Characterization of the High Molecular Weight Form of Epidermal Growth Factor*

(Received for publication, November 2, 1973)

JOHN M. TAYLOR,† WILLIAM M. MITCHELL,§ and STANLEY COHEN¶
From the Departments of Biochemistry, Microbiology, and Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

SUMMARY

In crude homogenates of the submaxillary gland of the male mouse, epidermal growth factor activity is found almost entirely in a high molecular weight complex. The complex has a molecular weight of about 74,000 and appears to be composed of 2 molecules of epidermal growth factor (6,045 molecular weight) and 2 molecules of a binding protein (29,300 molecular weight). The complex is stable only in the range of pH 5.0 to 8.0, and its behavior during isoelectric focusing, gel filtration, and ultracentrifugation suggests that it may be in a slow equilibrium with its subunits. In addition, the complex appears to undergo a concentration-dependent aggregation.

The biological activity of the high molecular weight complex is proportional to its fractional content of the low molecular weight factor. No evidence was found which indicated that the binding protein might affect the biological activity of the low molecular weight epidermal growth factor. It is postulated that the high molecular weight form of epidermal growth factor is an enzyme-product complex. The substrate is presumed to be the epidermal growth factor precursor which is recognized by a specific arginine esterase, the binding protein.

Epidermal growth factor is a polypeptide isolated from the submaxillary glands of adult male mice (1) which stimulates the proliferation and keratinization of various epidermal tissues in vivo and in vitro (2). Among the major metabolic events in epidermal tissue affected by EGF are the stimulation of protein and ribonucleic acid synthesis (3), polysome formation (4), and ornithine decarboxylase induction (5). Studies on the mechanism of action of EGF have recently been reviewed (6).

The major physical and chemical properties of EGF have been reported (7). EGF is a single chain polypeptide of 6045 molecular weight with an isoelectric point at pH 4.60. It is further characterized by the absence of lysine, alanine, and phenylalanine residues. It contains no free sulfhydryl groups and no carbohydrate side chain. The complete amino acid sequence of EGF and the location of the three disulfide linkages have been recently determined (8, 9).

Although the biologically active form of EGF is a relatively small polypeptide, Taylor et al. (10) have isolated a high molecular weight form of EGF from the submaxillary glands of adult male mice. The HMW-EGF has been shown to be composed of two components (10), the low molecular weight EGF, and an EGF-binding protein of molecular weight 29,300. The two components could be separated by ion exchange chromatography and then recombined to give a high molecular weight complex, similar in size to the native complex. In this communication the major properties of the HMW-EGF complex are described.

EXPERIMENTAL PROCEDURE

High Molecular Weight EGF—Native HMW-EGF was prepared from homogenates of the submaxillary glands of adult male albino mice as previously described (10). Recombined HMW-EGF was prepared from EGF and EGF-binding protein (10).

Gel Filtration—All gel filtration experiments were performed at 5° by the reverse flow methods with Sephadex G-100 (Pharmacia). The elution buffer consisted of 0.01 M sodium acetate, pH 5.9, containing 0.1 M sodium chloride. In a typical experiment, the sample, in a volume equal to 1 to 2% of the column bed volume, was added to the column by siphoning. All column eluates were monitored by an ISCO flow monitor at 280 nm.

For molecular weight estimations, the columns were calibrated according to the procedures described by Whitaker (11). The protein standards used for calibration were from Mann Research Laboratories, Kit S100A, with the exception of ribonuclease A (Sigma) and pancreatic trypsin inhibitor (Worthington). Column void volumes (V0) were determined by using blue dextran (Pharmacia). Individual sample elution volumes (V0) were measured by weight determinations of the eluate. The ratio of \( V0 : Vw \) was experimentally determined for each protein standard and plotted against the logarithms of the known molecular weights. The resultant calibration curve was then used to estimate unknown molecular weights from experimentally determined elution volumes.

