SYNTHESIS OF AN AFFINITY CHROMATOGRAPHY COLUMN DESIGNED FOR RECOVERY OF LABILE PROTEINS

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SUMMARY: An affinity chromatography column has been synthesized in which 11α-hydroxyprogesterone hemisuccinate is covalently bound to the stationary matrix through a disulfide linkage. This column was designed to explore the feasibility of recovering proteins which are not stable under conventional recovery conditions and which are stabilized by β-mercaptoethanol. 20β-Hydroxysteroid dehydrogenase (E.C.1.1.1.53) was adsorbed on a column of 11α-hydroxyprogesterone 11-hemisuccinyl-bis-β-aminoethyl disulfide-agarose in the presence of NAD^+. After washing the column free of non-specific protein, the enzyme was recovered simply by inclusion of β-mercaptoethanol in the buffer.

INTRODUCTION: Isolation of intact estrogen and progesterone receptor proteins from target tissue of mammalian or avian sources presents problems of instability when the protein is in crude form. Earlier reports indicated that the presence of thioglycerol in the buffer used for preparation of the crude cytosol containing chick oviduct progesterone receptor (1), significantly stabilizes the relatively labile protein. Similarly, calf uterine estrogen receptor is stabilized by β-mercaptoethanol (2,3) or dithiothreitol (4). Therefore, it seemed desirable to design an affinity chromatography column on which such proteins can be specifically adsorbed under optimum conditions of pH, ionic strength and temperature. Then following the elution of nonspecific pro-

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teins from the column, and under the same conditions, addition of a thiol to the same buffer, would release the protein from the column and also stabilize it. Affinity columns in which the specific ligand is bound to the stationary matrix through a disulfide linkage are expected to possess these features. This report describes the synthesis and application of a progesterone affinity column in which the steroid is bound to the stationary matrix (Sepharose 4B) via a disulfide linkage.

MATERIALS AND METHODS

11α-Hydroxyprogesterone 11-hemisuccinyl-bis-β-aminoethanethiol disulfide monoamide. To a solution of 698mg (3.1mmoles) of bis-β-aminoethyl disulfide dihydrochloride, synthesized from β-aminoethanethiol hydrochloride (Aldrich Chemical Co.) according to the method of Barnet (5), and 133mg (0.31mmole) of 11α-hydroxyprogesterone 11-hemisuccinate (Steraloids Co.) in 25ml of 50% (v/v) dioxane-water at 25°, was added with stirring 700mg (3.1mmole) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (Aldrich Chemical Co.). Progess of the reaction was monitored by applying 3-5μl of the reaction mixture on 2.5cm X 7.5cm thin layer chromatographic plates of silica gel containing a fluorescent indicator (Eastman No.6060) developed with chloroform-methanol (9:1), or n-butanol-acetic acid-water (12:3:5). The dried plates were first visualized under ultraviolet light (254 nm) to detect steroidal components, then were sprayed with ninhydrin reagent (Sigma Chemical Co.) and heated for 1 min at 110°. The diamine remained at the origin (blue spot) while the steroid hemisuccinate and monoamide product migrated with Rf values of 0.5 and 0.3, respectively (developed with chloroform-methanol). The latter two spots could readily be distinguished, not only by

the fact that as the reaction proceeded one increased intensity at the expense of the other, but the product spot absorbed ultraviolet light and also gave a positive (blue) ninhydrin reaction. After stirring for 2.5 hrs the reaction mixture was diluted with 60ml of η-butanol and sequentially washed with 10ml of saturated aq. NaCl then ten times with 10ml portions of water. This exhaustive washing removed all of the unreacted diamine, carbodiimide reagent and its urea by-product, and 90% pure product was obtained. The yield of monoamide was approximately 55%.

Structure of the monoamide. Thin layer chromatographic analysis of the butanol product solution showed it to be over 90% pure steroidal monoamide. Attempts were made to isolate the product by preparative thick layer (1.0mm) chromatography but during crystallization of the eluted product slow decomposition took place. Approximately 30mg of purified monoamide was treated with 1.0ml of acetic anhydride which rapidly converted the modified steroid to the corresponding N-acetyl derivative (II; Fig.1). Thin layer chromatography of the reaction mixture ($CHCl_3$-CH_3OH) showed a new spot (Rf 0.6), replacing that due to the monoamide, which absorbed ultraviolet light but did not give a positive ninhydrin reaction. Isolation of the acetyl derivative by preparative layer chromatography provided a homogenous oil which defied crystallization. The infrared spectrum of this material was consistent with the structure II (Fig.1); ν_{max}^{KBr} = 3390 cm^{-1} (amide N-H), 1550 cm^{-1}(amide II), 1735 cm^{-1}(C-11 ester), 1700 cm^{-1}(C-20 keto), strong 1680 to 1650 cm^{-1}(C-3 keto/amide I), 1260 cm^{-1} (amide III). The mass spectrum of the oil gave a molecular ion m/e of 607. Incubation of the steroid monoamide (I,Fig.1)(or N-acetyl derivative) with one equivalent of β-mercaptoethanol at 25° resulted in its conversion to four new products, as shown by thin layer

