Proteolytic Processing in the Biogenesis of the Neurosecretory Egg-Laying Hormone in *Aplysia*. 2. Analysis of Tryptic Fragments†

Robert W. Berry,* Michael J. Trump, and Justicia T. Baylen

ABSTRACT: Pulse–chase studies indicate that the peptide egg-laying hormone, ELH, of the neurosecretory bag cells of the mollusk *Aplysia* is generated by a complex multistep proteolytic processing sequence. Such data indicate that ELH and another secretory peptide, AP (acidic peptide), are generated from a 29 000-dalton precursor via a common intermediate and that this precursor also gives rise to an additional 13 000-14 500-dalton product. In the present study, we have adapted the procedure of Elder et al. [Elder, J. H., Pickett, R. A., Hampton, J., & Lerner, R. A. (1977) *J. Biol. Chem. 252*, 6510–6515] to obtain tryptic fragments of biosynthetically labeled bag cell proteins. Analyses of these fragments by isoelectric focusing and thin-layer chromatography/electrophoresis are consistent with the processing sequence inferred from pulse–chase data. Furthermore, the peptide maps have revealed the presence of an additional copy of the AP peptide within the 13 000–14 500-dalton product.

Kinetic studies have suggested that *Aplysia* egg-laying hormone (ELH)[1] is generated by the proteolytic cleavage of a larger precursor in a manner similar to a number of well-studied peptide hormones and secretory proteins (Arch, 1972; Loh et al., 1975; Berry, 1981). However, an adequate characterization of the processing sequence and the portions of the pathway leading to ELH and the other major secretory peptide, AP, require a determination of whether or not the amino acid sequence of the product is present in each of the putative precursors and intermediates, preferably by peptide mapping techniques. Unfortunately, as commonly applied, these techniques are not well suited to the study of transient intermediates in processing sequences because they require the isolation of pure protein in quantities sufficient to allow detection of the resulting proteolytic fragments and in the absence of detergents or denaturants in quantities which would impair the activity of the proteolytic enzyme used. Because of their rapid turnover rates, processing intermediates are not apt to be major quantitative constituents of cells, and although recent advances have extended the detection limit for peptides to the picomole range (Hare, 1977; Hunkapiller & Hood, 1980), losses involved in the purification of such minor tissue constituents would be expected to be considerable. Furthermore, some processing intermediates, such as the bag cell peaks I and II to be dealt with here, are insoluble without detergent treatment (Arch et al., 1976), probably because of an association with membranes.

Recently, Cleveland et al. (1977) and Elder et al. (1977) have each described peptide mapping methods that circumvent some of these difficulties. Both allow the production of proteolytic fragments from proteins isolated by $NaDodSO_4$–polyacrylamide gel electrophoresis, thus eliminating the problem of solubility and reducing the losses that would inevitably occur in a multistep isolation procedure. The procedure of Elder et al. (1977) has the further advantage that the proteolytic fragments are recoverable in a form suitable for two-dimensional separation and further analysis. In this technique, the protein of interest is separated from other components of a cell extract by analytical $NaDodSO_4$–polyacrylamide gel electrophoresis and radioiodinated while still in the gel. Following fixation of the protein with a solvent system that removes $NaDodSO_4$, trypsinization produces fragments that can be eluted from the gel at much higher efficiency than could the intact protein. This method has allowed the mapping of proteins contained in as little as 50 μg of cell extract.

As originally described, the method is not entirely suitable for the study of processing intermediates. In addition to the requirement that the fragments contain tyrosine, it requires that the protein of interest be a major component of the cell extract in order to achieve adequate separation from other species of similar molecular weight on $NaDodSO_4$–polyacrylamide gel electrophoresis. However, if detection were based not on radioiodination but on biosynthetically incorporated label, it would only be required that the starting protein be radiochemically pure. Since processing intermediates should have higher turnover rates than other cellular proteins, separation on $NaDodSO_4$–polyacrylamide gel electrophoresis might be expected to achieve sufficient radiochemical purity for peptide mapping.

Since we have previously shown that a high degree of radiochemical purity can be achieved for most members of the biosynthetically labeled ELH processing sequence by $NaDodSO_4$–polyacrylamide gel electrophoresis (Berry, 1981), we have adapted the method of Elder et al. to produce tryptic peptide maps of these proteins and thus to elucidate their processing relationships.

Materials and Methods

Starting material for these analyses consisted of members of the ELH processing sequence labeled by biosynthesis in the presence of [^{35}S]methionine or a mixture of 15 tritiated amino acids for varying periods and isolated by electrophoresis, all as described in the preceding report (Berry, 1981). Following removal of $NaDodSO_4$, the proteins of interest were trypsinized in the gels, and the fragments were eluted and separated by isoelectric focusing, thin-layer chromatography, or sequential

† From the Department of Cell Biology and Anatomy, Northwestern University School of Medicine, Chicago, Illinois 60611. *Received March 2, 1981.* This work was supported by National Institutes of Health Grants NS-11519 and RR-05370.

