An approach to the targeted attachment of peptides and proteins to solid supports

(corticotropin/avidin-biotin complex/biotinyl peptides/biotinyl proteins/hormone receptors)

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ABSTRACT   A novel approach to affinity columns is described that is based on the high avidity of biotinylated molecules for avidin attached to solid supports. Biocytin amide [N^ϵ-(+)-biotinyllysine amide] was coupled to the COOH-terminal carboxyl group of corticotropin(1–24) [ACTH(1–24)] to form [biocytin^{25}]ACTH(1–25) amide. The ability of this peptide to stimulate steroidogenesis of bovine adrenocortical cells was within experimental error identical to that of ACTH(1–24). The peptide also binds to avidin and avidin-Sepharose, forming stable complexes. Thus, with biotin as the anchor, the adrenocorticotropically active segment of the ACTH molecule was attached to a solid support in a targeted manner. The general applicability of this principle for the attachment of peptides and proteins to solid supports is discussed.

Affinity chromatography has become the method of choice for the isolation of biologically active molecules, and a great variety of techniques for the preparation of affinity columns is available (for a series of reviews, see ref. 1). In connection with investigations on the chemical nature of corticotropin(adrenocorticotropic hormone, ACTH) receptors, we required an affinity column in which ACTH(1–24), a biologically fully active fragment of the natural hormone, is covalently attached to a solid support. In order to exhibit maximal receptor affinity it appeared crucial that the linkage to the support be through the carboxyl-terminal end of ACTH(1–24), since this portion of the molecule is not essential for hormonal stimulation of the adrenal cortex.

Avidin, a constituent of egg white, exhibits high affinity for the B-complex vitamin biotin, and the resulting avidin–biotin complex is unusually stable ($K_D \sim 10^{-15}$). Avidin has been attached to solid supports without losing its avidity for biotin (for a review, see ref. 2).

It occurred to us that the covalent attachment of biotin to the COOH-terminus of ACTH(1–24) should generate a bivalent molecule that may be useful for studies of ACTH receptors. Such a substance can be expected to bind to avidin attached to Sepharose particles, thus coating their surface with accessible unmodified ACTH(1–24) molecules.

Various biotin enzymes are known (for a review, see ref. 3), and the high affinity of these proteins for avidin-Sepharose columns is well documented. In these molecules the biotin is covalently attached to the ϵ-amino group of lysine residues. These findings indicated that [biocytin^{25}]ACTH(1–25) amide (I) (Fig. 1) should be a bivalent molecule, exhibiting affinity both for ACTH receptors and for Sepharose-bound avidin. [Biocytin is N^ϵ-(+)-biotinyllysine.] This communication reports the synthesis of this compound and some of its properties.

Abbreviations: ACTH, corticotropin(adrenocorticotropic hormone); ACTH(1–24), the numbers in parentheses refer to the portion of natural ACTH to which the peptide analog sequence corresponds; biocytin, N^ϵ-(+)-biotinyllysine; Boc, tertiary butoxycarbonyl; OBu^t, tertiary butyl ester.

EXPERIMENTAL

Materials. N^α-Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH (VI), H-lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OBu^t, and ACTH(1–24) (Synacthen) were generously supplied by W. Rittel of Ciba-Geigy Corp., Basel, Switzerland. (Boc is tertiary butoxycarbonyl; OBu^t is tertiary butyl ester.) The (+)-biotin was obtained from W. E. Scott of Hoffmann-La Roche, Inc., Nutley, N.J. Avidin was purchased from Worthington Biochemical Corp., Freehold, N.J., and 4-hydroxyazobenzene-2'-carboxylic acid from Eastman Kodak. Sepharose 4B was supplied by Pharmacia Fine Chemicals.

