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Cross-linking of insulin with glutaraldehyde to form macromolecules

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In a search for a new type of sustained-release depot preparation for insulin, Mahbouba & Smith (1977) thiolated a proportion of the insulin units in the zinc-insulin hexamer and cross-linked the hexamer through disulphide bridges to form macromolecules (n = 410–708 monomer units) where native insulin was carried on a modified insulin skeleton. We describe here the formation of similarly structured macromolecules by the cross-linking of the zinc-insulin hexamer using glutaraldehyde.

Methods and results

Reaction between insulin and glutaraldehyde. An aliquot (25 μl) of glutaraldehyde solution (25%, Kodak Ltd) was mixed with a solution (20 ml) of zinc-insulin (50 mg BDH, 24·4 units mg^{-1}) in phosphate buffer (0·1 M) pH 8 and the mixture stored at room temperature (20 °C) for 3–5 min during which the extent of modification of the hexamer was 25–35%. The mixture was then passed down a column (40 cm × 2·6 cm i.d.) of Sephadex G-25 and eluted with the same buffer. Fractions (3 ml) were collected and examined at λ280 nm for the modified insulin. The protein fractions were pooled and the protein concentration was determined (Lowry et al 1951). The amino group titre of the protein fraction was determined immediately after the separation by mixing a solution (3 ml, 0·2–1·2 mg) with a solution of sodium trinitrobenzene sulphonate (TNBS) (25 μl, 0·0285 M) at 30 °C for 2 h (Habeeb 1966; Snyder & Sobocinski 1975) and comparing the colour developed at λ 420 nm with that obtained for similar concentrations of native insulin (3 amino groups mol^{-1}).

On storage of the glutaraldehyde—insulin reaction product (35% modification) at 4 °C in phosphate buffer, pH 8, the average molecular size (n) determined by light scattering (see Mahbouba et al 1974) increased from 3·8 to 17·8 hexamer units over seven days. A lower extent of modification using a shorter reaction time, gave smaller macromolecules. Highly modified (ca 70%) samples gave curved plots of Kc/R_θ vs $\sin^2\theta/2$ in the light scattering method for determining (n).

Characterization of the product. (a) *Confirmation of the hexamer structure.* Cu(II)-insulin was prepared by removing zinc from zinc-insulin using EDTA and then adding the calculated amount of copper (II) chloride solution to give 2 or 8 g atom Cu(II) $hexamer^{-1}$. The isolated Cu(II)—insulin was shown to contain 7·12 g atom Cu(II) and 0·72 g atom Zinc $hexamer^{-1}$ by analysis for protein (Lowry et al 1951), and Cu(II) and Zn ions by atomic absorptiometry (Mahbouba & Smith 1977). The Cu(II)-insulin was reacted with glutaraldehyde in the usual manner and the separated protein (45% modification) was found to have retained all its original copper and zinc content although 1·16 g atom Cu $hexamer^{-1}$ was present as Cu(1) as determined with 2,2′-biquinolin (Felsenfeld 1960). Attempts to replace the zinc in the glutaraldehyde-zinc insulin product by addition of Cu(II) ions was unsuccessful. Although the zinc was nearly all replaced by copper the latter was mainly in the Cu(I) state as inferred from e.p.r. studies.

E.p.r. spectra were recorded at 77K in a liquid nitrogen dewar with a Varian E-109 Spectrometer, with a Telmore Inst. subharmonic generator attached to give third harmonic spectra.

The spectra (Fig. 1) obtained from the native and modified insulin samples each containing six copper ions are very similar. There are two copper(II) sites in both samples as shown by the duplicate set of 'parallel' absorptions at the low field ends of the spectra. The third harmonic spectra demonstrate this much more clearly owing to their greater resolution. It is not possible in the perpendicular region to differentiate between two different axial copper(II) sites having different g perpendicular values and two rhombic species having nearly identical g_x and g_y values. Owing to this uncertainty it is only possible to measure $g_\perp$ and $A_\perp$:

Site 1, $g_\perp = 2{\cdot}285$, $A_\perp^{Cu} = 16{\cdot}5$ mT, Site 2, $g_\perp = 2{\cdot}23$, $A_\perp^{Cu} = 15{\cdot}5$ mT, (the $A_\perp^{Cu}$ value being an average of the two isotopic contributions).