[1] Abbreviations used: AP, acidic peptide, ELH, egg-laying hormone; M_r, relative molecular mass; MSH, melanocyte stimulating hormone; $NaDodSO_4$, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

0006-2960/81/0420-6206$01.25/0

chromatography and electrophoresis.

Enzymatic hydrolysis of the bag cell proteins in gel slices required only a minor modification of the procedure used by Elder et al. (1977), the first step of which involves immersion of the gels overnight in a solution of 25% 2-propanol/10% acetic acid to remove $NaDodSO_4$ while the proteins in the gel are being fixed. Although it has been reported that insulin (6000 daltons) is adequately retained by this treatment (Fairbanks et al., 1971), we found that it resulted in unacceptable losses of the lower molecular weight bag cell proteins. While gels kept overnight in this solution retained 90–100% of the radioactivity in peaks I and II as compared to gels kept overnight in cold 15% trichloroacetic acid, 48% of peak III and 75% of peak IV were lost. The methanol/acetic acid/water (50:10:60) solution we used was equally effective in $NaDodSO_4$ removal, as judged by the production of tryptic fragments, and retained 97% of peak III and 50% of peak IV in the gels.

Following $NaDodSO_4$ removal, the gels were sliced longitudinally into two unequal segments and sectioned transversely into 1-mm slices. The peaks of interest were identified by scintillation counting of the smaller slices. The two to three larger slices containing a given peak were minced with a scalpel blade, washed into a siliconized 6 × 50 mm culture tube with 200 μL of distilled water, and lyophilized. Lyophilization was repeated once more before enzymatic hydrolysis. For trypsinization, the minced gel was exposed to 200 μL of 0.05 M NH_4HCO_3, pH 8.0, containing 0.2 mg/mL TPCK-treated trypsin (Worthington), for 18–20 h at 37 °C. The supernatant was then replaced with an equal volume of the same buffer, and elution was allowed to proceed for an additional 18–20 h at 4 °C. The combined supernatant and wash solutions were lyophilized twice more from 200 μL of distilled water. In some cases, the eluted peptides were oxidized with performic acid (Hirs, 1967), although this was not done routinely, as it did not affect the number of peptides obtained. Although data on chymotryptic hydrolysates will not be presented here, a modification of the above procedure has yielded successful isofocusing maps with this enzyme as well. Hydrolysis is accomplished in 0.05 M ammonium acetate, pH 8.0, with 0.05 M $CaCl_2$. After lyophilization, the residue is treated with NH_4HCO_3 to precipitate Ca^{2+}.

The intact proteins are not appreciably soluble following the methanol/acetic acid treatment. No significant radioactivity could be eluted from any of the peaks with NH_4HCO_3 buffer. Elution with 1% Triton X-100 was similarly ineffective, but inclusion of 6 M urea in the Triton solution solubilized 80–100% of the radioactivity in each peak within 48 h at 4 °C. Elution efficiency was much higher for proteolytic fragments. The trypsinization and elution procedure described above released 84% of the radioactivity from peak I, 71% from peak II, 81% from peak III, and 86% from peak IV. Approximately 80% of this was recovered in the initial (18–20 h) buffer supernatant. Pilot studies indicated that no more than 10% of the total could be recovered if the elution was continued for an additional 72 h. Peptides recovered in the second elution step were indistinguishable in their isoelectric focusing profiles from those obtained from the first. However, there is evidence, to be presented below, of a consistent difference in elution efficiency between some of the fragments of protein IIIA.

Isoelectric focusing of tryptic hydrolysates was carried out in 5 × 75 mm cylindrical polyacrylamide gels containing 6 M urea, as described previously (Yates & Berry, 1981). After measuring the pH gradient with a surface electrode, the gels were sliced into 1-mm segments and subjected to liquid scintillation counting. For facilitation of comparisons between the different proteins, these data have been plotted on a linear pH scale (Figure 2). Because the pH gradient is nonlinear, this sharpens the acidic peaks and broadens the alkaline ones. Illustrations in the preceding report (Berry, 1981) are typical of the gradients obtained in this study.

