merck.

Binding of 2-deoxyglucose and glucose to bovine lens α -crystallin

This was quantified by periodic assays of the incorporation of radiolabel from D-2-deoxy[U-¹⁴C]glucose or D-[U-¹⁴C]glucose into trichloroacetic acid-precipitable protein by using liquid-scintillation counting. For further details, including the preparation of bovine α -crystallin, see Beswick & Harding (1984). Incubations were in 0.1 M-sodium phosphate buffer, pH 7.4, containing 0.05% NaN₃ at 37°C. In both experiments the final sugar concentration was 10 mM, including 5 μ Ci of the relevant radiolabelled sugar/ml, and the final protein concentration was 10 mg/ml.

Reaction of glycine with glucose

All incubations were in 0.1 M-sodium phosphate buffer, pH 7.4, at 37°C and were done under sterile

conditions in flasks with 0.22 μ m filters attached, so allowing gaseous exchange while maintaining sterility.

Method 1. The final incubation mixture contained 50 mM-D-glucose, 100 mM-glycine and 500 mM-sodium cyanoborohydride plus 1 μ Ci of D-[U-¹⁴C]glucose. Incubation was for 7 days, after which the reduced glucose-glycine adducts were isolated by ion-exchange and gel chromatography as in the method of Trueb *et al.* (1982), except that the Sephadex G-15 column was replaced by a Sephadex G-10 one.

Method 2. D-Glucose (50 mM) containing 1 μ Ci of D-[U-¹⁴C]glucose was incubated for 5 days. Glycine was then added to the incubation to give a final concentration of 100 mM, and the mixture incubated for a further 24 h before the addition of cyanoborohydride to give a final concentration of 500 mM. The resulting glucose-glycine adducts were isolated as in method 1.

Adducts obtained from methods 1 and 2 were analysed by using fast-atom-bombardment mass spectroscopy and proton n.m.r. to determine if the products derived from the addition of an aldehyde or a dicarbonyl to glycine.

Proton n.m.r.

Glucose-glycine adducts from method 1 were deuterium exchanged by dissolving/freezing-drying a sample in deuterium oxide three times before dissolution of the sample in deuterium oxide. The sample was then analysed on a Bruker WH300 spectrometer. The reference standard used was tetramethylsilane (external reference).

Mass spectroscopy

In order to identify the molecular ion, a sample of adduct was subjected to fast-atom bombardment (Barber *et al.*, 1981) from a glycerol plus oxalic acid matrix. The instrument used was a ZAB IF mass spectrometer (V.G. Micromass).

Results

Binding of glucose and 2-deoxyglucose to bovine lens α -crystallin

The rate of binding of 2-deoxyglucose does not differ markedly from that of glucose either in the presence or in the absence of sodium cyanoborohydride (Fig. 1). 2-Deoxyglucose was chosen for this comparison since it cannot undergo C1-C2 enolization (Scheme 1) and so cannot form α -ketoaldehydes (dicarbonyls) via the autoxidation mechanism proposed in Thornalley *et al.* (1984), nor can it stabilize via Amadori rearrangement. The observed 2-deoxyglucose binding must therefore be due to the open-chain aldehyde form. By comparison of the extent and rate of binding of the

Fig. 1. Comparison of the binding of glucose (▲) and 2-deoxyglucose (●) to bovine α -crystallin under identical conditions

For details see the text. After 2 days sodium cyanoborohydride was added (arrow on graph) to stabilize Schiff bases as they formed.

two sugars, it can be seen that open-chain-aldehyde binding could account for most of the observed reaction of glucose with bovine α -crystallin.

Reaction of glucose with the amino group of glycine

The reaction conditions were chosen to allow the maximum chance of detecting any dicarbonyl adducts while keeping close to physiological conditions. Free gaseous exchange was allowed to ensure sufficient oxygen for the autoxidation process. The use of cyanoborohydride as the reducing agent, rather than sodium borohydride, ensures that only the imine double bond of the Schiff base is reduced, not the carbonyl groups (Jentoft & Dearborn, 1979). The recovery of radioactive glucose in the isolated adducts indicated that more than 91% of the initial glucose had reacted after cyanoborohydride reduction with either of the described methods.