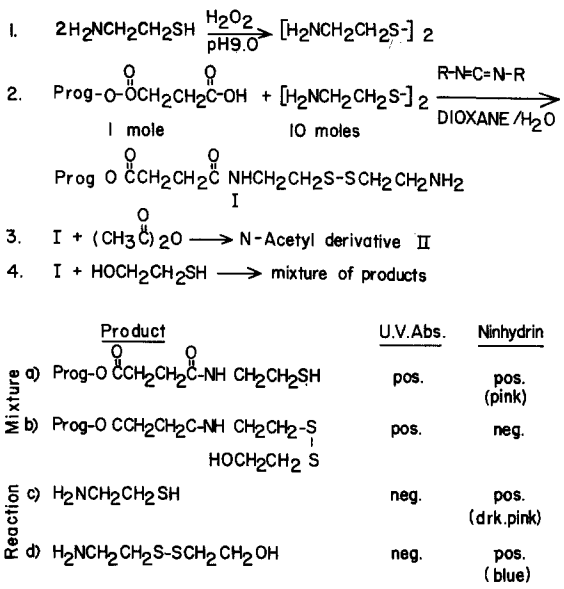

Fig.1. Synthesis and structure of 11α-hydroxyprogesterone 11-hemisuccinyl-bis-β-aminoethyldisulfide monoamide, I. Equation 4 represents products obtained (observed with thin layer chromatography) when I is reacted with β-mercaptoethanol.

chromatography (Fig.1). Two of the new spots adsorbed ultraviolet light and one of these reacted with ninhydrin producing a pink (thiol) spot. The other two spots do not absorb ultraviolet light but do give a ninhydrin reaction. Adjusting the pH to 9.0 and heating produced a mixture containing a single steroid component identified as 11α-hydroxyprogesterone. These reactions represented by equations in Fig.1, support the structure of the monoamide I and also demonstrate that the disulfide linkage is readily cleaved by β-mercaptoethanol.

11α-Hydroxyprogesterone 11-hemisuccinyl-bis-β-aminoethyl disulfide monoamide-agarose. The monoamide was prepared for conjugation with cyanogen bromide activated agarose by adding 40ml of water to 10ml of the above butanol solution of monoamide (containing ca. 20mg of the modified steroid) and concentrating the mixture to 10ml under reduced pressure at 40° (which removed all

of the butanol as a 50% azeotrope). An equal volume of dioxane was added to the aqueous residue and the resulting solution was added to a stirred suspension of 20ml of cyanogen bromide activated Sepharose 4B (4) in 50% (V/V) dioxane-0.05M phosphate buffer pH 7.0 for 18 hrs at 0-4°, then at 25° for 6 hrs. After filtration through a coarse fritted glass Buchner funnel the gel was washed exhaustively, then treated with glycine and washed as recommended by Sica, et al (4). Column bound steroid was determined by heating a measured volume of the gel in an equal volume of 0.1N NaOH, filtering, extracting the filtrate with ether, evaporating the ether extract, redissolving the residue in ethanol then determining hydroxyprogesterone content spectrophotometrically. Alternatively, an aliquot of gel was incubated with β-mercaptoethanol (0.15M) for 1 hr, filtered, and the filtrate was made alkaline (0.1N NaOH) and treated as above. The values for 11α-hydroxyprogesterone content was approximately 2×10^{-2}M (I.e.2×10^{-5} mole/ml of agarose), and values from each method agreed within 80%.

Purification of 20β-hydroxysteroid dehydrogenase (E.C.1.1.1.53)

An affinity column (4.0ml of gel; 8mm X 50mm) was equilibrated at 4° with 0.05M tris buffer pH 7.0 containing 1.4×10^{-3}M NAD^+. The enzyme (Sigma Chemical Co.) was dissolved in the same buffer (2mg in 1.0ml) and applied to the column. Activity was assayed as previously reported (6), and none was detected in the fractionated eluate from the column. After all of the protein was eluted (in the first 10ml) the column was washed with an additional, 20ml of buffer. Then the same buffer, but now containing 0.14M β-mercaptoethanol (β-ME), was incubated with the column (2 hrs at 4°) and the enzyme was eluted with the β-ME buffer.

RESULTS AND DISCUSSION: Initially, we synthesized an affinity column by the conventional method (4) of first reacting our bis-

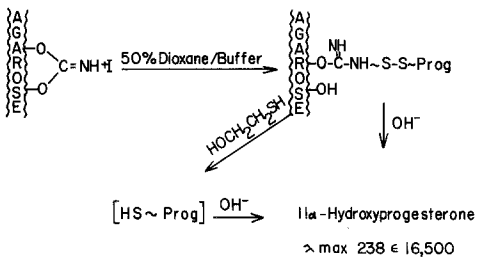

Fig.2. Synthesis and assay of 11α-hydroxyprogesterone 11-hemisuccinyl-bis-β-aminoethyl disulfide monoamide-agarose.

β-aminoethyl disulfide with CNBr activated agarose, then conjugating the steroid hemisuccinate with the resulting aminoalkyl-agarose. But a significant amount of non-specific protein was adsorbed, particularly with uterine progesterone receptor (7), presumably due to free amino groups on the column. To avoid this, the present method of column synthesis was employed in which the steroid monoamide was reacted directly with the activated agarose. The commercial hydroxysteroid dehydrogenase had a specific activity of 2-4 U/mg. After recovery from the column (with 60-75% efficiency) this was increased to 18-19 U/mg (literature value 19-21 U/mg (8)); this purity was confirmed by disc gel electrophoresis. NAD^+ inclusion in the buffer during chromatography was essential since omission of the cofactor resulted in complete loss of enzyme activity during chromatography.

The presently reported results with the enzyme, and subsequent results with sheep uterine progesterone and estrogen receptors (using appropriate columns), and antibody directed against 11α-hydroxyprogesterone 11-hemisuccinyl-BSA (7), have shown that inclusion of a disulfide linkage between the steroid and stationary matrix provides a column from which specifically adsorbed proteins can be readily and stably recovered with β-

mercaptoethanol. The general application of bis-β-aminoethyl disulfide or homologous diamines to synthesis of affinity chromatography columns, where recovery of sensitive proteins with thiols is desirable, seems promising.

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