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Mixed Dimers Formed by Crosslinking of Native and Glycated Proteins in the Absence of Free Sugar

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After a hyperglycaemic episode, glycated proteins remain in the body until removed by protein turnover. We have shown that in the absence of free sugar, such proteins can crosslink to native proteins, forming mixed dimers. They can also induce native proteins to crosslink into homodimers, presumably by release of a soluble crosslinking agent. Similar reactions *in vivo* could be responsible for the deposition of serum proteins in diabetic kidney, nerve and other tissues. Exposure to glycating sugar for brief periods, or at low concentration, still produced glycated protein capable of crosslinking to other proteins under sugar-free conditions. These crosslinks are nonfluorescent, unlike the advanced glycation endproducts usually observed.

Protein glycation following hyperglycaemia is generally agreed to contribute to diabetic complications and similar symptoms in ageing. The first stable protein-sugar adduct is an Amadori or Heyns product, depending whether the glycating sugar is glucose or fructose. Both are reactive compounds, which can produce fluorophores and other AGEs in the absence of free sugars. Particularly important are the sugar-derived crosslinks that may *in vivo* account for the stiffening of collagen (1), and the trapping of serum proteins in nerve, kidney and possibly other tissues (2–4). *In vitro*, sugar-derived crosslinking can be observed by electrophoresis as, for example, in ref. 5. In this paper we have simulated the post-hyperglycaemic state by removing free sugar from an *in vitro* glycated model protein, and then exposing it to a second unglycated protein. We found that under these conditions, even mildly glycated protein is able to crosslink to other proteins.

In vivo glycation by fructose has been clearly documented, but the Heyns product is hard to detect (6, and refs therein). Hence the potential contribution of fructose to disease is often overlooked, even though this sugar is a more powerful glycating agent than glucose (7). Fructose is a major dietary sugar, and serum concentrations are dependent on the amount ingested. For example, 1 g per kg of food produces 0.53 mmol/l serum fructose in healthy individuals (8), while up to 2 mmol/l concentrations have been reported in cirrhosis (9). Fructose is also derived from the polyol pathway which may be a major factor in AGE formation in the lens and other tissues involved in diabetic complications (10). Therefore in this paper, we have used fructose as the glycating sugar.

MATERIALS AND METHODS

In vitro fructation. Seven days fructation of lysozyme and β-lactoglobulin together (each 10 g/l) was performed in 0.1 M fructose and 0.1 M sodium phosphate buffer at pH 7.4, with 3 mM sodium azide. Brief fructation of lysozyme alone (10 g/l), was carried out for 0.5–12 h at 37°C in 0.5 M fructose in the 0.1 M phosphate buffer, or for 12 h at 4°C in 5 mM fructose in 0.5% ammonium bicarbonate adjusted to pH 7.4 with HCL. Free sugar was removed by exhaustive dialysis at 4°C against 0.5% bicarbonate. The samples were stored frozen at −20°C.

In vitro crosslinking in sugar-free conditions. Lysozyme (10 g/l) fructated to different extents, was incubated at 37°C in the 0.5% bicarbonate solution with native β-lactoglobulin (10 g/l) for 7 days. (The control was mixed with native lysozyme.) Under these conditions, all protein remained soluble.

Electrophoresis. in sodium dodecylsulphate on 12% or 15% polyacrylamide gels was performed on protein pre-treated

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Abbreviation: AGE, advanced glycation endproduct.

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with mercaptoethanol, according to the method of Laemmli (11). Protein was detected by staining with colloidal Coomassie brilliant blue (12).

Chemicals. were from BDH, Poole, UK or from Sigma. The hen eggwhite lysozyme from Sigma contains traces of a nondisulphide-linked oxidation dimer of 28 kD. The 98% pure β-lactoglobulin from Sigma is a mixture of A and B isozymes, with traces of a lactose-derived AGE dimer. Monomer Mr values are 14 kD for lysozyme and 18 kD for β-lactoglobulin.