Analytical Sedimentation Equilibrium—High speed sedimentation equilibrium determinations were performed with a Spinco

* This study was supported by United States Public Health Service Grants HD 00760 and AM 09823. This work was taken in part from a dissertation by J. M. T. submitted to the Graduate School, Vanderbilt University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† National Institutes of Health Graduate Trainee (2-T01-AM-5441). Present address, Department of Pharmacology, Stanford University School of Medicine, Palo Alto, California 94305.
§ Recipient of a Career Development Award from the National Institute of Arthritis and Metabolic Diseases.
¶ To whom reprint requests should be addressed.
1 The abbreviations used are: EGF, epidermal growth factor; HMW-EGF, high molecular weight epidermal growth factor.
model E analytical ultracentrifuge equipped with Rayleigh interference optics according to the meniscus depletion method of Yphantis (13). Measurements were made at 15o with protein concentrations ranging between 0.2 and 0.8 mg per ml. The centrifuge runs were made for 20 to 24 hours. Molecular weights were calculated on mass distribution as a function of the accumulated fringe displacement in the liquid column at any point r. Low speed sedimentation equilibrium studies were performed with a split-beam photoelectric scanning system and a multiplex accessory. Scans were made at 280 nm using the slow scanning speed. Solutions of HMW-EGF were exhaustively dialyzed against 0.1 M sodium acetate (pH 5.60), and a 3-mm liquid column of sample was layered over perfluorotributylamine (FC43, Minnesota Mining and Manufacturing Co.) in the solution section of the cell with dialysate being added to the solvent sector. Determinations were made at 20o with a rotor velocity of 11,000 rpm. Runs were made for 24 to 36 hours before scanning. Different protein solutions were examined over a 4-fold concentration range.

The apparent weight average molecular weights were calculated in the usual way (12, 14) assuming a partial specific volume of 0.74.

Additional Physical Methods The extinction coefficient, sedimentation velocity, diffusion coefficient, and circular dichroic spectra were determined as previously described (7, 14).

Protein Determination—The protein concentrations of various solutions were estimated by the procedure of Lowry et al. (15) with bovine serum albumin (Armour) as a standard. Concentrations were also measured by using the experimentally determined extinction coefficient.

Cellulose Acetate Electrophoresis—Sepharose III cellulose acetate strips (Gelman) were used according to the manufacturer's directions (Technical Bulletin 7076-B). Protein samples in 10-μl volumes were subjected to 200 volts of constant voltage for 2 hours at room temperature. The proteins were stained with 0.2% ponceau S (Colab) in 3% trichloroacetic acid.

Isoelectric Focusing Isoelectric points were determined by the method of isoelectric focusing (7). The pH of the HMW-EGF solution was kept within the range of pH 5 to 7 during the preparatory steps; this solution was not allowed to come into direct contact with the electrode solutions.

Assay for EGF Activity—EGF activity was measured both in vivo, by means of the eyelid-opening assay described by Cohen (1), and in vitro, by its ability to induce ornithine decarboxylase in epidermal organ cultures as described by Stastny and Cohen (5).

RESULTS

Cellulose Acetate Electrophoresis—The stability of HMW-EGF under different pH conditions was investigated by electrophoresis on cellulose acetate strips. Samples containing 20 μg of protein were examined at pH 4.4, 5.1, 8.0, and 8.8. The results of these experiments are illustrated in Fig. 1. Only a single, sharp band of protein was observed at pH 5.1 and pH 8.0, suggesting that the HMW-EGF was stable over the pH range of 5 to 8. However, at pH 4.4 and pH 8.8, two bands of protein were observed, suggesting an apparent dissociation of the sample. In both cases, comparison with known standards identified the thin leading band as low molecular weight EGF, and the broader trailing band as the EGF-binding protein.

Isoelectric Point—The isoelectric point of HMW-EGF was determined by the isoelectric focusing technique. The experiment was performed with a 4.0-mg sample in a pH gradient of 5 to 7 at 5o. The result, as shown in Fig. 2, indicated that the HMW-EGF had undergone partial dissociation. The major peak at pH 5.40 was HMW-EGF and formed a precipitate with antibody to the low molecular weight EGF. The minor peak at pH 5.60 was assumed to be dissociated EGF-binding protein by virtue of its isoelectric point identity. This minor peak had little ability to precipitate the EGF antibody; the slight reaction which occurred was probably due to contaminating HMW-EGF. No other peaks above background absorbance were observed. In previous experiments, it was shown that isoelectric focusing of HMW-EGF in different pH gradients, where the average pH of the ampholyte solution was below pH 5, resulted in complete dissociation of the protein into its subunits (10).

Estimation of Molecular Weights by Gel Filtration—Gel filtration experiments for molecular weight studies employed a column of Sephadex G-100 which had been previously calibrated with proteins of known molecular weight (7, 11, 14). In separate experiments the molecular weights of both native and recombinant HMW-EGF were estimated to be approximately 55,000 to 60,000, assuming a typical globular configuration.