Peptide Syntheses. The amino acids were of the L-variety except for glycine. The systems used for thin-layer chromatography were $R_f{}^1$ n-butanol, acetic acid, water (3:1:1) and $R_f{}^2$ n-butanol, acetic acid, water, pyridine (30:6:24:20). The key steps in the synthesis of [biocytin^{25}]ACTH(1–25) amide (I) are illustrated on Fig. 2. The partially protected peptide H-Lys-(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys-(Boc)-Val-Tyr-Pro-OBu^t was converted to (III). This compound was coupled to biocytin amide (II) [acetate; analysis: calculated: C, 50.1; H, 7.7; N, 16.2; S, 7.4; found: C, 50.3; H, 7.6; N, 16.5; S, 7.7 melting point 141–143°; $[\alpha]_D{}^{25}$ = +55.9° (C 0.37 in MeOH); $R_f{}^1$ = 0.2] to give (IV) [amino acid ratios in 24-hr acid hydrolysate, Lys$_{5.1}$Arg$_{2.0}$Pro$_{3.1}$Gly$_{0.9}$Val$_{3.0}$Tyr$_{0.9}$; average recovery of amino acids, 85%; $[\alpha]_D{}^{26}$ = −29.4° (C 0.68, in MeOH); $R_f{}^1$ = 0.4; $R_f{}^2$ = 0.7]. The trifluoroacetyl group was removed with aqueous piperidine (4). The ensuing peptide (V) was acylated with (VI) to afford crude (VII), which was purified by chromatography on carboxymethylcellulose (5) (amino acid ratios in 24-hr acid hydrolysate, Lys$_{4.9}$His$_{0.9}$NH$_{3\ 1.4}$Arg$_{2.9}$Ser$_{1.8}$Glu$_{1.1}$Pro$_{3.0}$Gly$_{2.1}$Val$_{3.3}$Met$_{1.0}$Tyr$_{2.1}$Phe$_{1.0}$; average recovery of amino acids, 88%; $R_f{}^1$ = 0.3; $R_f{}^2$ = 0.7). For conversion to (I), this material was deprotected with trifluoroacetic acid. The ensuing trifluoroacetate salt was converted to the acetate and this was reduced with thioglycolic acid (6) [amino acid ratios in 24-hr acid hydrolysate, Lys$_{5.1}$His$_{0.9}$NH$_{3\ 0.9}$Arg$_{2.9}$Ser$_{1.8}$Glu$_{1.0}$Pro$_{3.2}$Gly$_{2.1}$Val$_{3.3}$Met$_{0.9}$Tyr$_{1.9}$Phe$_{1.0}$; average recovery of amino acids, 86%; biotin content, 95% (dye assay) and 90% (microbiological assay); peptide weight corrected for recovery by amino acid analysis; $[\alpha]_D{}^{25}$ = −67.5° (C 0.44, in 1% acetic acid); $R_f{}^2$ = 0.4]. Details of this synthesis will be published elsewhere.

Biological Assays and Avidin-Biotin Binding Measurements. The steroidogenic activity of (I) was determined with bovine adrenocortical cells (7). Biotin was determined spectrophotometrically (8) and by microbiological assay using Lactobacillus plantarum ATCC 8014 as the test organism (9). Samples of [biocytin^{25}]ACTH(1–25) amide were autoclaved for 2 hr at 110° in 3 M sulfuric acid for liberation of biotin. Avidin-Sepharose 4B was prepared and assayed according to
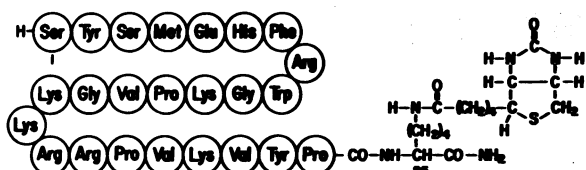
FIG. 1.   Structure of [biocytin[25]]ACTH(1–25) amide (I).

Bodanszky and Bodanszky (10). An avidin-Sepharose 4B column saturated with 4-hydroxyazobenzene-2'-carboxylic acid on titration with (+)-biotin had a binding capacity of 0.024 μmol/ml of settled resin in 0.1 M sodium acetate buffer pH 4.0 at room temperature. Under identical conditions the resin bound 0.074 μmol of [biocytin[25]]ACTH(1–25) amide. In the presence of 0.188 μmol of ACTH(1–24) to reduce nonspecific adsorption, the binding capacity of the resin was 0.032 μmol of [biocytin[25]]ACTH(1–25) amide. ACTH(1–24) (0.09 μmol/ml of resin) did not displace the dye from the avidin-Sepharose.

RESULTS AND DISCUSSION

Many of the presently available procedures for attaching peptide hormones to solid supports are not targeted and result in the formation of ill-defined, inhomogeneous complexes in which the positions of the covalent bonds between peptide and support are not established. Although such preparations have been used successfully in isolation work, it is desirable to have available more selective, targeted procedures for attaching peptide hormones to solid supports for receptor studies. Affinity columns suitable for this purpose should satisfy the following criteria: (*i*) they should exhibit maximal binding capacity for

the receptor, i.e., the hormone should be attached to the support via a single site that is not involved in its biological function; (*ii*) the synthetic route used for anchoring the hormone to the support should be unequivocal and adaptable to scaling up; and (*iii*) the ultimate step involved in attaching the hormone to the support should proceed in high yield. The route to an affinity column described in this communication satisfies these criteria.

