Mechanism of coupling periodate-oxidized nucleosides to proteins

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This paper describes a mechanism of coupling periodate-oxidized nucleosides to proteins. Each of the dial-dehyde groups of a periodate-oxidized nucleoside is shown to couple to lysine residues on different protein molecules through Schiff bases, thereby cross-linking different protein molecules, forming a polymer. This is in contrast to the previous model in which nucleosides were suggested to couple to proteins through a morpholine structure. The cross-linked structure of the nucleoside-antigen, significantly different when compared to the native protein, may affect the specificity and the efficiency of antibody production.

Nucleoside-antigen   *Periodate oxidation*   *Schiff base*   *Protein cross-linking*   *Polymerization*
Antibody production

1. INTRODUCTION

Protein conjugates of nucleosides and nucleotides are used as immunogens for the induction of nucleic acid reactive antibodies [1–4] and in the analysis of antibody specificity [5–10]. Various methods have been reported for covalently coupling nucleosides, nucleotides and oligonucleotides to protein molecules [11–14]. However, the periodate procedure of Erlanger and Beiser [11] has been the most extensively used [1–11]. In this method a nucleic acid containing free 2'- and 3'-ribose hydroxyl groups can be covalently coupled to a protein carrier by oxidizing the ribose with periodate. Erlanger and Beiser [11] postulated that the 2 dialdehyde groups generated by periodate oxidation of a nucleoside molecule formed a morpholine derivative on the protein by coupling to a single ε-amino group of lysine. They supported their view by analyzing a model compound

prepared by the reaction of periodate-oxidized AMP with methylamine [15]. We report here that each of the 2 aldehyde group of a periodate-oxidized nucleoside link to different lysines through Schiff bases, thereby cross-linking different protein molecules. The cross-linked nucleoside-antigen structure is significantly different from that of a native protein, and may be responsible for the increased efficiency of antibody production by these antigens.

2. MATERIALS AND METHODS

Nucleosides, nucleotides, bovine serum albumin (BSA), thyroglobulin and Sephadex G-200 were from Sigma.

Conjugates of nucleosides with bovine serum albumin were prepared according to Erlanger and Beiser [11]. In this method, the nucleoside was first oxidized with periodate and its dialdehyde product was then coupled to BSA at pH 9.5, as described below. The nucleoside (50 μmol) was added to sodium periodate (100 μmol), dissolved in water (2 ml) and stirred in the dark for 60 min. Excess periodate was destroyed by adding 5 μl distilled

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ethylene glycol. After about 5 min, the reaction mixture was added dropwise to a solution of BSA (50 mg in 2 ml distilled water) adjusted to a pH between 9.0 and 9.5 with K_2CO_3, under constant stirring. After 60 min at room temperature, 2.0 ml of freshly dissolved sodium borohydride (1.25 mmol) were added and the reaction mixture was left in the dark for 12–16 h. Finally, the reaction mixture was diluted to 10 ml with distilled water and dialyzed against tap water and lyophilized. Control BSA was prepared by a similar procedure, except that no nucleoside was added. Nucleoside trialcohols were prepared by reducing the periodate oxidized nucleosides with a 25-fold molar excess of $NaBH_4$ for 2 h.

Nucleotide-BSA conjugates were prepared using carbodiimide as the coupling reagent according to Halloran and Parker [12]. Nitrocellulose filter binding assay was carried out as described [9].

3. RESULTS AND DISCUSSION

If periodate-oxidized nucleosides coupled to the ϵ-amino groups of lysines of BSA through a morpholine derivative, then a single nucleoside molecule would be coupled to a single lysine side-chain. Thus a nucleoside-BSA conjugate would be a unit protein molecule, with the lysines on its surface linked to individual nucleosides. However, if a nucleoside coupled to a lysine through a Schiff base, then a nucleoside dialdehyde could cross-link 2 protein molecules. This could be tested by investigating if the conjugates were still unit protein molecules or had cross-linked structures. For this, the molecular mass of the conjugates were determined by gel filtration and gel electrophoresis.