The elution profiles of native HMW-EGF and recombinant HMW-EGF showed significantly skewed peaks. All portions of
Fig. 3. Gel filtration elution profile of high molecular weight epidermal growth factor. A sample containing 4 mg of HMW-EGF in 2 ml of elution buffer (0.01 M sodium acetate, pH 5.9, and 0.1 M sodium chloride) was applied to a Sephadex G-75 column (1.5 X 55 cm) equilibrated with the same buffer. The flow rate was 2.0 ml per cm² per hour and 3-ml fractions were collected. The elution profile of the native HMW-EGF is illustrated. The three tubes immediately to the left of the dashed line were pooled and termed Leading Edge a. The four tubes immediately to the right of the dashed line were pooled and termed Trailing Edge b. Inset, rechromatography of the Leading Edge a yielded Peak a; rechromatography of the Trailing Edge b yielded Peak b'. When Peak a' was divided into Leading Edge and Trailing Edge fractions, rechromatography of the Leading Edge yielded an elution peak having the same skewed character.

**TABLE I**

Molecular weight of high molecular weight epidermal growth factor by sedimentation velocity and diffusion coefficients

| Concentration | Observed sedimentation coefficient | Observed diffusion coefficient | Molecular weight |
|---------------|-----------------------------------|-------------------------------|-----------------|
| mg/ml         | $S_{20,w} \times 10^{12}$ s⁻¹      | $D_{20,w} \times 10^{10}$ cm² s⁻¹ |                 |
| 6.6           | 4.68                              | 5.59                          | 77,800          |
| 4.3           | 4.69                              | 5.55                          | 77,900          |
| 3.3           | 4.73                              | 6.00                          | 73,000          |
| 2.2           | 4.77                              | 6.50                          | 67,900          |
| 0°            | 4.81                              | 6.70                          | 66,500          |

* Infinite dilution (extrapolated to zero concentration).

the skewed peaks possessed the ability to form a precipitate with the antibody to the low molecular weight EGF. In separate experiments, employing a calibrated column of Sephadex G-75, rechromatography of either the leading edge or the trailing edge of the HMW-EGF eluate yielded peaks having the same skewed character with essentially the same elution volumes (illustrated in Fig. 3). These data suggested that HMW-EGF underwent an apparent reversible dissociation under the conditions employed for gel filtration. The exact nature of this dissociation phenomenon is not clear.

**Extinction Coefficient**—The values for $E_{280}^{1%}$ at 280 nm of the native HMW-EGF and of the recombined HMW-EGF were determined to be 19.1 and 19.6, respectively. The protein concentrations were estimated from the interference fringe counts in the ultracentrifuge synthetic boundary cell.

**Sedimentation Velocity**—High speed sedimentation velocity measurements on native HMW-EGF over the concentration range of 2.2 to 6.6 mg per ml showed a symmetrical peak with a value of 4.81 ± 0.02 S for the $S_{20,w}$ (Table I). However, careful examination of the schlieren base-line revealed a trace of more slowly sedimenting material. Over this limited concentration range, no major dependence of sedimentation coefficient on protein concentration was observed, indicating a symmetrical sedimenting mass.

**TABLE II**

Molecular weight of native and recombined high molecular weight epidermal growth factor by high speed sedimentation equilibrium analysis

|                  | Native HMW-EGF* | Recombined HMW-EGF⁺ |
|------------------|-----------------|---------------------|
| Concentration    | Molecular weight| Concentration        | Molecular weight |
| mg/ml            |                 | mg/ml               |                 |
| 0.6              | 73,600          | 0.8                 | 74,500          |
| 0.4              | 74,900          | 0.6                 | 67,200          |
| 0.3              | 75,500          | 0.5                 | 70,100          |
| 0.2              | 75,200          | 0.4                 | 77,100          |
| Average          | 74,800 ± 700    | Average             | 72,200 ± 3,800  |

* Average speed was 23,160 rpm.
⁺ Average speed was 26,980 rpm.

Fig. 4. Typical high speed sedimentation equilibrium plot of ln concentration against $r^2$ for the high molecular weight epidermal growth factor.

**Diffusion Coefficient**—Diffusion coefficient measurements on HMW-EGF over the concentration range of 2.2 to 6.6 mg per ml yielded a value of $6.70 \times 10^{-7}$ cm² s⁻¹ for the $D_{20,w}$ (Table I). A slight dependence of diffusion coefficient on protein concentration was observed, with increasing values for $D_{20,w}$ at decreasing sample concentrations.

