ACTH DIAZOTIZED TO AGAROSE: EFFECTS ON ISOLATED ADRENAL CELLS

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ACTH coupled in an azo linkage to an agarose support induces steroidogenesis in free adrenal cells in the same manner as does free ACTH. Observation of incubates of adrenal cells and ACTH-agarose indicates that 1) agarose-ACTH is not adherent to the cell surface, 2) entrance of ACTH into the cell may not be a prerequisite to the initiation of steroidogenesis, 3) the continuous presence of ACTH is not necessary to maintain maximal steroid production in cellular incubates, and 4) induction does not alter the functional integrity of the bound corticotropin.

The chemical binding of proteins and peptide hormones to inert support materials such as cellulose (1,2,3), agarose (5,6,7,8,9,10), polyacrylamide (11,12), starch and cross-linked dextran (2) may be effected with the retention of the bioactive characteristics of the free molecules. These macroscopic compounds can be used as instruments to explore the interactions of biologically potent agents with the cell surface. This work is concerned with the nature of the interaction between ACTH-agarose and isolated adrenal cells.

MATERIALS AND METHODS

20 mg ACTH (Armour & Co. 20 IU/mg porcine) is diazotized to 7 ml settled volume of 3-[3-(4-aminobenzamido)-propylamino]-propylamine agarose (derived from 3-(3-aminopropylamino)-propylamine, AF 102 8-9 μM amine/ml; Affitron Corp., 4737 Muscatel Ave., Rosemead, Calif. 91770) at pH 7 (13). After 20 min the gel is washed with 1 liter of 0.1% bovine albumin in 0.1M phosphate buffer pH 7.0 at 4°C, 4 liters of 0.1M phosphate buffer pH 7.6 at 22°C, and 20 ml of 0.1N HCl containing 0.9% NaCl and 0.5% bovine albumin (ACTH vehicle) at 4°C, and is stored as a slurry in 0.1M phosphate buffer

pH 7.6 at 4°C. The final gel color is light orange at pH 7 (pale yellow pH 9, bright red-pink pH 2).

A bioassay of the buffer washes of ACTH-agarose indicates that essentially no freely elutable ACTH remains adsorbed to the gel matrix (Table I). All biological activity exhibited by the gel is attributed to chemically linked ACTH.

TABLE I. Bioassay of buffer washes of ACTH-agarose

ACTH (μU)	Net Corticosterone (μg $\pm$ 10%)
0	0.00
5	0.08
10	0.21
100	0.37
0.1 ml Aliquot	
Reaction mixture (total volume 13 ml) after diazotization	1.24
Filtrate: 1 liter 0.1M phosphate buffer pH 7.0 with 0.1% bovine albumin	0.91
Filtrate: 4 liters 0.1M phosphate buffer pH 7.6	0.03

Weights of gel aliquots are determined as wet weights of filtered slurry. The degree of gel hydration contributes considerable error to the weights given.

The beaded gel has a minimum diameter approximately three times that of the free adrenal cells. It is assumed that gel particles do no enter the cells.

The *in vitro* bioassay for corticosteroidogenic activity consists of the incubation of 0.1 ml of test material with 0.9 ml of a suspension of trypsinized adrenal cells in Krebs Ringer Bicarbonate Glucose at 37°C for 2 hours under 5% CO_2/95% O_2 in a Dubnoff incubator (60 RPM)(14,15). Male Sprague-Dawley rats (250-400g) are used as adrenal sources.

The adrenal cell assay manifests a limitation in that consecutive cell harvests do not exhibit identical steroidogenic potentials: fixed numbers of cells do not reproducibly generate the same quantity of corticosterone on

Figure 3 shows the association constants of various codon-anticodon complexes as functions of the inverse absolute temperature. The most striking result of these experiments is the fact that the binding between complementary tRNA anticodons is about three orders of magnitude stronger than the binding of complementary trinucleotides to tRNA anticodons and that it displays a far smaller temperature dependence.[2] The binding between complementary trinucleotides in aqueous solution is not observable.[10]

In as much as Figure 3 yields good linear relationships one is justified in interpreting these results in terms of the free energy of binding (ΔF) which is given by the changes of enthalpy (ΔH) and entropy (ΔS) upon binding:

$$\Delta F = -RT \ln K = \Delta H - T\Delta S \ . \qquad (1)$$

In this way one obtains the following thermodynamic parameters (in kcal M^{-1} at 10°C) for codon-anticodon binding*

System	ΔF	ΔH	$T\Delta S$
$tRNA^{phe}$:UUC	-3.7	-14.3	-10.6
$tRNA^{phe}$:$tRNA^{glu}$	-7.2	- 5.2	+ 2.0

The errors are between 1 and 2 kcal M^{-1}. It is seen that the free energy of binding is almost doubled when UUC has a suitable conformation imposed on it by the secondary and tertiary structure of $tRNA^{glu}$. This change in ΔF is the result of ΔH decreasing by 9 kcal M^{-1} and $T\Delta S$ increasing by 13 kcal M^{-1}. While a detailed understanding of these changes in enthalpy and entropy cannot be obtained without including the contributions of water of solvation, it is safe to say that the great increase in the entropy term for the $tRNA^{phe}$:$tRNA^{glu}$ system reflects the fact that the two anticodons have well defined complementary conformations. The fact that $T\Delta S$ is positive means that before binding occurred the anticodons were surrounded by well ordered water.