The Sephadex G-200 gel filtration profiles of adenosine-BSA, AMP-BSA and thyroglobulin are given in fig.1a. The patterns of isopentenyl adenosine-BSA (i⁶A-BSA), IgG and BSA are shown in fig.1b. Most of the adenosine-BSA and i⁶A-BSA conjugates were excluded from the gel indicating that the molecular masses were greater than 600 kDa (the exclusion limit of the gel), compared to the molecular mass of BSa which is 60 kDa. This indicated that the conjugates were in a polymeric form. The nucleoside-BSA conjugates were heterogeneous in size. Control BSA, treated with periodate and borohydride without the addition of nucleoside, gave a profile similar to that of BSA.
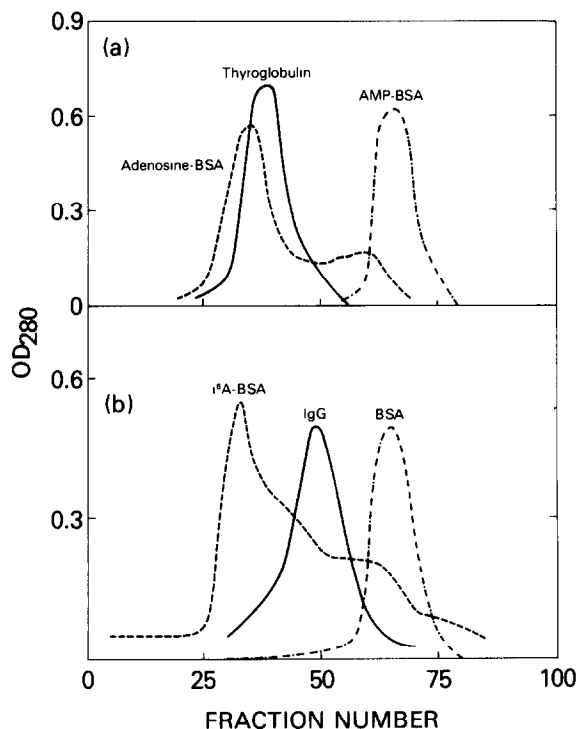

Fig.1. Sephadex G-200 chromatography of nucleoside-BSA conjugates. The proteins and nucleoside-BSA conjugates (20 mg) in 0.4 ml TBS (0.01 M Tris, pH 7.5, 0.14 M NaCl) were separately filtered through a Sephadex G-200 column (2 × 50 cm) equilibrated with TBS. Reaction mixtures (1.2 ml) were collected and monitored for UV absorption at 280 nm. Profiles of absorption at 280 nm are given for (a) adenosine-BSA, thyroglobulin and AMP-BSA, and (b) i⁶A-BSA, IgG and BSA.

Thyroglobulin and IgG were used as size markers.

Carbodiimide is widely used as a reagent for coupling nucleotides to proteins. This reagent is supposed to couple the phosphate group of a nucleotide to the ϵ-amino group of lysines of a protein through a phosphoramidate bond and is not expected to cross-link protein molecules. To test this, AMP-BSA, prepared using carbodiimide as the coupling reagent, was also chromatograped on Sephadex G-200. It gave a profile identical to that of BSA, indicating that no cross-linking of protein molecules had occurred in this reaction.