RESULTS

When lysozyme and β-lactoglobulin are incubated together in fructose for 7 days, and then subjected to polyacrylamide gel electrophoresis, three different dimers can be observed (Fig. 1). The traces of lysozyme and β-lactoglobulin homodimers already present (see bands of Mr 28 kD and 36 kD in lane A) have intensified slightly; but the major change is formation of a β-lactoglobulin-lysozyme heterodimer of Mr 32 kD, labelled MD in lane B. (At the heavy loading needed to demonstrate dimer formation, minor contaminants may also be seen. These are present from the beginning, and do not change during incubation at 37°C.)

There is no formation of heterodimer if fructation is carried out in HEPES buffer rather than phosphate (lane C), or if 50 mM aminoguanidine or 1 mM DETAPAC are included in the phosphate buffer (lanes D and E). This suggests that crosslinking of the glycated protein involves metal-catalysed oxidation, and is therefore enhanced by the traces of copper and iron in phosphate buffer (13,14), and inhibited by free radical scavengers or by metal chelators such as DETAPAC. Inhibition by the hydrazine aminoguanidine suggests that carbonyl groups are required for crosslinking.

Having demonstrated the presence of heterodimer when mixtures of proteins are glycated together, we then glycated lysozyme on its own, dialysed it free of unbound sugar, and reincubated it with native β-lactoglobulin. Fig. 2 shows that in the absence of free sugar, fructated lysozyme can still crosslink with native β-lactoglobulin to form the 32 kD heterodimer observed earlier. The most intense heterodimer band is seen in lane E, where lysozyme fructation had been carried out under optimum conditions, i.e. by incubating in 0.5 M fructose for 4 hours at 37°C. However even 30 min at 37°C (lane C), or 12 hours at 4°C in only 5 mM fructose (lane B), appear to have glycated the lysozyme protein enough for it to crosslink to unglycated protein under sugar-free conditions.

An unexpected finding in Fig. 2 is the β-lactoglobulin homodimer BD, whose intensity increases steadily from lanes B-D, as the protein is exposed to more extensively glycated lysozyme.

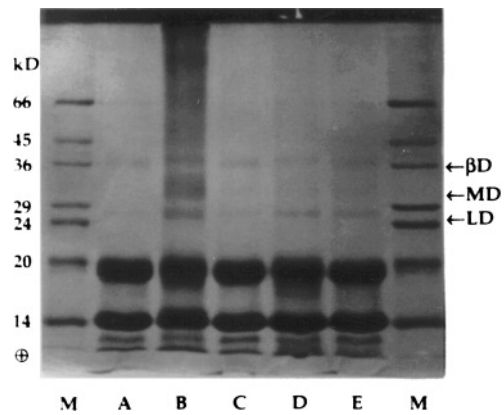

FIG. 1. Electrophoresis in 12% polyacrylamide, of β-lactoglobulin and lysozyme glycated together at 37°C for 7 days in 0.1 M fructose and 0.1 M phosphate buffer (lane B). Phosphate was replaced by HEPES (lane C) or mixed with 50 mM aminoguanidine (lane D) or with 1 mM DETAPAC (lane E). The control (lane A) was prepared as for lane B but immediately frozen. The Mr values of marker proteins (lane M) are shown on the left. Arrows on the right indicate positions of β-lactoglobulin dimer (βD), mixed β-lactoglobulin-lysozyme dimer (MD), and lysozyme dimer (LD).

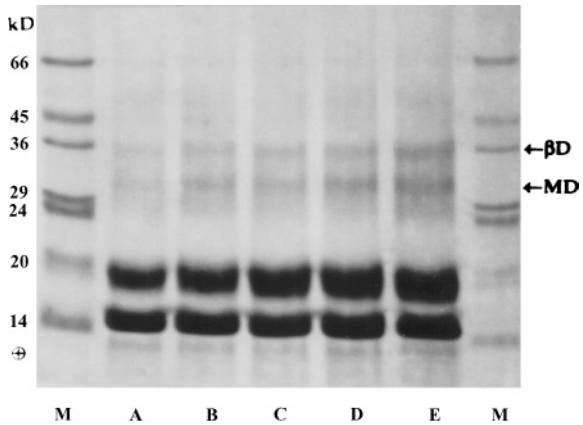

FIG. 2. Electrophoresis in 15% polyacrylamide of β-lactoglobulin incubated in sugar-free solution for 7 days with lysozyme previously fructated under different conditions, i.e., zero glycation (lane A), *in vitro* fructation at 4°C for 12 h (lane B), *in vitro* fructation at 37°C for 0.5 h (lane C), 2.5 h (lane D) and 4 h (lane E). Markers and dimers are labeled as in Fig. 2.