The uniquely specific binding between a peptide hormone and its receptor depends on interactions between two molecules at multiple sites, and it is this complementarity that distinguishes the specific affinity of the hormone for the receptors from nonspecific binding. Reactions that result in a family of hormone-support complexes are undesirable since each species in such a mixture can be expected to possess a different binding affinity for the receptor, thus decreasing the selectivity of the adsorption-desorption process.

A great deal of information is available (for a review, see ref. 11) regarding those structural features of the ACTH molecule that are essential for adrenocorticotropic activity, and it has been well established that ACTH(1–24) exhibits the full spectrum of biological activities of the natural hormone. It is also known that biological activity and binding affinity for adrenocortical membranes are lost when the ϵ-amino groups of the lysine residues 11, 15, and 16 in ACTH(1–23) are protected by formyl groups (12). Thus, any involvement of these groups in binding to a support could be expected to lower or destroy receptor affinity. In general it has been shown that modifications in the NH$_2$-terminal region of ACTH lower or eliminate biological activity, whereas modifications in the COOH-terminal region are compatible with hormonal function.
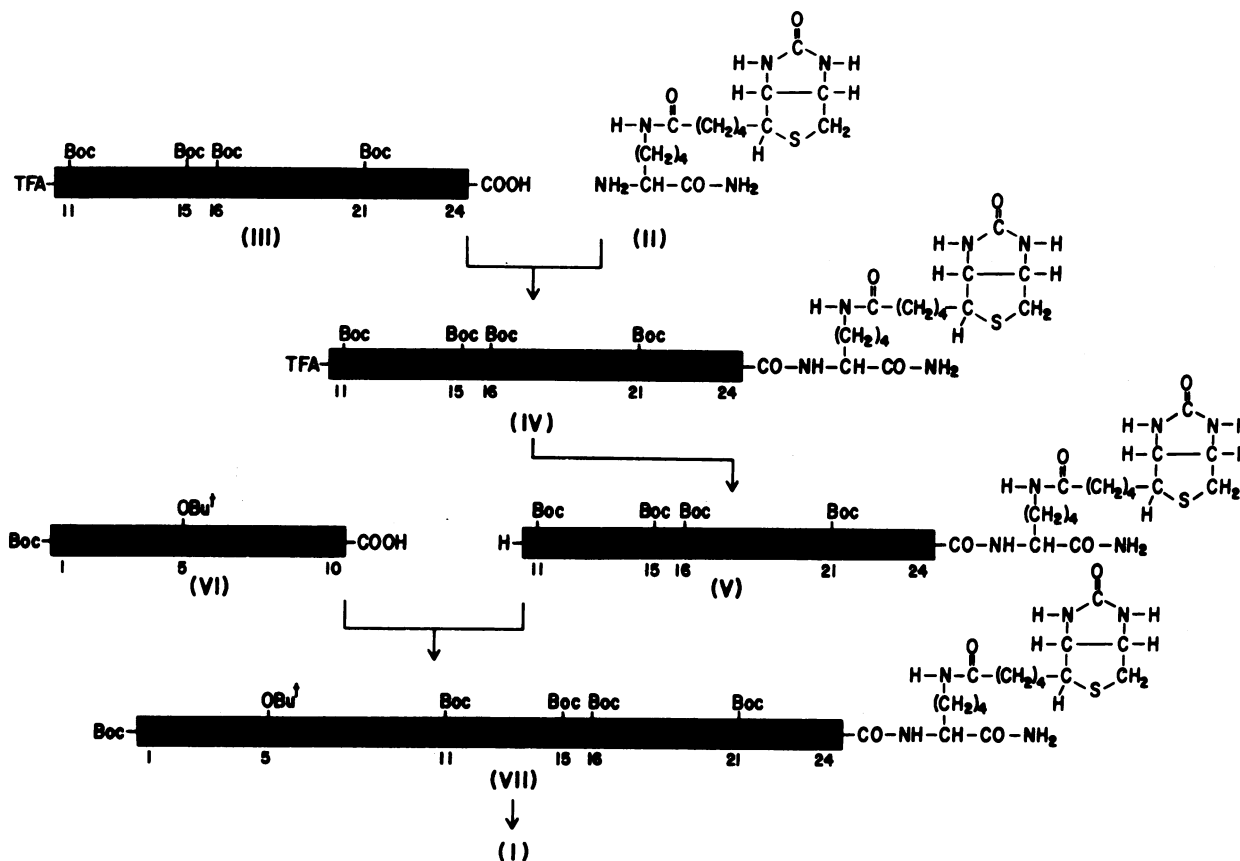