(b) *Aldehyde groups.* 0·5 ml of a solution of 3-methyl-2-benzothiazolone hydrazone (0·4%) (Sawicki et al 1961) was added to the sample (0·5 mg ml^{-1}) in phosphate buffer (0·01 M, 1 ml) pH 8. The mixture was left for 30 min, a solution of ferric chloride (2·5 ml, 0·2%) added and, after a further 15 min at room temperature, the mixture centrifuged and the absorbance of the supernatant measured at λ 670 nm against a blank put through the same procedure. The aldehyde content was determined from a standard curve obtained for butanal by the same means. The ultraviolet absorption curves obtained for butanal and the product had the same shape in the λ 600–700 nm region. A product with 28% modification had 2·05 aldehyde groups $hexamer^{-1}$.

(c) *Lysine residues.* Amino acid analysis of the product (22% modification, 1·84–CHO groups $hexamer^{-1}$) and native insulin was carried out by the method of Spackman et al (1958) and showed that only the original lysine content had been reduced (by 20%) in the

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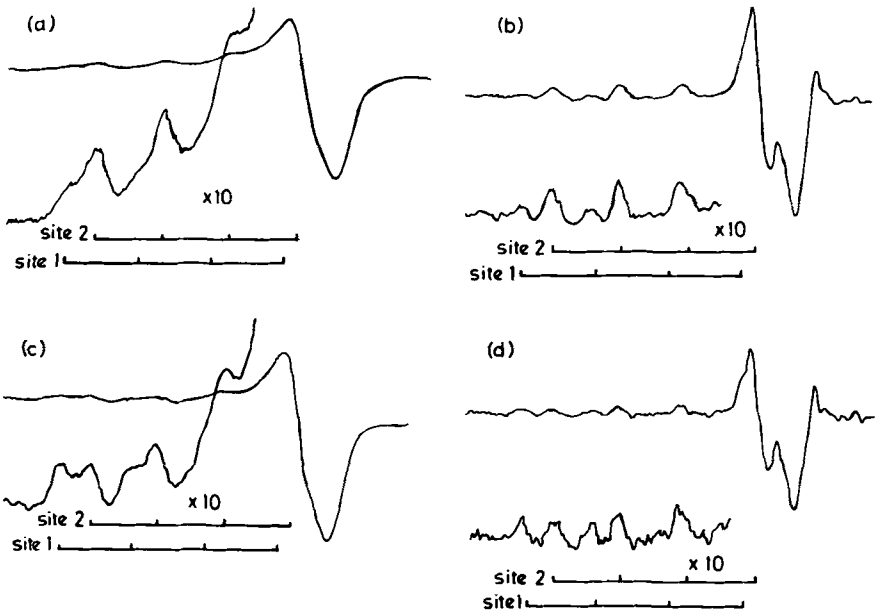

FIG. 1. E.p.r. spectra of insulin containing six copper ions at 77K. (a) First harmonic spectrum of native insulin. (b) Third harmonic spectrum of native insulin. (c) First harmonic spectrum of modified insulin. (d) Third harmonic spectrum of modified insulin.

product. A 22% modified product would be expected to show zero or 66% reduction if the reagent was completely present in either the monomer or polymer form respectively.

Further cross-linking with diaminodecane. A glutaraldehyde zinc-insulin product (23% modification, 90 mg) in phosphate buffer (20 ml, 0·1 M) pH 8 was divided into two portions and, after adjustment of one portion with hydrochloric acid (1 M) to pH 6·5, they were stored at 4 °C for 24 days. The solutions were examined for aggregation and percentage modification at intervals (Table 1). In a subsequent experiment, diaminodecane (1 mg) was added to each portion (10 ml) before storage (Table 1).

Table 1. Storage of glutaraldehyde—insulin product at pH 6·5 and 8·0 in presence and absence of diaminodecane.

Storage period (days)	Glutaraldehyde-insulin product: Percentage modification pH 6·5	pH 8	Aggregation number pH 6·5	pH 8	Glutaraldehyde-insulin product in presence of diaminodecane: Aggregation number pH 6·5	pH 8
2	51	30	10·7	2·0	22	2·4
5	59	41	—	3·1	37	3·3
8	55	37	12·3	3·5	—	—
24	52	—	19·8	—	31	—

