Assignment of Disulfide Bonds in Corticotropin-releasing Factor-binding Protein*

We have previously isolated, cloned, and characterized a protein that specifically binds and inactivates the peptide corticotropin-releasing factor. The integrity of the disulfide bonds in the binding protein is essential for this activity as reduction abolishes the protein's ability to bind corticotropin-releasing factor. The disulfide arrangement of the 10 cysteines present in the mature protein was established by analysis of proteolytically cleaved protein and sequence analysis of cystine containing fragments. A pattern is observed where each cysteine is connected to the next one in a sequential manner. Inspection of the genomic DNA encoding for this protein reveals that four of the domains defined by disulfide linkage coincide with four different exons.

Corticotropin-releasing factor-binding protein (CRF-BP) was initially purified from human serum (Behan et al., 1989) and subsequently cloned from human liver and rat brain cDNA libraries (Potter et al., 1992). Both purified and expressed CRF-BPs bind to CRF with high affinity and neutralize the peptide's biological activity. In human beings, the only species thus far shown to express CRF-BP in the liver, circulating CRF-BP has been proposed to protect the maternal endocrine system from the high levels of CRF (Campbell et al., 1987; Goland et al., 1986; Linton et al., 1987; Sassaki et al., 1984) produced by the placenta (Petraglia et al., 1987) during pregnancy. Rats, primates, and other species examined express CRF-BP in the brain and pituitary gland, where it may serve to modulate the neuroendocrine and neural actions of CRF (Vale et al., 1981). From both human and rat cDNAs, 322 amino acid precursors and 298 amino acid mature proteins containing 10 cysteines are predicted, suggesting the presence of five disulfide bonds. To gain insight into the structural properties of the protein we have now determined the disulfide bond arrangement of the expressed human CRF-BP.

MATERIALS AND METHODS

Expression of CRF-BP in Stably Transfected Chinese Hamster Ovary Cells—The full-length 1.9-kilobase pair CRF-BP cDNA containing its own polyadenylation signal sequence was cloned into expression vector pSG5 (Stratagene) downstream from a strong SV40 promoter as described previously (Potter et al., 1991). Chinese hamster ovary cells were transfected with this construct and a Rous sarcoma virus neomycin construct by the calcium phosphate procedure. Stable cell colonies were selected over a period of 2 weeks by growing the cells in complete media (Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, 2 mM glutamine) containing 1 mg/ml G418. Colonies were subsequently isolated and grown in complete media as separate cell lines. Media from each of the lines were then screened for the presence of the CRF-BP by virtue of their ability to inhibit binding of a CRF antibody to radiolabeled CRF trace, and the one producing the highest quantity of CRF-BP was then aliquoted and stored in liquid nitrogen to be used for all subsequent expression studies. For large scale protein expression, cells were grown to confluence in 500 ml of complete medium on large tissue culture plates. Two liters of binding protein medium was then percolated through a 1-ml CRF-solid phase (which was prepared by coupling 1 mg of rat/human CRF to 1 ml of Affi-Gel-10) overnight with the aid of a peristaltic pump. Bound recombinant CRF-BP was then eluted in 50 ml sodium acetate, formate, 20% acetonitrile buffer, pH 3.0, essentially as already described (Behan et al., 1989). Both the media and the cells from the purified CRF-BP were found to exhibit the same characteristics as the natural material from human plasma in that they inhibited CRF-induced ACTH secretion from rat anterior pituitary cells in vitro and hindered binding of a CRF antibody to radiolabeled CRF trace with identical dose-response characteristics.

Trypsin Digest of CRF-BP—Affinity-purified CRF-BP (approximately 10 mg) was further purified by reversed phase HPLC. (A gradient from 18 to 54% acetonitrile was run within 30 min at a flow rate of 0.2 ml/min.) The protein eluting at 28 min was collected and concentrated to dryness in a Savant Speed-Vac concentrator. Trypsin (sequencing grade, 1 pg) dissolved in 50 ml of TES buffer (0.1 M, TES, pH 8, adjusted with NaOH) was added. After 2 h of incubation at 37 °C, 20% Tween 20 was added to a final concentration of 0.5%. The sample was divided into two equal aliquots. One aliquot was mixed with 75 ml of 0.5 M aqueous acetic acid and subjected to HPLC separation. To the other aliquot, 55 ml of ammonium acetate buffer (0.1 M, pH 4.5) and TCEP (0.5 M in dH2O; 20 ml) was added, followed by incubation for 30 min at room temperature. The sample was kept frozen at −20 °C until injected into the HPLC.