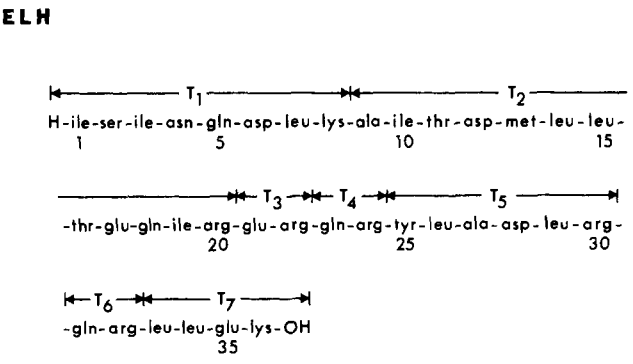

FIGURE 1: Amino acid sequence of ELH according to Chiu et al. (1979), with the tryptic fragments expected from complete hydrolysis indicated.

Chromatography was carried out on 20 × 20 cm plastic-backed thin-layer sheets of MN 300 cellulose (Brinkman, Westbury, NY) in butanol/pyridine/acetic acid/water (13:10:2:8). For two-dimensional maps, chromatography was followed by electrophoresis in pyridine/acetic acid/water (1:10:89, pH 3.5) for 1 h at 900 V. The plates were either rinsed with 7% 2,5-diphenoxyazole in ether (Randerath, 1970) or sprayed with En^{3}hance (New England Nuclear, Boston, MA) and subjected to autofluororadiography on preflashed Kodak X-RP film at −80 °C (Laskey & Mills, 1975). With diphenoxyazole, the detection limit for 5-mm diameter spots was 10^3 dpm for tritium and 10^2 dpm for ^{35}S at an exposure time of 7 days. The latter reagent reduced the tritium detection limit from 10^4 dpm to 10^3 dpm in 3-day exposures and enhanced ^{35}S detectability, although this effect was not quantitated.

Results

Tryptic Fragments of ELH. The amino acid sequence of ELH is shown in Figure 1, along with the expected sites of cleavage by trypsin. It is apparent that complete cleavage would produce seven fragments, only one of which would contain methionine. With the amino acid mixture used in the present study, the dipeptides T_3, T_4, and T_6 would probably not contain detectable radioactivity, while the remaining fragments should be labeled approximately equally. However, it can be anticipated that complete cleavage might not be attained, owing to the inhibitory effect of the glutamate residue adjacent to arginines-20 and -22 (Kasper, 1975). Thus, one might expect tryptic hydrolysis to produce a variable mixture or T_2–T_4 fragments, and, therefore, as many as three methionine-containing peptides might be observed.

Figure 2 shows typical examples of the ELH fragments produced by tryptic hydrolysis as revealed by isoelectric focusing. The results are in agreement with the predictions above. A single acidic fragment (p*I* 3.9–4.1), containing methionine, was observed in four of eight cases. In the other four experiments, two fragments having p*I*'s of 3.9 and 4.1, were produced, both containing methionine. The ratio of label in the two fragments varied from run to run, suggesting that these peptides were the result of incomplete cleavage at residue 20 or 22. The isoelectric points of these fragments suggest that incomplete cleavage occurs only at arginine-20 and that