The molecular masses of the conjugates of adenosine, uridine, cytidine and guanosine with BSA were further analyzed by SDS-polyacrylamide gel electrophoresis (fig.2a). A smear of protein was
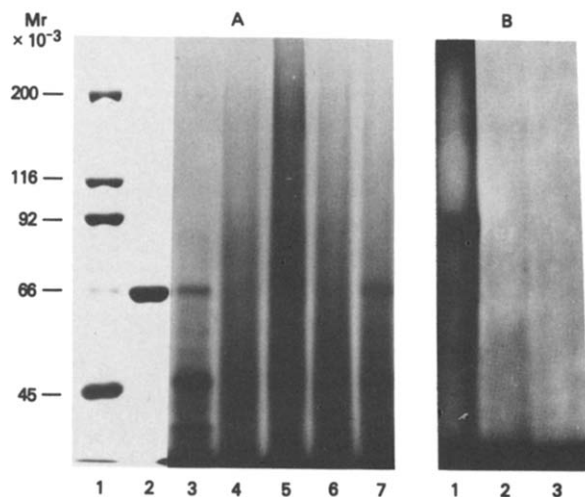
Fig.2. 7.5% SDS-polyacrylamide gel electrophoresis of nucleoside conjugates of BSA. (A) Nucleoside conjugates of·BSA, mock-treated BSA and untreated BSA were subjected to SDS-polyacrylamide gel electrophoresis, stained with Coomassie blue and destained. Lane 1, marker proteins, myosin (M_r 200 000), β-galactosidase (M_r 116 250), phosphorylase b (M_r 92 500), BSA (M_r 66 200) and ovalbumin (M_r 45 000); lane 2, BSA; lane 3, mock-treated BSA; lane 4, adenosine-BSA; lane 5, uridine-BSA; lane 6, cytidine-BSA; lane 7, guanosine-BSA. (B) The uridine-BSA conjugate was prepared as described in section 2, except that the conjugation reaction was carried out with serially diluted uridine dialdehyde. Polyacrylamide gel electrophoresis was carried out as for A. Lane 1, 10^{-2} M; lane 2, 10^{-3} M; lane 3, 10^{-4} M uridine dialdehyde.

observed ranging in molecular mass from 30 to 300 kDa in the nucleoside conjugates of BSA (lanes 4, 5, 6 and 7, fig.2a), indicating that the conjugates existed in a polymer form of varying molecular mass. In contrast, polymerization was not observed in mock-treated BSA (lane 3, fig.2a). The only possible way by which the BSA was polymerized in the nucleoside conjugates of BSA was by cross-linking different BSA molecules by the periodate-oxidized nucleosides. Protein degradation was seen in nucleoside-BSA conjugates, and in mock-treated BSA, as demonstrated by the protein smear in much smaller molecular mass regions. Discrete bands were observed in the low molecular mass range (less than 45 kDa) when the conjugates were subjected to electrophoresis on an SDS 5–10% gra-

dient polyacrylamide gel (not shown), indicating that the degradation occurred in a non-random manner. The mechanism of breakdown of a·peptide chain by borohydride is not well understood [16]. However, the data indicated that in addition to cross-linking by nucleoside bridges, the structure of the polymer conjugate may also be affected by borohydride reduction of its peptide chains.

That the periodate-oxidized nucleosides caused intermolecular cross-linking of proteins was also demonstrated by the fact that dilution of the periodate-oxidized nucleosides in the conjugation reaction resulted in a reduction of the mean molecular mass of the nucleoside-protein conjugates. Fig.2b shows polyacrylamide gel electrophoresis of BSA cross-linked with serial dilutions of periodate-oxidized uridine. Polymerization was reduced several-fold (lanes 1–3, fig.2b) when the nucleoside was serially diluted in the reaction mixture.

Khym [15] originally reported that the reaction product between periodate-oxidized AMP and methylamine was a morpholine derivative in which only 1 mol of methylamine residue was involved in the reaction with 1 mol of periodate-oxidized AMP. Based on this, Erlanger and Beiser [11] proposed the formation of a morpholine derivative in the conjugates of periodate-oxidized nucleosides with proteins. The present data indicate that the coupling of nucleoside dialdehydes to protein cross-links the protein molecules to a polymer form. It is possible that each dialdehyde group of a periodate-oxidized nucleoside coupled to the ϵ-amino group of a lysine through a Schiff base. Amines are well known to react with aldehydes to form N-alkylaldimines called Schiff bases [17–19], which are easily reduced by the addition of hydrogen across the NC double bond [18–20]. Thus the 2 dialdehyde groups of a nucleoside can cross-link 2 different proteins through Schiff bases. Because several molecules of a nucleoside couple to a protein molecule, the cross-linking process can lead to a huge nucleoside-protein polymer.

