Purification and Crystallization of the Polypeptide Hormone Human Chorionic Somatomammotropin*

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We have purified the large polypeptide hormone human chorionic somatomammotropin to near electrophoretic homogeneity by a new purification scheme. The hormone crystallized from polyethylene glycol in a form suitable for high resolution x-ray analysis; the crystals are monoclinic, space group C2, a = 123.9, b = 30.3, c = 53.8 Å, β = 119° 30 min, with 1 molecule/asymmetric unit. The growth-promoting activity of homogeneous hormone and of dissolved crystals was measured through their effect on committed erythroid precursor cells in tissue culture assays. Both homogeneous and redissolved crystalline hormone had activities comparable to that of hormone purified by standard procedures. The latter preparations yielded either no crystals, or disordered crystals unsuitable for x-ray analysis.

Human chorionic somatomammotropin, also called placental lactogen and chorionmammotropin, is a multifunctional placental polypeptide hormone whose chemical and biological properties have been reviewed in detail (1). It exhibits both growth-promoting and lactogenic activities, similar to the activities displayed by the homologous pituitary proteins growth hormone and prolactin, and modulates maternal insulin activity. However, its true biological function is not known. HCS1 is secreted by the placenta into the maternal blood stream, but there are at least two reports (2) of successful pregnancy and delivery in which no HCS could be detected in either the maternal circulation or the placenta by a sensitive immunochemical assay. These results suggest that HCS may not be absolutely required for normal fetal development. Kaplan (3) postulated that HCS has the general function of ensuring the developing fetus an adequate supply of fuels, notably glucose, even when the mother is fasting. An understanding at the molecular level of polypeptide hormone action will require knowledge of the three-dimensional structure (or structures) of the hormone, its membrane-bound receptor, and the hormone-receptor complex. The only polypeptide hormones whose structures have been determined by macromolecular x-ray crystallographic techniques are insulin (4), glucagon (5), and avian pancreatic polypeptide (6).

Our initial goal is to obtain crystals of HCS, human growth hormone, and prolactin and to determine the three-dimensional structures of one or all of them. HCS has been purified by workers in several laboratories (see for example, Refs. 1 and 8–10) usually by a series of batch (organic, acid, and salt precipitations) and chromatographic (ion exchange, gel exclusion) steps. Almost all hormone preparations used for chemical and biological studies are heterogeneous as judged by isoelectric focusing or polyacrylamide gel electrophoresis at pH 8 (for example, Ref. 10). Heterogeneity may arise from spontaneous deamidation, irreversible aggregation, trace proteolysis of the protein during purification, or intron mis-splicing. Moreover, exposure of hormones to organic solvents may cause a partial denaturation, thus preventing crystallization, without affecting the biological activity.

Such heterogeneity also leaves open the question of whether the numerous biological activities reported for HCS (1) are intrinsic to the molecule or whether some may be artifacts arising from the contaminants. Tarli et al. (10) reported differing biological activities of partially separated components of heterogeneous HCS.

Despite this chemical heterogeneity, crystallization of HCS has been reported by workers from several laboratories (7, 11–14). Such crystals were found to be extensively disordered when subjected to x-ray analysis (13, 14). We believe that this disorder results from chemical heterogeneity, rather than from some intrinsic property of the hormone such as unusual molecular flexibility (13, 14). We report here a new purification scheme for HCS which avoids the use of organic solvents, high pH, and elevated temperature. These conditions are known to promote deamidation and other causes of heterogeneity. This scheme yields hormone which is nearly homogeneous as judged by isoelectric focusing, which (for the first time) forms single crystals suitable for high resolution x-ray analysis and which has growth-promoting activity comparable to HCS purified by standard techniques.