In vitro, AGEs are often detected by an assay simpler than electrophoresis, using their fluorescence rather than their polymerisation. The data in Fig. 3 suggest that this approach could underestimate the full extent of AGE formation. When fructated lysozyme is re-incubated with β-lactoglobulin in sugar-free solution, fluorescent AGEs are not formed unless the lysozyme has been fructated for at least 3 hours (in 0.5 M fructose at 37°C). Yet laser densitometer readings from gels identical to that in Fig. 2 show clearly that lysozyme fructated for only 30 min. under these conditions is capable of forming AGE crosslinks.

DISCUSSION

It has already been shown that glucated ribonuclease (5,7) continues to form homopolymers in the absence of free sugar. We have extended these observations to show that under these condi-

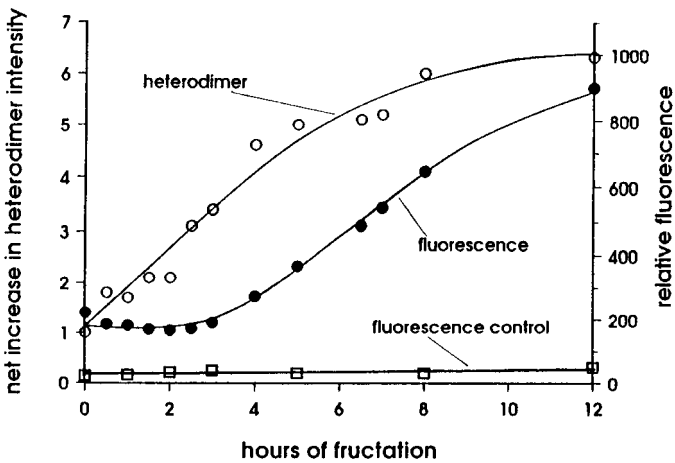

FIG. 3. Formation of AGE crosslinks and fluorophores, by β-lactoglobulin incubated for 7 days in sugar-free solution, with lysozyme previously fructated at 37°C for different times. Open circles show the net increase in staining of heterodimer band, as a percentage of the total stain in that lane. Closed circles show the increase in fluorescence of these same samples (at Ex = 325 nm, Em = 375nm). Controls (squares) show the fluorescence of fructated lysozyme samples mixed with β-lactoglobulin and immediately frozen.

tions, glycated proteins can also attack unglycated proteins, forming heterodimers. The mechanisms appear to be similar, involving metal-catalysed oxidation and aminoguanidine-sensitive carbonyl groups. Taken together, these reports suggest a mechanism whereby glycated serum protein could, in the absence of free sugar, become irreversibly bound to structural protein by sugar-derived crosslinks. This could be relevant to the deposition of serum proteins in diabetic nerve and kidney (2–4).

Dimers of β-lactoglobulin were seen when the protein was incubated with glycated lysozyme (Fig. 2); this suggests that glycated lysozyme releases a soluble reagent capable of crosslinking other proteins. A probable candidate is a dicarbonyl compound equivalent to 3-deoxyglucosone; this can crosslink native lysozyme *in vitro* (15), and is liberated from model Amadori compounds and glycated proteins under physiological conditions (16,17). Our findings show that such a compound could react directly with nearby proteins if produced *in situ.* This route to sugar-derived crosslinking would be obscured in most *in vitro* work by the continual presence of free sugar. *In vivo* it could provide another mechanism for glycated proteins to promote unwanted crosslinking of tissue protein.