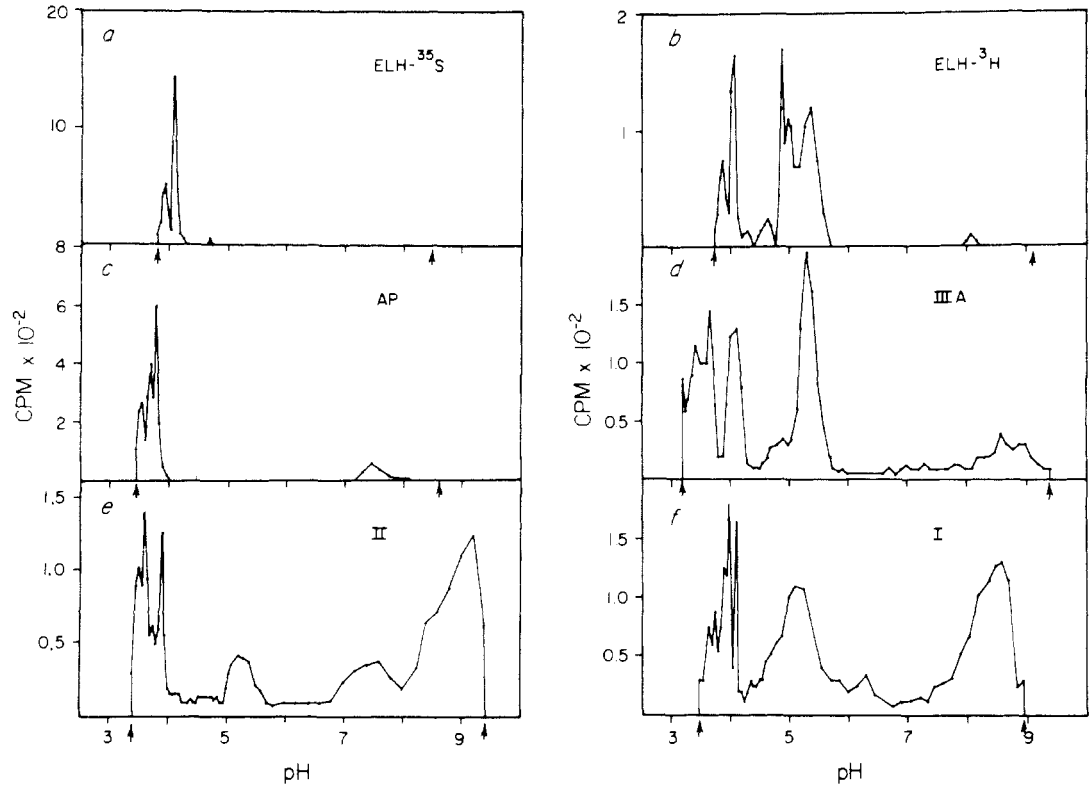

FIGURE 2: Analysis of tryptic fragments by isoelectric focusing. (a) Fragments of ELH from bag cells labeled for 4 h with [^{35}S]methionine and chased for 16 h. (b) Same as (a), but labeling was with tritiated amino acids. (c) Fragments from AP, labeled as in (b). (d) Fragments from protein IIIA; labeling was with tritiated amino acid mix for 4 h. (e) Fragments from peak II, labeled for 8 h with tritiated amino acids. (f) Fragments from protein I, labeled as in (e). Arrows indicate pH at the top and bottom of the gels.

arginine-22 is always subject to cleavage under our conditions of hydrolysis: the p*I* of T_2 estimated by the method of Edsall & Wyman (1958) is 4.7 and that of $T_2 + T_3$ is 5.0, while that of $T_2 + T_3 + T_4$ would be 6.4. It must be noted here that the observed isoelectric points of all ELH fragments are more acidic than the calculated values. However, the minimal separation of the two observed fragments suggests that they correspond to T_2 and $T_2 + T_3$.

In addition, as many as three additional non-methionine-containing fragments can be resolved (Figure 2b) in the p*I* 5–6 region, although these frequently appear as a broad unresolved band on our gels. These undoubtedly correspond to T_1, T_5, and T_7, each of which has a calculated isoelectric point of 6.4.

Isoelectric Focusing of Tryptic Fragments of Members of the ELH Sequence. Having identified those tryptic fragments that are internal to the amino acid sequence of ELH, we next searched for them in isoelectric focusing profiles of tryptic hydrolysates of the other members of the ELH processing sequence. Typical examples are shown in Figure 2.

AP, the acidic bag cell product peptide, yielded four fragments, all of which contained methionine (Figure 2c). The acidic fragments were all more acidic than the T_2 peptide of ELH, and the neutral ELH fragments were absent. Thus, as expected, AP cannot be an ELH precursor. Protein IIIA, the 6000-7000-dalton peptide which was postulated (Berry, 1981) on kinetic grounds to serve as a common precursor to ELH and AP, yielded four major fragment groups (Figure 2d). Each contained methionine. The acidic ELH fragments were present, confirming its assignment as an ELH precursor, although the disperse nature of its p*I* 5-6 fragments did not allow a determination of whether the more neutral ELH peptides were also present. In accordance with the suggestion that this protein also serves as an AP precursor, several p*I* 3–4 peptides were present as well. The basic fragment was consistently more basic (p*I* = 8.5) than the basic fragment of AP (p*I* = 7.5), but this could represent the N- or C-terminal fragment of AP, which could be modified during cleavage from the precursor.

Peak II, a heterogeneous group of 13 000–20 000-dalton proteins (Berry, 1981), yielded six tryptic fragments or groups (Figure 2e). Only the p*I* = 7.5 material did not contain methionine. In all cases except the one illustrated, the acidic fragments were more acidic than pH 3.9, and we never observed a p*I* = 4.1 peptide. Furthermore, the p*I* = 5–6 material always contained less radioactivity than would be expected of ELH fragments T_1, T_5, and T_7. This is in agreement with the kinetic evidence that peak II does not contain an ELH precursor (Berry, 1981). Kinetic evidence also indicates that peak II does not contain a precursor to AP. Thus it was surprising to find a close correspondence in p*I* between the acidic peak II fragments and AP peptides. This relationship will be discussed in more detail below.

Tryptic fragments of protein I, the 29 000-dalton putative precursor to other members of the sequence, are shown in Figure 2f. All of these fragments were found in methionine-labeled material. It is clear that this pattern contains fragments common to those of all of the other proteins examined.