Endoprotease Asp-N Digest of CRF-BP—Protein (approximately 10 mg) was purified by HPLC and dried down as described above. Endoprotease Asp-N (sequencing grade, 1 pg) dissolved in sodium phosphate buffer (25 mM, 5% acetonitrile, 0.2% Nonidet P-40, pH 8) was added, and digestion was allowed to proceed for 16 h at 37 °C. The sample was divided into two aliquots. One aliquot was injected into the HPLC after mixing with 75 ml of 0.5 M acetic acid; the other aliquot was reduced using TCEP as described above.

Reversed Phase HPLC—A Hewlett-Packard 1090L Liquid Chromatograph equipped with a Vydac C-18 column (2.1 x 150 mm; particle size: 5 μm; pore size: 300 Å) was used. The flow rate was 0.2 ml/min. Samples were collected manually based on absorbance at 210 nm.

Sequence Analysis—Sequence analysis was performed on an Applied Biosystems 470A protein sequencer equipped with an on-line phenylthiohydantoin-derivative analyzer (ABI 120A). Samples were applied to polybrene-coated glass fiber filters and sequenced using the standard program supplied by the manufacturer.

RESULTS

CRF-BP (sequence in Fig. 1) expressed by stably transfected Chinese hamster ovary cells was treated with two highly specific proteases, trypsin and endoprotease Asp-N. The resulting fragments were separated by reversed phase HPLC. Ali-
DISCUSSION

The strategy utilized here to determine the disulfide arrangement in CRF-BP involved digestion of the protein with specific proteolytic enzymes and resolution of the fragments by reversed phase HPLC with and without treatment with reduc-
Disulfide Arrangement in CRF-BP

TABLE I
Sequence analysis results

The sequence signal observed for the proteolytic fragments is given and mapped to the known sequence of hCFRBP. The numbering of the residues follows the one published (Potter et al., 1991). For each fragment the residues observed in sequencing cycle are listed in the order they elute on the phenylthiohydantoin analyzer. The fragments of CRF-BP to which these residues could be mapped are listed underneath the observed sequence.

| Fragment No. | Residues observed | Mapped to hCRF-BP |
|--------------|-------------------|-------------------|
| A1           | 35-61             | DPFLKCAAPPISPFETIKY |
|              |                   |                   |
| A2           | 177-219           | DPFLKCAAPPISPFETIKY |
|              |                   |                   |
| T1           | 60-111            | CDMFLKCAAPPISPFETIKY |
|              |                   |                   |
| T2           | 137-147           | YIDFCSLESK |
|              |                   |                   |
| T3           | 177-183           | YIDFCSLESK |
|              |                   |                   |
| T4           | 194-216           | YIDFCSLESK |

Fig. 3. Schematic representation of the proteolysis strategy. Horizontal bold lines represent the protein and the peptide fragments thereof. Open ovals represent cysteine residues. The signal peptide/propeptide portion is labeled SP. A1 and A2, endoproteinase Asp-N digest fractions; T1–T4, trypsin digest fractions. The determined disulfide connectivity is shown in the top portion of the schematic. Arrows indicate the location of introns in the corresponding gene.

Fig. 4. Ligand immunoradiometric assay analysis of binding of CRF to nonreduced and reduced CRF-BP. The binding of [125I]-o-Tyr8-hCRF to nonreduced (○) and reduced (□) CRF-BP is expressed as the ratio of the counts bound to the total counts (50,000 cpm) added. The complex of CRF-BP and bound CRF was precipitated with a specific anti-CR1-BP antibody. Details of this assay are described elsewhere (Behan et al., 1983b).

When the ability of reduced CRF-BP to bind CRF was tested,
it was found that this ability was abolished. It is concluded that the stabilization of the structure by disulfide bonds is essential for biological activity.

The reducing agent used in this study, TCEP (Burns et al., 1991; Fischer et al., 1993), has several advantages over conventional reducing agents such as dithiothreitol or β-mercaptoethanol. It is highly efficient at low temperatures (21–37°C), and incubation times are short (30 min or less). The reagent is specific toward disulfide bonds in proteins; it is not reactive to other functional groups found in proteins. Another major advantage is the absence of contaminants absorbing at 210 nm, which simplifies comparison of HPLC profiles of reduced and nonreduced materials.