EXPERIMENTAL PROCEDURES

HCS was purified from frozen placentas obtained from Tompkins County Hospital. External membranes and large blood vessels were removed as the placenta (approximately 500 g) thawed in saline. The
placenta was homogenized in 1500 ml of 0.1 M NH₄HCO₃, pH 7.8. The slurry was stored for 4 h at 4 °C and then clarified by centrifugation. All subsequent manipulations were performed at cold room temperatures. HCS in the supernatant (crude; Table I) was adsorbed to 120 ml of packed DEAE-cellulose that was previously equilibrated with 0.1 M NH₄HCO₃. DEAE-cellulose was washed in a sintered-glass funnel until free of nonsorbed proteins. HCS was eluted with a single step gradient to 0.4 M NH₄HCO₃. The elute (DEAE-cellulose; Table I) was brought to 50% saturation of (NH₄)₂SO₄ and HCS was collected by centrifugation. The pellet was dissolved in about 30 ml of 0.1 M NH₄HCO₃ and clarified by centrifugation, and the supernatant ([NH₄]₂SO₄; Table I) was applied directly to a Sephadex G-100 column (6 x 95-cm) equilibrated in the same buffer and eluted at 50 ml/h. Fractions containing HCS were pooled (G-100; Table I), frozen, and lyophilized. The preparation was dissolved in 0.1 M NH₄HCO₃ and rechromatographed on the same column. Fractions containing HCS were pooled (G-100 repeat; Table I), frozen, and lyophilized.

HCS was dissolved in 2 ml of 25 mM imidazole-Cl, pH 7.0, and layered on a preparative discontinuous polyacrylamide gel. The gel (320 mm²) consisted of a 20-ml gel (in 100 mM imidazole-Cl, pH 7.0; total acrylamide monomer, (w/v), 7.7%; ratio of N,N'-methylenebisacrylamide to total acrylamide monomer, 2.4%) and a 4-ml stacking gel (in 50 mM imidazole-Cl, pH 7.0; total acrylamide monomer, (w/v), 2.4%; ratio of N,N'-methylenebisacrylamide to total acrylamide monomer, 1.3%) cast in a Calanco "prep-disc" apparatus. Electrode buffer was 50 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH adjusted to 7.0 with imidazole. HCS was electrophoresed at 20 mA and collected as it ran off the end of the gel. Fractions containing HCS were pooled (Prep-disc; Table I), chromatographed through Sephadex G-25 into 0.1 M NH₄HCO₃, frozen, and lyophilized.

During purification, HCS was monitored by analytical gel electrophoresis (system as described above) and quantified using Pharmacia (Uppsala, Sweden) Phadebas hCS (hPL) radioimmunoassay test kit.

Final recovery of HCS was normally 15 to 25% of the amount initially present in the crude homogenate.

Standard preparations of HCS and human growth hormone, purified as previously described (8, 15) were kindly supplied by C. H. Li (Hormone Research Laboratory, University of California, San Francisco). In order to distinguish his HCS preparation from ours, we will refer to them as HCS (std) and HCS (Hunt).

Antiserum to HCS (std) was a gift from S. L. Kaplan (Department of Pediatrics, University of California, San Francisco).

For crystallization, HCS was initially dissolved in 10 mM NH₄HCO₃ at a concentration of 10 mg/ml and was sterilized by Millipore (0.22 μm) filtration. HCS was crystallized using a variety of conditions by the hanging-drop, vapor-diffusion method (13, 14). Single crystals were routinely grown by dissolving HCS in an equal volume of 0.1 M sodium phosphate, pH 6.5, containing polyethylene glycol 1000, at a protein concentration of 2.5 or 5 mg/ml, and equilibrating droplets composed of 0.1 M sodium phosphate, pH 6.5, containing polyethylene glycol 1000. Crystals were mounted in glass capillaries and X-ray precession photographs were taken with CuKa radiation.

Growth-promoting activity of HCS was performed on cultured, committed erythroid precursor cells. Peripheral blood was obtained with the informed consent of healthy volunteers. Nucleated cells were collected by centrifugation in Wintrobe tubes and washed in culture medium. The effect of HCS on these cells was assessed by duplicate or triplicate assays of large erythroid colonies in the methylcellulose culture system using 5% fetal calf serum or a serum-substituted method (16-18). All cultures contained 0.1 to 0.5 unit/ml of human urinary erythropoietin. In some experiments a microtitr-plate method was employed (19). The growth-promoting effect of HCS was also assessed in a serum-substituted colony assay utilizing K-562 cells (20). All activity is reported as percentage of colony count compared to control cultures lacking hormone.