Fragment Analysis by Thin-Layer Chromatography and Electrophoresis. Despite the fact that isoelectric focusing of ^{3}H-labeled material has the potential for visualizing peptides that lack methionine, it is possible that nonidentical fragments may have closely similar or identical isoelectric points, and it is apparent from Figure 2 that some of the observed peaks are heterodisperse. Therefore, we confirmed these results by separating methionine-labeled tryptic fragments by thin-layer chromatography and electrophoresis. A one-dimensional chromatographic separation is shown in Figure 3.

In 6 of 13 cases, ELH yielded a single methionine-containing

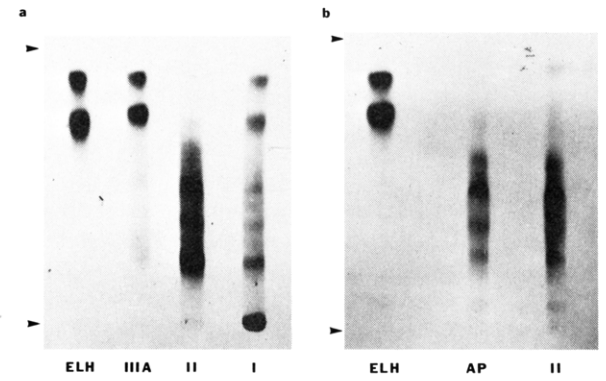

FIGURE 3: Thin-layer chromatography of methionine-labeled tryptic fragments. (a) Fragments of protein I, peak II, and protein IIIA, labeled for 4 h, and ELH, labeled for 4 h with a 16-h chase. (b) Fragments from peak II, AP, and ELH, labeled for 4 h with a 16-h chase. Both plates were sprayed with En^{3}hance and exposed to Kodak X-RP film for 4 days. Arrows mark the origin and solvent front.

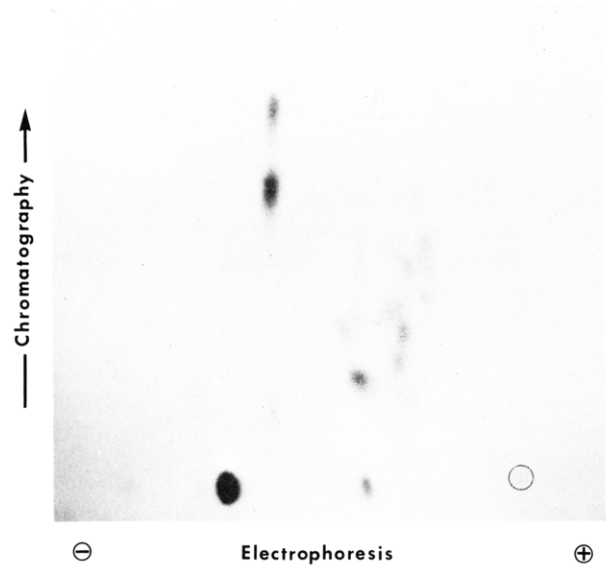

FIGURE 4: Thin-layer chromatography and electrophoresis of protein I, labeled for 4 h with [^{35}S]methionine. Circle denotes the origin. An additional six fragments are visible on the original chromatogram.

fragment of high chromatographic mobility. Two such fragments were visible in the remainder of the experiments, such as that illustrated here. It is obvious that these fragments are also present in proteins I and IIIA but are absent from peak II and AP, thus confirming the isofocusing results. As in the isofocusing analyses, tryptic hydrolysates of AP yielded four fragments. Three of these comigrated with protein I material, which is consistent with the precursor role postulated for this protein. All four fragments were also present in protein IIIA, although they are very weak in the experiment illustrated. The ratio of ELH to AP fragments in IIIA material varied widely from experiment to experiment, probably reflecting variable losses of the AP peptides. Again, as in the isofocusing experiments, all four of the AP fragments are present in peak II material.

Finally, to confirm that the ELH fragments separated by chromatography were indeed homogeneous, we subjected tryptic hydrolysates to two-dimensional separation by thin-layer chromatography and electrophoresis. In all cases, each chromatographic ELH spot was electrophoretically homogenous. A protein I hydrolysate is illustrated in Figure 4; the same result was obtained with fragments derived from ELH and protein IIIA.

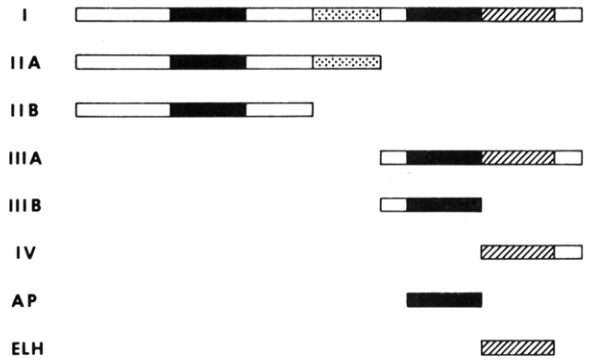

FIGURE 5: Schematic representation of the distribution of AP and ELH fragments within members of the processing sequence. The assignment of proteins and cleavage fragments relative to the N- and C-termini is arbitrary.

