STOICHIOMETRIC ENZYME–PEPTIDE CONJUGATION OF AN AFFINITY MATRIX

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1. Introduction

The design and development of sensitive competitive enzyme immunoassays for peptides depends upon the availability of techniques for the synthesis of stable and stoichiometric enzyme–peptide conjugates. Since most peptides of biological interest are available pure only in minute quantities it is also important that the conjugation procedure of choice involve minimal loss of the peptide or permit maximal recovery of chemically unaltered peptide.

The majority of enzyme immunoassays for peptide hormones so far reported have been designed for insulin [1–5]. The stoichiometry of the conjugates used in these assays has never been reported and a general, reproducible and efficient method for the synthesis of suitable enzyme–peptide conjugates is lacking; as a result no enzyme immunoassays are as yet available for the majority of biologically important peptides.

The reversible immobilisation of a protein at a critically low density (below which intermolecular conjugation of the immobilised protein cannot occur) on a suitable affinity matrix greatly facilitated the formation of protein–protein monoconjugates between two enzymes [6] and between an enzyme and an antibody [7]. This matrix principle has been extended to the conjugation of a radiolabelled peptide to an enzyme, and the stoichiometry of the resultant conjugate was analysed.

2. Materials and methods

Invertase (EC 3.2.1.26) was a product of Boehringer Mannheim, porcine insulin was from Novo, carrier-free $Na^{125}I$ and insulin binding reagent (guinea pig anti-insulin complexed with anti-guinea pig gamma globulin) were from the Radiochemical Centre, Amersham; glutaraldehyde, crystalline bovine serum albumin, glucose oxidase, peroxidase and *o*-dianisidine were obtained from Sigma and sucrose was from Pfanstiehl.

Protein was estimated by the Lowry method [8] using crystalline bovine serum albumin as standard. Invertase was assayed by the one step method [9]. I^{125} radioactivity was assayed on an ECIL well-type gammacounter with a counting efficiency of 66% for this isotope. Plastic tubes and siliconised glassware were used throughout.

Porcine insulin (1 mg) was labelled with 2 mCi $Na^{125}I$ using the chloramine T method as in [10]. The labelled peptide was separated on Sephadex G-50 (1 × 18 cm) equilibrated with 50 mM Hepes (pH 7.5) containing 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$. The specific activity of the labelled peptide (total vol. 4.01 ml) was 1.24 mCi/mg. A 10 μl aliquot was diluted to 2.5 ml in 50 mM Hepes (pH 7.5) with 2% w/v of bovine serum albumin.

Succinyl Con A–Sepharose was prepared as in [6] and equilibrated with 50 mM Hepes (pH 7.5) containing 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$. All operations outlined below were at 4°C. Succinyl Con A-Sepharose 4 ml (packed vol.) was mixed with 4 ml of the above buffer and 2 ml of a 0.25 mg/ml (0.125 mg protein/ml) solution of invertase were added dropwise and the mixture was stirred overnight. The gel was packed into a column and 4 ml of a 1.5% (v/v) solution of glutaraldehyde in the above buffer were passed into the column which was closed for 18 h. The column was washed with 5 bed vol. of

the above buffer and 4 ml ^{125}I-labelled insulin (1 mg) were passed into the column. The column was closed for a further 18 h then washed with 50 mM Hepes (pH 7.5) with 5 mM ethanolamine, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$ until negligible radioactivity was detected in the washings. The column was brought to room temperature and eluted with 300 mM α-methyl mannoside in 50 mM Hepes (pH 7.5) with 5 mM ethanolamine and 150 mM NaCl. Radioactivity was monitored and peak fractions (total vol. 8 ml) were pooled. The conjugate (10 μl) was diluted to 2.5 ml in 50 mM Hepes (pH 7.5) containing 2% w/v bovine serum albumin, and assayed for invertase and for ^{125}I radioactivity. The pooled conjugate (4 ml) was concentrated using Aquacide III (Calbiochem), dialysed exhaustively against 50 mM phosphate (pH 7.5) and assayed for protein.

Insulin binding reagent (100 μl) was added to separate tubes set up in duplicate containing:

(i) Conjugate (50 μl) + 50 μl Hepes (pH 7.5) with 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mM $MnCl_2$;
(ii) Conjugate (100 μl);
(iii) Dilute I^{125} labelled insulin (100 μl).