In addition to the cross-linking reaction through Schiff bases, the formation of morpholine derivatives of the nucleosides on the protein molecules cannot be excluded. Thus several possible reactions could take place between the periodate-oxidized nucleosides and the protein molecules such as (see fig.3): (i) the 2 aldehyde groups of a
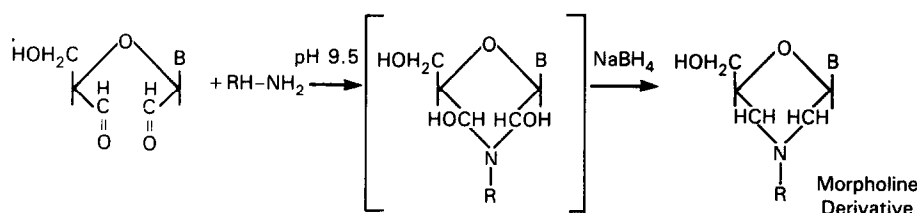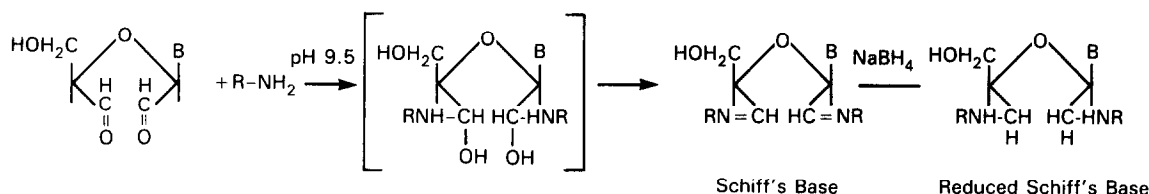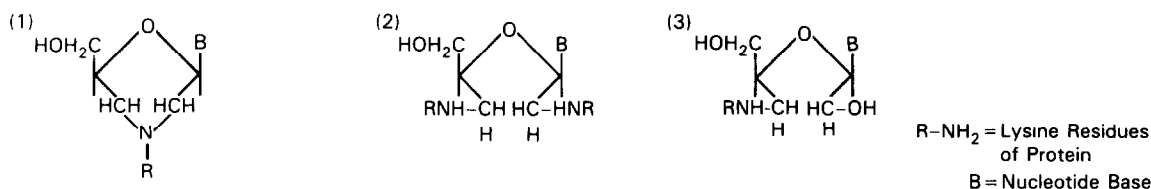
(A) MORPHOLINE DERIVATIVE FORMATION

(B) SCHIFF'S BASE FORMATION

(C) POSSIBLE STRUCTURES ON THE NUCLEOSIDE-PROTEIN CONJUGATE

Fig.3. The 2 possible reactions occurring during the conjugation of periodate-oxidized nucleosides to proteins are shown schematically. The 2 different reactions can couple a nucleoside dialdehyde respectively (A) to a single lysine forming a morpholine structure and (B) to 2 different lysines through Schiff bases cross-linking different proteins. (C) The 3 different possible structures of a nucleoside on the nucleoside-BSA conjugate are shown schematically.

periodate-oxidized nucleoside react with one lysine side-chain forming a morpholine derivative; (ii) the 2 aldehyde groups couple with 2 different lysine side-chains by Schiff base formation, causing intra- and intermolecular linkages in proteins; and (iii) one of the aldehyde groups of a dialdehyde couples with a lysine side-chain, and the other is reduced to an alcohol by borohydride.

The fact that the structure of the nucleoside-BSA conjugate is a large polymer may have bearing on the efficiency and specificity of antibody production by this type of antigen. The antibodies elicited against a nucleoside-BSA conjugate had 1000-fold more affinity to the nucleoside trialcohol than to the unmodified nucleoside, as assayed by nitrocellulose filter binding [21]. The reason for this was not clear earlier. The present data indicated that the nucleoside molecules on the surface of the conjugates contained a free alcohol group either at the 2'- or at the 3'-position of ribose, and this form of the nucleoside could have served as an antigenic determinant. The antigens prepared by the periodate method are generally more efficient immunogens than those prepared by the carbodiimide method (Jacob et al., unpublished). This may be because the structure of the cross-linked protein was drastically different from that of the native BSA molecule, whereas the structure of antigens prepared by using carbodiimide was not significantly different from that of a native protein. The drastically altered antigen structure may contribute to the efficient elecitation of antibodies.

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