The characteristics of the complex between transfer RNA's with comple-

* The binding of the wobble codon UUU for which data are shown in Fig. 3 is 6 times weaker than that of the codon UUC, but the values of ΔH and $T\Delta S$ (-15 and -12 kcal M^{-1}, respectively) are not very different.[2]

exogenous calcium, as is that of free ACTH.

The agarose-ACTH can be sequestered during cellular incubation by the use of nylon screens (nylon monofilament cloth 400-37; Tobler, Ernst & Traber, Inc. 37 μ mesh opening) which gives the cells access to the agarose beads, but allows removal of the beaded ACTH from the incubate at a given time. 3 mg samples of ACTH-agarose were incubated with identical cell aliquots for 35 min, then two gel samples were separated from their cell incubates, and a third control gel was allowed to remain in place. Incubation was continued for a total incubation time of two hours (Table II). Maintenance of maximal steroid production in 2 hour incubates (as described by incubate in constant contact with the ACTH-gel) may be achieved after exposure of the adrenal cells to ACTH-agarose for only the first 35 min of incubation.

TABLE II. 3mg Aliquots of gel incubated with cells

Gel Contact Time (min)	Total Incubation Time (hours)	Net Corticosterone (μg ± 10%)
35	2	0.133
35	2	0.113
120	2	0.083

A microscopic examination of the ACTH-agarose (50-150 μ diameter) and adrenal cell (10-15 μ diameter) incubate reveals a random distribution of particles; the two particle types are not adherent. Since the incubate is subject to constant shaking, which prevents prolonged contact between non-adherent particles, this suggests that steroidogenesis can be rapidly initiated in the cell after transient contact with corticotropin.

Several tests were made on the ACTH-agarose to determine its stability and degree of biochemical similarity with free ACTH.

The ACTH-gel (0.1 g) was incubated with phosphate buffer 0.1M pH 7.6 at 37°C for 2 hours with shaking to determine its stability under the conditions of the corticosterone assay. After incubation, the gels were washed with ACTH vehicle and tested for steroidogenic capacity. No detect-

TABLE III. ACTH-agarose stability to 0.1M phosphate buffer pH 7.6 37°C 2 hours

Gel Weight (mg)	Net Corticosterone (μg ± 10%)
Pre-incubated gel	
0.63	0.52
0.90	0.67
Untreated gel	
0.65	0.49
1.73	1.26

able loss of activity occurred in the pre-incubated gel (Table III).

Free ACTH is unaffected by 0.1N HCl (17). The biological integrity of the ACTH-agarose was tested after washing it with the ACTH vehicle for 29 hours at 4°C. The gel-bound ACTH maintained dose-related activity after this treatment (18).

A 0.1 g aliquot of ACTH-gel was washed 10x with 20 ml of ACTH vehicle and then incubated in 1 ml of the same at 37°C for 2 hours. The gel was washed in ACTH vehicle and re-incubated with isolated adrenal cells. The gel retains graduated steroidogenic activity even after warm acid treatment (18).

The gel-bound ACTH is susceptible to proteolytic enzyme attack. It exhibits no steroidogenic activity after pre-incubation (10 mg) with trypsin (30 mg 220 U/mg) for 2 hours (Table IV).

TABLE IV. ACTH-agarose activity after trypsin digest

Gel Weight (mg)	Net Corticosterone (μg ± 10%)
Untreated gel	
0.70	0.14
3.11	0.64
Trypsin digested gel	
1.60	-0.25
3.20	-0.28

Samples of ACTH-agarose were pre-incubated with adrenal cells in order to determine whether or not a specific interaction related to inductive

contact (e.g. scission of the corticotropin from the gel matrix before induction, or proteolytic destruction after contact) occurs during incubation which would inactivate the gel. Aliquots of ACTH-agarose were pre-incubated with adrenal cells for 15 min or 100 min, washed twice with 0.1M phosphate buffer pH 7.6, washed twice with ACTH vehicle and stored in the same at 4°C. 24 hours later, they were assayed for steroidogenic potential versus untreated gel (Table V). A very slight, non-specific decline in steroidogenic capacity was observed with increased pre-incubation time. This is believed to be due to lytic principles in cell debris deriving from the cell separation procedure. No specific effect related to induction is in evidence.

Equilibration of anti-human ACTH antibody (1:25,000 B/F = 1) with the ACTH-gel for 3 days resulted in a total loss of steroid activity by the gel.

We have been able to obtain similar results (18) with β 1-24 corticotropin bound to agarose by the reaction scheme described above (13).

TABLE V. Pre-incubation of ACTH-agarose with 0.9 ml adrenal cells

Pre-incubation time with cells (min)	Net Corticosterone (μg ± 10%)
0	0.96
0	1.07
15	0.88
15	0.90
100	0.80
100	0.86

CONCLUSIONS

ACTH linked to an agarose support exhibits characteristics identical with those of the free molecule: it induces steroidogenesis in free adrenal cells; its activity is calcium dependent; it is resistant to 0.1N HCl treatment; it is susceptible to tryptic digestion.

Observation of incubates of the ACTH-gel and adrenal cells suggest that there is no specific adherence between the two particles. The minimum ACTH-gel size is 3x that of the adrenal cells, therefore induction must occur at the cell surface without passage of the corticotropin into the cell. The

adrenal cells elaborate maximum levels of steroid during 2 hour incubations even though exposure to the ACTH-gel is limited to 35 min, thus continuous stimulation of the cells is not necessary to maintain steroidogenesis. The ACTH-gel does not lose activity after incubation with adrenal cells, suggesting that destruction of the corticotropin is not an adjunct of the induction process.

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