Discussion

Our experience with this modification of the peptide-mapping procedure of Elder et al. (1977) indicates it to be a sensitive and relatively convenient method for the structural analysis of quantitatively minor components of complex cell homogenates. We have obtained interpretable maps from insoluble proteins separated by $NaDodSO_4$–polyacrylamide gel electrophoresis which contain less than 9000 ^{3}H dpm by isofocusing and approximately 6000 ^{35}S dpm in the thin-layer system. The actual quantity of protein that this represents is difficult to determine with precision, but a rough estimate can be made. The starting material for electrophoresis consists of a pair of bag cell clusters containing approximately 50 μg of total protein. Densitometry of $NaDodSO_4$ gels stained with Coomassie blue indicates that peak III represents about 5% of the total staining density. If staining is assumed to be proportional to protein concentration, then the starting material for maps of peak III is about 2.5 μg, or 0.5 nM. Peaks I and II represent less material. It would therefore appear that this technique is approximately as sensitive as those employing radioiodination or fluorescent detection methods (Elder et al., 1977; Stephens, 1978). Since it can be used in any cellular system capable of incorporating approximately 10^4 dpm of tritium into the protein of interest regardless of the quantity of that protein that is present, the method should be particularly useful for structural studies of proteolytic processing sequences.

The results of the peptide mapping analyses of bag cell proteins obtained with this method are summarized in Figure 5. They confirm and complement the results of the kinetic analyses in the preceding report (Berry, 1981). In particular, they confirm the scheme of ELH processing introduced there: since internal tryptic fragments of ELH are found in, and only in, proteins I and IIIA, the route of ELH biogenesis must be I → IIIA → ELH. Although we did not investigate peak IV specifically, the kinetic data unambiguously assign it an intermediate role between IIIA and ELH. Conversely, both AP and the peak II proteins lack the ELH peptides and cannot lie on the pathway leading to this product.

Taken alone, the peptide mapping data are more ambiguous with respect to AP generation. AP fragments are present in protein I, peak II, and protein IIIA. However, IIIA cannot be derived from peak II because it is a single molecular species (Berry, 1981) which contains both ELH and AP fragments, the former of which are not present in peak II. While these data could reflect the existence of parallel pathways for AP generation whereby both peak II and protein IIIA are used as precursors, this is inconsistent with the kinetic data which indicate that AP and ELH, or rather their immediate pre-

cursors, IIIB and IV, arise simultaneously and in equimolar amounts. We are thus forced to the conclusion that the route of AP generation follows that of ELH up to a point, namely, I → IIIA → IIIB → AP.

The entire processing sequence can be regarded as consisting of two early cleavage steps followed by three later ones. The early steps serve to generate an intermediate form of each of the final products (IIA, IIIB, and IV) while the later steps convert each intermediate into the final product form (IIB, AP, and ELH). This scheme suggests a rationale for the existence of multistep processing, as the number of steps identified here is precisely the minimum number that would be required to generate the three products, provided that each must pass through an intermediate form. The question of the functional significance of the intermediate forms is not addressed directly by the present data. It may be that the cleavage points of the protein I precursor lie within regions that have no functional significance for the products and that these "tails" must be removed for full product function. A more intriguing possibility is that the "tails" represent "signal" sequences for intracellular segregation or transmembrane insertion (Blobel, 1980).

An additional explanation is required for the prolonged existence of the IIIA intermediate as compared to the relatively rapid cleavage of the IIA species. This may have to do with the subcellular disposition of the IIIA products which enter neurosecretory granules for axonal transport and, ultimately, secretion. The prolonged existence of this molecular species may reflect a role in the differential translocation of its products to a subcellular compartment from which they can gain access to the interior of granules. This would be consistent with the notion that peptides smaller than 65–70 amino acids are not capable of transmembrane insertion (Steiner et al., 1980). By this line of reasoning, the multistep processing sequence described here is entirely compatible with the extended form of the "signal" hypothesis (Blobel, 1980) provided that two translocation or segregation steps are required to package the secretory products.

The most unexpected finding in the present study was the discovery of multiple copies of the AP peptide in the precursor. It is clear that the copy residing in the IIIA domain is processed to a final product form, but the fate of the second copy remains in doubt. The majority of peak II protein or proteins in which this copy resides do not appear to be subjected to axonal transport [Arch, 1972; but see Loh et al. (1975)], and their kinetic behavior suggests that they form a large pool in the cell body which turns over slowly (Berry, 1981). It seems likely that peak II represents an end product which is degraded, rather than processed, but the data do not rule out the possibility of processing to the AP form at a rate that would not be visible in the pulse–chase studies done to date. The latter hypothesis is readily testable in that it would predict a steady-state bag cell content of AP twice that of ELH.