Method 1. Mass spectroscopy of the reduced adducts. This type of fast-atom bombardment of the sample produces very little fragmentation of the molecules. As a result the most abundant species is the molecular ion. The glucose-glycine

Fig. 2. Fast-atom bombardment of adducts produced by method 1

For details see the text. The molecular ion is at m/z 240 and corresponds to the relative molecular mass of the adduct. The insets show the predicted structures and relative molecular masses of the adducts (a) aldehyde product and (b) dicarbonyl product.

adducts formed by using method 1 produce a molecular ion with m/z 240 (Fig. 2). As indicated by the Figure inset, this corresponds to the relative molecular mass of the adduct formed by reaction of the open-chain aldehyde of glucose with the amino group of glycine (inset structure *a*). The m/z of the dicarbonyl product (inset structure *b*) would be 238. There is no significant contribution from such a species.

Proton n.m.r. A deuterium-exchanged sample of the same adduct was analysed in order to count the number of non-exchangeable protons associated with the adduct. This will differ depending on whether the free aldehyde (ten non-exchangeable protons) or the dicarbonyl (nine non-exchangeable protons) is bound to the amino group (Fig. 3 inset). The n.m.r. spectrum (Fig. 3) indicates that the signal is derived from ten protons, consistent with the aldehyde form of glucose being involved in adduct formation, not the dicarbonyl.

Method 2. In this method glucose is preincubated alone for 5 days to allow any autoxidation products that may form to accumulate, so increas-

ing the chances of their detection. Hexose dicarbonyls have been extracted from tissues and analysed by t.l.c. (Szent-Györgyi *et al.*, 1967), and therefore the compounds must be reasonably stable, although the dicarbonyls in these studies may have been artifacts of the extraction procedure (Otsuka & Együd, 1968). The glycine was then added and the mixture incubated a further 1 day to ensure time for all species present to form Schiff bases. The sterility of the incubation mixture was confirmed by subculture on to nutrient agar before reduction of adducts with cyanoborohydride.

Again the analysis by mass spectroscopy of reduced adducts revealed a molecular ion at m/z 240, with very little material at m/z 238 (spectrum not shown). This indicates that even under these conditions the main adduct is formed from the open-chain aldehyde, not the dicarbonyl autoxidation product.

Incubation of glucose alone

If glucose autoxidizes appreciably via an enediol, new products would be formed (De Wit *et al.*,

Fig. 3. Proton-n.m.r. spectroscopy of the adducts produced by method 1

For details see the text. The integrated area under the spectrum indicates that the adduct possesses ten non-exchangeable protons. The inset explains the basis for the difference in the predicted number of non-exchangeable protons depending on whether the aldehyde or the dicarbonyl adduct is formed. Exchangeable protons are removed from the structural formulae to allow easy comparison of the number of non-exchangeable protons, predicted for either case, that are responsible for the observed n.m.r. signal.

1979). Incubation of glucose in 0.1M-phosphate buffer, pH 7.4, for 20 days at 37°C under aerated sterile conditions revealed no new products detectable by gas chromatography. The retention times for the incubated glucose were unlike those for a sample of glucose dicarbonyl (glucosone).

Discussion

Experiments to test the specific question addressed here have not been previously described. However, evidence exists to support the idea of free aldehyde rather than dicarbonyl binding of glucose and other aldoses to amino groups. Robins & Bailey (1972), while studying collagen cross-links, allowed lysine to react with glucose and with mannose for 4h at pH 7.9 and 37°C. The Schiff bases formed were then reduced with potassium borohydride. The glucosyl adduct gave a single product that had a different retention time on amino acid analysis from that of the single product obtained from mannose. This result could only occur if the configuration at C-2 was maintained throughout the reaction; any formation of an enediol or dicarbonyl would lead to loss of the configuration at C-2 and the formation of a mixture of glucosyl and mannosyl products, regardless of which was used initially. Secondly, dicarbonyl compounds react preferentially with arginine residues rather than amino groups (Yankeelov, 1972) to give a product stable to acid hydrolysis. Eble *et al.* (1983) incubated ribonuclease with glucose until most of the lysine had disappeared, but no decrease in the arginine content of the protein was observed. This would not be expected if glucose dicarbonyl were present in significant amounts. Lastly, incubations of haemoglobin with glyceraldehyde leads to modification of amino groups with no change to arginine residues (Acharya & Manning, 1980).

We therefore conclude that the production of dicarbonyls by autoxidation (Thornalley *et al.*, 1984) is not a significant factor in the non-enzymic glycosylation of proteins under conditions that approximate to those found *in vivo*.

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