RESULTS AND DISCUSSION

HCS (Hunt), purified according to the scheme summarized in Table I, is nearly homogeneous as judged by nondenaturing gel electrophoresis at pH 7.0, in contrast with HCS (std) which is clearly heterogeneous (Fig. 1a). When both samples were subjected to isoelectric focusing over a pH range from 4.0 to 6.5 (Fig. 1b), our HCS preparation, although much more homogeneous than the standard preparation, exhibited slight heterogeneity. We estimate that this preparation is at least 95% pure.

Four hormone preparations were subjected to crystallization attempts by vapor diffusion: HCS (Hunt), HCS (std), and two samples of HCS (std) that were previously subjected to preparative gel electrophoresis at pH 7.0. This final step separated the two main electrophoretic components in the standard preparation (Fig. 1a); each was homogeneous when examined by analytical gel electrophoresis at pH 7.0 (data not shown). Numerous crystallization conditions were examined, at pH values on either side of the isoelectric point of HCS (pH 6.15). The best crystals from our new preparation were obtained from protein concentrations ranging from 2.5 to 5 mg/ml in 0.1 M sodium phosphate, pH 6.5, with polyethylene glycol 1000 at 2, 4, or 8% (w/v) as precipitant. Bullet-shaped crystals (Fig. 2) appeared in 1 day and grew to a maximum size of 0.25 x 0.3 x 0.5 mm in 3 to 4 days at room temperature. None of the other three hormone preparations yielded any crystals under identical solution conditions in parallel experiments. Only disordered crystals could be obtained from HCS (std) (13), under somewhat different solution conditions.

The 12° precession photographs and core axis photographs of each of the principal zones were obtained, which showed that the crystals were monoclinc; systematic absences for h
primitive erythroid precursor (burst-forming unit) is also responsive to low concentrations of HCS (25). We first tested the various HCS preparations at 100 ng/ml for bioactivity in the erythroid burst-forming unit assay with 10% fetal calf serum and found that the redissolved crystals had activity comparable to the partially purified material but less activity than the highly purified HCS (Table II). Dose-response curves were then constructed for the preparations which revealed that 100 ng/ml was not an optimal concentration at which to test bioactivity. Rather, maximal responses of 150 to 170% of control were seen at 10 to 50 ng/ml in both serum-containing and serum-substituted cultures (Fig. 3).

When tested in serum-substituted culture using human erythroleukemia K-562 cells as target, HCS (crystal) was as active as HCS (std) and HCS (Hunt) (Table II). Thus, we conclude that our crystalline material has a prominent growth-promoting effect in vitro similar to that of purified noncrystallized hormone. Whether the observed differences in activity (Table II; Fig. 3) of the hormone preparations reflect true differences in bioactivity or result from normal bioassay variability remains to be established.

These data confirm that the crystals contain intact, authentic HCS which possesses appreciable growth-promoting activity as judged by these tissue culture assays, and that our new purification scheme yields HCS which is of at least comparable activity to that of standard HCS. Structure determination of our HCS crystals is progressing by standard macromolecular crystallographic techniques (26).

**TABLE II**

| Preparation      | K-562 |
|------------------|-------|
| HCS (std)        | 115 ± 4 | 126 ± 0 |
| HCS (Hunt)       | 130 ± 1 | 135 ± 2 |
| HCS (crystal)    | 114 ± 1 | 127 ± 1 |
| Human growth hormone | 142 ± 1 | 134 ± 2 |
| Diluent          | 100 ± 1 | 100 ± 1 |

*Stimulation is expressed as per cent of colony count over controls lacking hormone ± standard error of the mean.*

**FIG. 3.** Dose-response curves for HCS (std) (a), HCS (Hunt) (b), and HCS (crystals) (d), as assayed by colony formation stimulation of erythroid burst-forming unit cultured in (a) 10% fetal calf serum, 0.1 unit/ml of erythropoietin or (b) serum-substituted medium, 0.5 unit/ml of erythropoietin. Replicate plates differed by less than 4%. Control cultures were identical except that they lacked HCS.
Crystals of Chorionic Somatomammotropin

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