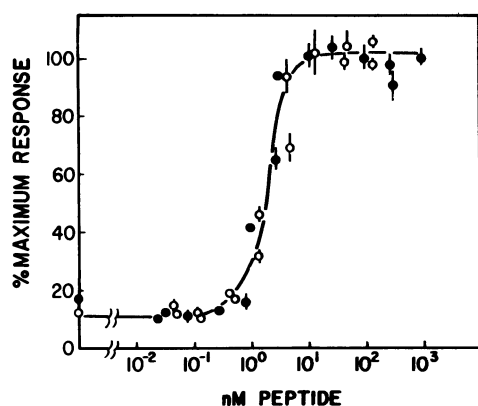FIG. 2.   Synthetic route to [biocytin[25]]ACTH(1–25) amide. TFA, trifluoroacetyl.

FIG. 3. Stimulation of steroidogenesis in bovine adrenocortical cells by ACTH(1–24) (O) and [biocytin[25]]ACTH(1–25) amide (●). Stimulation was 5- to 10-fold above baseline. Vertical bars indicate standard deviation.

ACTH has been attached to solid supports, and the resulting complexes exhibited some steroidogenic activity. Thus, Schimmer *et al.* (13) reacted ACTH with diazotized *p*-aminobenzoylcellulose and found that the ensuing complex or complexes stimulated steroidogenesis of ACTH-sensitive mouse adrenal tumor cells in tissue culture. Selinger and Civen (14) attached porcine ACTH to diazotized 3-[3′-(4-aminobenzamido)-propylamino]-propylamine-Agarose and observed that the ensuing complex stimulated steroid production in isolated rat adrenal cells. Histidine[6,] tyrosine[2], and tyrosine[23] are likely sites where interaction between the diazotized support and the hormone would occur. The observation that these ACTH complexes elicited steroidogenesis was taken as an indication that the hormone acts on the surface of the cell and does not penetrate into the interior.

With the aim of developing an affinity column for ACTH receptor isolation, Fauchère and Pelican (15) attached cysteine to the NH_2-terminus of ACTH(5–24) and coupled this peptide via the sulfhydryl group to polyacrylamide and Sepharose beads. This targeted approach leaves no doubt regarding the bond linking the peptide to the support but has the disadvantage that a section of the ACTH molecule exhibiting low biological activity (16) was used.

The realization that the attachment of a solid support to the NH_2-terminal region of ACTH(1–24) is likely to interfere with hormonal activity, and presumably with receptor binding, prompted us to selectively attach (+)-biocytin amide (II) to the COOH-terminal carboxyl group of this peptide. The expectation that this modification of ACTH(1–24) would not affect adrenocorticotropic potency, and hence affinity for the cell receptors, was verified experimentally. The ability of peptide (I) to stimulate steroidogenesis in bovine adrenocortical cells (Fig. 3) was, within experimental error, identical to that of ACTH(1–24).

By the dye binding assay (8) it was determined that peptide (I) binds to avidin and avidin-Sepharose 4B, forming stable

complexes. Based on these results we conclude that [biocytin[25]]ACTH(1–25) amide is indeed a bifunctional molecule that shows affinity both for ACTH receptors on adrenocortical cells (receptor affinity) and for avidin (avidin affinity).

Attempts to use avidin-Sepharose columns for the affinity chromatographic separation of biotinyl enzymes have been only partially successful (3) because these enzymes bind to avidin columns with great avidity and frequently can only be desorbed by the use of denaturing reagents. The high affinity for avidin columns of [biotinyl[25]]ACTH(1–25) amide (I), and most likely other biotinyl peptides and proteins, is a highly desirable property as concerns preparation of affinity columns.

The application of the biotin-avidin affinity for the anchoring of molecules to solid supports, an example of which has been presented in this study, is by no means restricted to ACTH(1–24), but can be applied to other peptides as well as to artificially created biotinyl proteins. Such biotinyl proteins should be readily accessible via acylation of proteins under mild conditions with carboxyl-activated biotin derivatives.

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