It was found that the disulfide arrangement in CRF-BP follows a pattern where each cysteine is joined to the next one in a sequential manner. This sequential disulfide arrangement is reminiscent of that found in members of the immunoglobulin superfamily (Williams, 1987). One characteristic of this class of protein is the presence of domains defined by 5 sequential cystines. A sequence comparison between CRF-BP and members of the immunoglobulin superfamily, however, did not reveal any significant homologies at the amino acid sequence level. The loop size, i.e. the number of amino acid residues between 2 cysteines, is smaller in CRF-BP (20–46 residues) than that of the members of the immunoglobulin family (approximately 70 residues). The difference in loop size and the absence of sequence homology make a prediction of tertiary structure based on the known structures of immunoglobulins infeasible.

For the immunoglobulin superfamily it has been proposed that the domains defined by disulfide linked loops arose from gene duplication of a single ancestral gene (Williams, 1987). One argument in favor of this hypothesis is the fact that in most members of that superfamily these domains are found on separate exons. Analysis of the genomic CRF-BP sequence reveals that four of the domains defined by disulfide loops are found on single exons, whereas the domain defined by cysteine 104 and cysteine 141 is disrupted by an intron (Behan et al., 1993a).

To test whether the loops formed by the disulfide bonds in CRF-BP define domains that might have arisen from gene duplication as has been postulated for the immunoglobulin family, a comparison for internal homologies was carried out by the Dotplot program (University of Wisconsin Genetics Computer Group (UWGGC) program package). This program compares the sequence to itself at a given stringency marking homologous regions by a dot. Two regions were identified that exhibited moderate homology to each other. A computer alignment analysis of this region using the GAP program (UWGGC program package) revealed that CRF-BP 101–112 and CRF-BP 234–246 are 50% identical and show 83% similarity allowing a 1 amino acid gap. These two regions are defined by the second and fourth disulfide loop and are each contained on a separate exon (exon 3 and exon 6 (Behan et al., 1993a)).

It is speculated that the five-loop motif found in CRF-BP, in analogy to the immunoglobulin structure, provides a framework in which the disulfide bonds are at the core of the structure and the loops are arranged to form pockets or clefts on the surface for substrate binding. Considering the size of the loops found in CRF-BP, it is hypothesized that at least two loops are involved in binding interactions, whereas additional loops stabilize the structure. In the absence of three-dimensional structure information, this hypothesis can now be tested by expression of shortened forms of CRF-BP containing pairs of loop domains and by determining the affinity for CRF of these analogs.

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REFERENCES
Behan, D. P., Linton, E. A., and Lowry, P. J. (1989) J. Endocrinol. 122, 23–31
Behan, D. P., Potter, E., Lewis, K. A., Jenkins, N. A., Copeland, N., Lowry, P. J., and Vale, W. W. (1990a) Genomics 14, 63–68
Behan, D. P., Potter, E., Sutton, S., Fischer, W., Lowry, P. J., and Vale, W. W. (1993b) Ann. N. Y. Acad. Sci. 697, 1–8
Burns, J. A., Butler, J. C., Moran, J., and Whitesides, G. M. (1991) J. Org. Chem. 56, 2646–2650
Campbell, E. A., Linton, E. A., Wolfe, C. D. A., Scraggs, P. R., Jones, M. T., and Lowry, P. J. (1987) J. Clin. Endocrinol. Metab. 64, 1054–1059
Fischer, W. H., Rivier, J. E., and Craig, A. G. (1990) Rapid Commun. Mass Spectrom. 7, 225–228
Goldstein, H. B., Norton, S. L., Stark, R. I., Brown, Jr., and Frantz, A. G. (1986) J. Clin. Endocrinol. Metab. 63, 1522–1535
Linton, E. A., Maclean, C., Nieszen, A., Kreisman, C., Tilders, F. J., van der Veen, E. A., and Lowry, P. J. (1987) J. Clin. Endocrinol. Metab. 64, 1047–1053
Petraglia, F., Sawchenko, P. E., Rivier, J., and Vale, W. (1987) Nature 328, 717–719
Potter, E., Behan, D. P., Fischer, W. H., Linton, E. A., Lowry, P. J., and Vale, W. W. (1991) Nature 348, 423–426
Potter, E., Behan, D. P., Linton, E. A., Lowry, P. J., Sawchenko, P. E., and Vale, W. W. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4192–4196
Sasaki, A., Liotta, A. S., Luckey, M. M., Margioris, A. N., Suda, T., and Krieger, D. T. (1984) J. Clin. Endocrinol. Metab. 59, 812–814
Vale, W., Spiess, J., Rivier, C., and Rivier, J. (1981) Science 213, 1394–1397
Williams, A. F. (1987) Immunol. Today 8, 298–303