The existence of multiple copies of a given peptide within the sequence of its precursor and the differential processing of those copies is not unprecedented. A strictly analogous situation exists in the case of proopiomelanocortin, which contains three (nonidentical) copies of the peptide MSH (Nakanishi et al., 1979) not all of which are processed to product peptides (Roberts et al., 1978; Mains & Eipper, 1980). Furthermore, gene duplication provides a readily conceivable mechanism by which such a precursor sequence could arise. In this respect, it is interesting that the data in Figure 3 suggest that all of the AP fragments may be present in unaltered form in peak II. This would imply that the entire structure of AP is present and, thus, that it is likely to be internal to the sequence of a peak II protein. Since both AP and ELH coexist in protein IIIA, which cannot be much larger than the sum of these two peptides, the transfer of the AP sequence must have been accomplished with some degree of precision.

One possible reason for such a precise transfer could be that neurons other than the bag cells possess proteins that are similar to those of peak II and effectively process them to yield AP or some variant thereof. This would be analogous to the differential processing of proopiomelanocortin in different regions of the pituitary (Roberts et al., 1978; Mains & Eipper, 1980). In this regard, it is of interest that a number of neurosecretory cells of the abdominal ganglion synthesize and process precursor molecules of about the same molecular weight as peak II (Berry, 1978). If this is the case, then some mechanism must exist in the bag cells to prevent or delay cleavage of peak II. While there are several conceivable ways by which such a processing block might be accomplished, the charge heterogeneity of peak II proteins suggests that this could be due to a modification reaction, such as phosphorylation, glycosylation, or acetylation.

The data presented here and in the preceding report provide a relatively straightforward outline of ELH processing at the molecular level. However, the picture that emerges is a complex one, and several issues need to be resolved, including the functional significance of intermediates, the fate of the second AP copy, subcellular sites of cleavage and translocation, and the structural relationships between these proteins and other *Aplysia* neurosecretory peptides. Detailed examination of these problems should throw light on the mechanisms and functional significance of multistep proteolytic processing systems in general.

Acknowledgments

We thank S. Arch for helpful discussion, A. Telser for generous advice and for comments on an earlier version of the manuscript, and Bill Goosens and Y. Geinisman for photographic assistance.

References

Arch, S. (1972) *J. Gen. Physiol. 60*, 102–119.
Arch, S., Smock, T., & Earley, P. (1976) *J. Gen. Physiol. 68*, 211–225.
Berry, R. W. (1978) in *Biochemistry of Characterised Neurons* (Osborne, N. N., Ed.) pp 283–308, Oxford, Pergamon Press.
Berry, R. W. (1981) *Biochemistry* (preceding paper in this issue).
Blobel, G. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1496–1500.
Chiu, A. Y., Hunkapiller, M. W., Heller, E., Stuart, D. K., Hood, L. E., & Strumwasser, F. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6656–6660.
Cleveland, D. W., Fischer, S. G., Kirschner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.
Edsall, J. T., & Wyman, J. (1958) *Biophysical Chemistry*, pp 507–510, Academic Press, New York.
Elder, J. H., Pickett, R. A., Hampton, J., & Lerner, R. A. (1977) *J. Biol. Chem. 252*, 6510–6515.
Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.
Hare, P. E. (1977) *Methods Enzymol. 47*, 3–18.
Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 247–259.
Hunkapiller, M. W., & Hood, L. E. (1980) *Science (Washington, D.C.) 207*, 523–525.

Kasper, C. B. (1975) in *Protein Sequence Determination* (Needleman, S. B., Ed.) 8th ed., pp 114–161, Springer-Verlag, New York.
Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335–341.
Loh, Y. P., Sarne, Y., & Gainer, H. (1975) *J. Comp. Physiol. 100*, 283–296.
Mains, R. E., & Eipper, B. A. (1980) *Ann. N.Y. Acad. Sci. 343*, 94–110.
Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C. Y., Cohen, S. N., & Numa, S. (1979) *Nature (London) 278*, 423–427.
Randerath, K. (1970) *Anal. Biochem. 34*, 188–205.
Roberts, J. L., Phillips, M., Rosa, P. A., & Herbert, E. (1978) *Biochemistry 17*, 3609–3168.
Steiner, D. F., Quinn, P. S., Chan, S. J., Marsh, J., & Tager, H. S. (1980) *Ann. N.Y. Acad. Sci. 343*, 1–16.
Stephens, R. E. (1978) *Anal. Biochem. 84*, 116–126.
Yates, M. E., & Berry, R. W. (1981) *Gen. Comp. Endocrinol. 43*, 308–314.