The tubes were incubated at 4°C for 4 h, centrifuged at 4000 × *g* for 25 min and decanted. The ^{125}I radioactivity in the precipitates and supernatants of each tube and the invertase activity in the supernatants of (i) and (ii) were checked.

3. Results and discussion

1. As can be seen from table 1, insulin and invertase are present in the conjugate in approximately equimolar amounts. The results of the precipitation experiments (table 2) demonstrate similar proportions of radioactivity and catalytic activity in the precipitates and corresponding supernatants, indicating that no uncoupled enzyme is present in the conjugate.

We suggested that the large molecular sizes of urease [6] and IgG [7] made it sterically impossible for more than one molecule of these proteins to be coupled to a single molecule of immobilised invertase. Invertase is a glycoprotein containing 50% by wt of carbohydrate and has 60 lysine residues [11]. On reacting a large (~100-fold) molar excess of ^{125}I-labelled insulin with immobilised invertase we theo-

Table 1
Stoichiometry of conjugate

	Invertase (1 mg weighed)	Insulin (1 mg weighed)	Conjugate (Total pooled)
Enzyme activity (Katals)	2.5×10^{-5}	–	0.8×10^{-5}
Protein content (Lowry with albumin standard)	582 μg	–	178 μg
^{125}I radioactivity (cpm)	–	2725×10^6	21.04×10^6
Molecular weight	270 000 (glycoprotein containing 50% carbohydrate)	5700	
	135 000 (protein moiety)		
nmol insulin	–	175.44	1.35
nmol invertase (On basis of weighed glycoprotein)	3.70	–	1.19
nmol invertase (on basis of protein moiety)	4.31	–	1.32

Table 2
Precipitation of conjugate and ^{125}I-labelled insulin by insulin binding reagent

	Conjugate (50 μl) + buffer (50 μl)	Conjugate (100 μl)	^{125}I-labelled insulin (100 μl)
Radioactivity in precipitate	43.1 × 10^3 cpm	93.0 × 10^3 cpm	41.66 × 10^3 cpm
Radioactivity in supernatant	96.3 × 10^3 cpm	164.0 × 10^3 cpm	88.61 × 10^3 cpm
% Radioactivity precipitated	31.1%	36.2%	31.5%
Enzyme activity total	0.5 × 10^{-7} katals	1.0 × 10^{-7} katals	–
Enzyme activity supernatant	0.36 × 10^{-7} katals	0.62 × 10^{-7} katals	–
% Enzyme activity precipitated	28%	38%	–

retically expected a conjugate of defined stoichiometry but containing at least a few peptide molecules coupled to every enzyme residue. It is possible that the use of a greater molar excess of insulin may result in a different stoichiometry. It is also theoretically possible that when this glycoprotein enzyme is immobilised on succinyl Con A–Sepharose only a single 'cluster' of lysine residues is sterically available for conjugation. This possibility deserves investigation.

2. The peptide radioactivity (98%) was recovered in the first 2 bed vol. during washing. In radioimmunoassay systems very minute quantities of peptide may be quantitatively iodinated. Previous enzyme–peptide conjugation systems have been in comparison considerably wasteful of peptide [1–5]. In this matrix technique the peptide is not directly exposed to free coupling reagent and in direct contrast to other methods uncoupled peptide may be almost quantitatively recovered in a chemically unmodified form. It is possible that by recirculating a small amount of peptide through an immobilised invertase column, much smaller initial concentrations of peptide may be utilised with similar results.
3. One of the primary limitations of enzyme-linked assays even for large macromolecular antigens was the absence of a technique for the reproducible stoichiometric synthesis of enzyme–antigen and enzyme–antibody conjugates. This limitation can largely be overcome by the use of matrix conjugation techniques [6,7]. The proven stability of glutaraldehyde conjugates [12] particularly so in comparison with reagents which couple proteins through sulphydryl groups [13] makes the use of this bifunctional dialdehyde a preferable alternative.

It has been suggested that enzyme immunoassays for peptides are unlikely to replace radiolabelled methods basically because the peptide moieties in enzyme–peptide conjugates are likely to be sterically relatively inaccessible for combining with antibody [14]. The precipitation results outlined in table 2 indicate that the enzyme-attached insulin is sterically available and antigenically as reactive as 'free' radioactive insulin.

The use of matrix synthesised enzyme–peptide conjugates in enzyme-linked immunoassay is in progress and will be reported separately.

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