Protein-peptide conjugation by a two-phase reaction

The protein-protein conjugation method of Carlsson *et al.* (1978) has been extensively used for, among other things, the linking of catalytic toxins to recognition proteins in the construction of selective cytotoxic agents (Bourdon *et al.*, 1984). We are presently attempting to synthesize a selective cytotoxic agent for a subclass of pituitary cells that produce gonadotropic hormones. In the course of this work we made an observation which expands the possible applications of the method of Carlsson *et al.* (1978).

The hypothalamus secretes a decapeptide, LH-RH, which, through a membrane-receptor-mediated reaction, stimulates the synthesis and secretion of luteinizing hormone and follicle-stimulating hormone from a subclass of pituitary cells (Ory, 1983). We are chemically linking LH-RH to cytotoxic agents in an effort to produce a cytotoxic agent for those pituitary cells that produce gonadotropins. This may constitute a new approach to the regulation of fertility in a variety of animals. In one series of experiments, we used the method of Carlsson *et al.* (1978) to perform the conjugation to cytotoxic proteins and, consequently, employed a lysine-containing analogue of LH-RH (Seprodi *et al.*, 1978) to provide an amino group for the linking reaction.

The protein-protein conjugation method of Carlsson *et al.* (1978) uses the heterobifunctional reagent SPDP in a two-step reaction sequence. PDP derivatives of protein are first formed by a reaction between protein amino groups and the succinimidyl residue of the reagent. The linking reaction occurs between the pyridyl disulphide portion of PDP-derivatized protein and native, or introduced, thiol groups of a second protein. It was noted in the description of the original method that extensively PDP-derivatized proteins often precipitated. The precipitation was explained either by a change in conformation due to derivatizing an extensive number of protein free amino groups or by the introduction of hydrophobic PDP groups (Carlsson *et al.*, 1978).

We found that, upon allowing the analogue of LH-RH ([D-Lys⁶], des-Gly¹⁰-LH-RH ethylamide;

Seprodi *et al.*, 1978) to react with SPDP, the PDP-derivatized decapeptide precipitated quantitatively. The derivatized peptide redissolved upon exposure to aqueous solutions of mercaptoethanol. Since removal of the pyridyl residue allowed the derivatized peptide to redissolve, the increased hydrophobicity of the PDP-peptide was apparently the sole cause of the precipitation. The PDP-peptide, when dispersed in aqueous solution by sonic oscillation, would react quite well with protein in solution to form peptide-protein conjugates. For example, in 60 min at room temperature (approx. 22°C) with a 4-fold molar excess of PDP-peptide, 90% of diphtheria toxin A chains (intrinsic thiol group) were conjugated with peptide as measured by absorbance at 343 nm (pyridine-2-thione absorbance). Also, under similar conditions, 85% of PDP-pokeweed antiviral protein chains (chemically introduced thiol group) were conjugated with peptide. The reactions were confirmed by purifying the peptide-protein conjugates by molecular sieve chromatography and demonstrating (by induced secretion of luteinizing hormone in sheep) that the conjugate was bioactive.

The fact that the two-phase reactions occur so readily allows the Carlsson *et al.* (1978) conjugation procedure to be used with small peptides, most of which we would expect to precipitate upon reaction with SPDP for the same reason that the analogue of LH-RH precipitated. Further, the two-phase reaction conditions make separation of products from reactants much less laborious, for both types of reactions, because simple centrifugation removes the unreacted material from the reaction mixture.

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Abbreviations used: LH-RH, luteinizing hormone-releasing hormone (luliberin); SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; PDP, pyridyl disulphide propionate.