Steroid–Protein Interactions. Human Corticosteroid Binding Globulin: Some Physicochemical Properties and Binding Specificity†

Kenneth E. Mickelson,*,‡ June Forsthoefel, and Ulrich Westphal

ABSTRACT: Reducing agents (dithiothreitol and β-mercaptoethanol) significantly decrease the affinity constants of the human corticosteroid-binding globulin (CBG)–cortisol complex in proportion to their concentration; the resulting K_a values are more consistent than those obtained in the absence of the reductants. The effect is reversible. The equilibrium association constants of the CBG complexes with cortisol and progesterone show a relatively broad pH maximum between pH 8 and 11. In this pH range, cortisol was found to be bound more strongly than progesterone; this relationship is reversed around pH 6. The van't Hoff plot of the temperature effect on K_a of the CBG–cortisol complex (4–41 °C) exhibits a nonlinear, possibly biphasic temperature dependency. The shape of the van't Hoff plot was similar in the presence of mercaptoethanol. The association of cortisol and progesterone to human CBG at 4 and 37 °C is enthalpy driven, compensating for the unfavorable change in entropy. Studies with 47 steroids served to elucidate the influence on binding affinity of polar and nonpolar groups and other structural alterations. The contribution of specific structural changes in the steroid molecule to the free energy of binding can be calculated from the results. Important structures for optimal binding are the 20-oxo group, a 10β-methyl group, and a double bond at the 4 position. A complementary image of the binding site with respect to the nature of binding at various locations is proposed.

The corticosteroid-binding globulin (CBG)[1] of human blood serum was the first high-affinity steroid-binding protein to be reported (Daughaday, 1956; Bush, 1957; Sandberg & Slaunwhite, 1958). CBG has been extensively purified and characterized from the sera of human, rabbit, and rat [for review, see Westphal (1971); Rosner, 1976; Ballard, 1979; Westphal, 1980] and, more recently, from guinea pig serum (Mickelson & Westphal, 1979). Guinea pig CBG has an affinity constant for cortisol that is 18 times larger than that for progesterone (Mickelson & Westphal, 1980). Human CBG, in contrast, binds both cortisol and progesterone with similar affinity (Stroupe et al., 1978). In the present study, the influence of reducing agents, temperature, and pH on the binding affinity has been investigated. Furthermore, the affinity constants of human CBG complexes with a number of steroids have been determined in order to define the structural features producing strong or weak interaction at various locations in the steroid molecule. A complementary image of the binding site may be deduced from the results. This information is expected to contribute to a systematic investigation of the nature of the binding site, an effort that has entered a new phase recently by the crystallization of the cortisol complex of guinea pig CBG and α_1-acid glycoprotein (McPherson et al., 1980), opening the way for direct visualization of a high-affinity steroid binding site by X-ray diffraction analysis. These crystallizations follow the preparation of a crystalline lead salt of α_1-acid glycoprotein (Schmid, 1953) and the crystallographic studies on the progesterone-binding uteroglobin (Buehner & Beato, 1978; Mornon et al., 1980).

Materials and Methods

Term human pregnancy serum was obtained from Norton-Children's Hospital (Louisville, KY). Amberlite XAD-2 was from Rohm and Haas; it was washed 10 times with an equal volume of methanol and then in a similar way with distilled water at room temperature. Endogenous steroids were removed by gently shaking 1 L of pregnancy serum with 200 mL (settled volume) of Amberlite XAD-2 resin for 8 h at room temperature. The serum was then filtered through a fritted disk funnel (coarse), dialyzed for 40 h against 50 mM sodium phosphate–0.5 M KCl (pH 9.0, 4 °C, 0.02% sodium azide),

† From the Department of Biochemistry, University of Louisville School of Medicine, Health Sciences Center, Louisville, Kentucky 40292. *Received March 31, 1981.* Our studies were supported by a grant from the National Institute of Arthritis, Metabolism and Digestive Diseases (AM-06369) and a research career award (U.W.) from the Division of General Medical Sciences (GM-K6-14138) of the U.S. Public Health Service. This is Report No. 44 in the series Steroid–Protein Interactions; for the preceding paper, see Mickelson & Westphal (1980). Part of this work has been presented previously (Mickelson et al., 1980).

‡ Present address: Immunochemistry Laboratory, Ames Division, Miles Laboratories, Inc., Elkhart, IN 46515.

[1] Abbreviations used: BME, β-mercaptoethanol; CBG, corticosteroid-binding globulin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; PBG, progesterone-binding globulin; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/81/0420-6211$01.25/0