For much *in vitro* work, proteins are heavily glycated under unphysiological conditions. Yet results reported here suggest that proteins need be only mildly glycated to show potentially far-reaching changes in biological activity. Indeed it is to be expected that even small post-translational changes will alter the higher-order structure of a protein and so may affect its activity. A problem with much glycation work is to detect such changes in proteins glycated under near-physiological conditions. The most sensitive methods, using antibody binding and catalytic activity (18,19), suggest that exposure to only millimolar sugar concentrations can bring about significant changes in protein structure and activity. Our work suggests that the ability to crosslink irreversibly with native proteins may be one such change.

When β-lactoglobulin and glycated lysozyme were incubated together, the early crosslinks were nonfluorescent. This is consistent with Baynes' observation (20) that less than 1% of the AGE crosslinks in *in vitro* glycated ribonuclease are fluorescent. Similarly McPherson *et al.* found that crosslinking precedes fluorescence in ribonuclease (7), while Makita *et al.* (21) have shown that immunogenic AGEs are formed well before fluorescent AGEs, during the *in vitro* glycation of albumin. Given that most post Amadori and post Heyns reactions are irreversible, and that changes to low-turnover proteins are cumulative, these findings suggest that *in vivo,* even brief hyperglycaemia resulting in minimal protein glycation could contribute to tissue damage, via unwanted protein crosslinking.

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REFERENCES

1. Bailey, A. J., and Kent, C. M. J. (1989) *in* The Maillard Reaction in Aging, Diabetes and Nutrition (J. W. Baynes and V. M. Monnier, Eds.), pp. 109–122, A.R. Liss, New York.
2. Brownlee, M., Vlassara, H., and Cerami, A. (1986) *Diabetes* **35,** 999–1003.
3. Miller, K., and Michael, A. F. (1976) *Diabetes* **25,** 701–708.
4. Michael, A. F., and Brown, D. M. (1981) *Diabetes* **30,** 843–846.
5. Eble, A. S., Thorpe, S. R., and Baynes, J. W. (1983) *J. Biol. Chem.* **258,** 9406–9412.
6. Ahmed, N., and Furth, A. J. (1992) *Clin. Chem.* **38,** 1301–1303.
7. McPherson, J. D., Shilton, B. H., and Walton, D. J. (1988) *Biochemistry* **27,** 1901–1907.
8. MacDonald, I., Keyser, A., and Pacy, D. (1978) *Am. J. Clin. Nutr.* **31,** 305–1311.
9. Avgerinos, A., Harry, D., Bousboukas, S., Theodossiadou, E., Komesidou, V., Pallikari, A., Raptis, S., and McIntyre, N. (1922) *J. Hepatol.* **14,** 78–87.
10. Cohen, M. P. (1987) The Polyol Paradigm and Complications of Diabetes. Springer-Verlag, New York.
11. Laemmli, U. K. (1970) *Nature* **277,** 680–5.
12. Neuhoff, V., Arnold, N., Taube, D., and Ehrhardt, W. (1988) *Electrophoresis.* **9,** 255–262.

13. Fu, M., Knecht, K. J., Thorpe, S. R., and Baynes, J. W. (1992) *Diabetes* **41** (Suppl. 2)**,** 42–48.
14. Cochrane, S. M., and Furth, A. J. (1993) *Biochem. Soc. Trans.* **21,** 97S.
15. Kato, H., Shin, D. B., and Hayase, F. (1987) *Agric. Biol. Chem.* **51,** 2009–2011.
16. Kato, H., Cho, R., Okitani, A., and Hayase, F. (1987) *Agric. Biol. Chem.* **51,** 683–689.
17. Zyzak, D. V., Richardson, J. M., Thorpe, S. R., and Baynes, J. W. (1995) *Arch. Biochem. Biophys.* **316,** 547–554.
18. Ganea, E. (1992) *S. Cer. Biochem.* **35,** 21–26.
19. Ganea, E., and Harding, J. J. (1995) *Eur. J. Biochem.* **231,** 181–185.
20. Dyer, D. G., Blackledge, J. A., Thorpe, S. R., and Baynes, J. W. (1991) *J. Biol. Chem.* **266,** 11654–11660.
21. Makita, Z., Vlassara, H., Cerami, A., and Bucala, R. (1992) *J. Biol. Chem.* **267,** 5133–5138.