Discussion

Glutaraldehyde is one of the most extensively used protein cross-linking reagents (Bishop & Richards 1968; Bowes & Cater 1966; Habeeb & Hiramoto 1968; Schejter & Bar-Eli 1970) although its chemistry has been the subject of much debate. Commercial solutions of "25% glutaraldehyde", pH about 3, contain 79% water, 3% glutaraldehyde and 18% derivatives of higher molecular weight which can be broken down to glutaraldehyde (Monsan et al 1975). Hardy et al (1969) concluded that commercial glutaraldehyde solutions contain the dialdehyde in equilibrium with its cyclic monohydrate, and open chain monohydrate and dihydrate. At near neutral or alkaline pH, where the cross-linking reaction is generally carried out, the dialdehyde undergoes an aldol condensation with itself, followed by dehydration to give α-,β-unsaturated aldehyde polymers, $OHC\text{-}CH_2\text{-}[CH_2]_2\text{-}CH{=}[C(CHO)\text{-}[CH_2]_2\text{-}CH]_n{=}C(CHO)[CH_2]_2CHO$ (Richards & Knowles 1968; Monsan et al 1975). Cross-linking with protein occurs by reaction of an aldehyde group in the polymer with the ε-amino group of lysine to give an unsaturated Schiff's base which is stable to strong acid. Further reaction between the conjugated Schiff's base and another amino group may occur by Michael addition to a limited extent. A polymer with $n = 6$ could bridge across two suitably placed lysine residues in a protein to give a 4:1 ratio for glutaraldehyde: protein as observed with collagen (Korn & Filachione 1967). In acid media the monomer reacts to form a non-conjugated Schiff's base which is unstable to acid. Insulin contains three amino groups: the ε-amino group of B29-lysine and the α-amino groups of A1-glycine and B1-phenylalanine. The reaction between glutaraldehyde and zinc-insulin at pH 8 gave about 25–35%

modification of the 18 amino groups present in the hexamer within 3–5 min. The product contained 2–3 aldehyde groups per hexamer. The low aldehyde content of the product with respect to the degree of modification suggested that reaction had occurred largely with glutaraldehyde monomer rather than the polymer alone. Amino acid analysis of the product showed that only the lysine content had been reduced, that 30% of the modified lysine was acid-stable due to Schiff's base formation with glutaraldehyde polymer. However this calculation assumes that modification with glutaraldehyde occurs only at the lysine residues of proteins, although this situation is unlikely here since aldehyde groups on the introduced glutaraldehyde (either monomer or polymer, n = O) are suitably placed to undergo an intramolecular reaction with Al glycine (cf. Brandenberg et al 1977) during the separation process and this reaction could contribute to the extent of modification. In this way the very low free aldehyde content of the product is accounted for although the calculated extent of modification of the insulin by the glutaraldehyde monomer relative to the polymer is reduced. Despite the difficulties of quantifying the relative importance of the monomer reaction, it does occur to a significant extent. Its observation here for the first-time in near neutral media is due to the method of mixing of the reactants and the short reaction period before the glutaraldehyde monomer is appreciably depleted by polymer formation, with subsequent reaction of the polymer alone with the protein.

The glutaraldehyde zinc-insulin product increased in molecular size when a solution was stored at pH 8 and 4 °C. The cross-linking reaction responsible for this growth could be due either to an intermolecular aldol and dehydration reaction between two aldehyde chains or Schiff's base formation between an aldehyde and an amino group. Large molecules are formed during storage in a slightly acidic media, pH 6·5. Here the reaction is more likely to be formation of a Schiff's base since reaction between the carbonyl group and base is acid-catalysed and an aldol reaction is encouraged by basic media since the mechanism involves carbanion formation (Sykes 1965). An even larger macromolecule formed when the product was stored in the presence of excess diaminodecane at pH 6·5, although little effect was observed at pH 8. The only intermolecular reaction that could rationally occur would be by Schiff's base formation between an aldehyde group on the protein and the amino group of the diaminodecane, both groups on the amine giving further cross-links. This mechanism would be expected to be catalysed in slightly acid media as observed experimentally.

The spectra in Fig. 1 show that cross-linking has little effect on the paramagnetic copper(II) sites present in the native insulin sample containing six copper ions and that the hexamer structure exists in the macromolecule. The relative proportions of the two sites however are changed, site 1 being more prominent in the cross-linked material. In previous work (Evans et al 1979), on freeze dried insulin, the insulin copper(II) site occurring in all samples whether modified, native or wet native was found to have e.p.r. parameters of 16·5 mT for $A_{\parallel}^{Cu}$ and 2·285 for $g_{\parallel}$, values which suggests that there is a core site relatively unperturbed by modification of the outside of the hexamer. This change in relative proportions of the two sites is probably a reflection of the fact that some of the copper ions are more deeply buried within the insulin hexamer and that any reduction to copper(I) (which is undetectable by e.p.r.) will affect the outer copper(II) sites more than the inner ones. In the event that the copper(II) ions at the centre of the hexamer were to be reduced this would result in the disintegration of the hexamer.

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