Substitution into the classical Svedberg equation ($M_w = RTs/(1 - v\phi)D$) of the values for $S_{20,w}$ and $D_{20,w}$ observed at different protein concentrations yielded values for the molecular weight which decreased with more dilute protein concentrations (Table I). These data suggested that HMW-EGF may be dissociating into lower molecular weight forms in dilute solutions.

**High Speed Sedimentation Equilibrium**—The results of molecular weight determination by the meniscus depletion method of sedimentation equilibrium analysis are shown in Table II. The HMW-EGF was observed to have an average molecular weight of 74,800 ± 700 and the recombined HMW-EGF had an average molecular weight of 72,200 ± 3,800. These data indicate a close similarity in molecular size. Typical plots of log concentration...
Table III
Molecular weight of native and recombinant high molecular weight epidermal growth factor by low speed sedimentation equilibrium analysis

| Concentration (mg/ml) | Native HMW-EGF | Recombined HMW-EGF |
|-----------------------|----------------|-------------------|
|                       | Whole column   | Meniscus  | Base       | Whole column | Meniscus | Base       |
| 0.4                   | 60,300         | 51,500   | 65,500     | 61,100       | 49,100   | 67,800     |
| 0.3                   | 58,900         | 51,800   | 67,400     | 58,000       | 53,500   | 65,400     |
| 0.2                   | 61,200         | 54,400   | 70,700     | 57,600       | 49,500   | 65,600     |
| 0.1                   | 54,500         | 46,600   | 65,500     | 54,400       | 46,600   | 65,500     |
| Average               | 58,700         | 51,100   | 67,300     | 58,000       | 51,100   | 66,000     |

Fig. 5. Typical low speed sedimentation equilibrium plot of In concentration in terms of optical density against $r^2$ for the high molecular weight epidermal growth factor.

against $r^2$ revealed no significant heterogeneity for either complex (Fig. 4).

Low Speed Sedimentation Equilibrium—The results of molecular weight determinations on the native HMW-EGF and on the recombinant HMW-EGF by low speed sedimentation equilibrium analysis at 11,000 rpm using photoelectric scanning optics are shown in Table III. Graphs of log concentration against $r^2$ were analyzed in detail (Fig. 5). Values for the apparent molecular weight over the entire liquid column were approximately 58,000 for both the native and recombinant HMW-EGF, indicating a close similarity in molecular size. However, for both proteins, values for the apparent mass at the meniscus were approximately 7,000 daltons lower, and at the base of the liquid column the values were approximately 7,000 daltons higher than the average value over the entire liquid column. These data suggested that HMW-EGF was dissociating into lower molecular weight forms under the experimental conditions employed. The observation that both the native and recombinant forms of HMW-EGF underwent the same dissociation phenomenon suggests a close similarity in quaternary structure and composition.

Circular Dichroism—The circular dichroic spectra of HMW-EGF and the recombinant HMW-EGF are shown in Fig. 6. Both spectra are essentially identical, indicating a close similarity in secondary structure. The most significant CD feature is the minimum occurring at 201 nm having an ellipticity of ~8200 deg-cm$^2$ per dmole, suggesting a predominantly nonhelical conformation. The decreased strength of this band, as compared to randomly coiled model polypeptides, indicates the probable existence of some helical structure. This is supported by the negative dichroism near 220 to 225 nm with an ellipticity of ~1500 deg-cm$^2$ per dmole at 222 nm. The origin of this weak dichroic activity cannot be assigned adequately, however, since the spectra is not adequately resolved. The slight difference in the circular dichroic behavior between the two proteins at longer wavelengths is probably within experimental error.

Biological Activity of High Molecular Weight Epidermal Growth Factor in Vivo—By means of the eyelid-opening assay described by Cohen (11), HMW-EGF was observed to be biologically active (Table IV). Approximately 6-fold greater amounts of HMW-EGF were required to produce the same effect as originally observed with the low molecular weight EGF. This observation was in agreement with the physically determined results which suggested that the low molecular weight EGF was approximately one-sixth by weight of the high molecular weight complex (10). The EGF-binding protein alone possessed no EGF activity. Tentatively, it would appear that the presence of the
TABLE IV
Effect of injection of high molecular weight epidermal growth factor into newborn mice

| Time of eyelid opening | Dose required |
|-----------------------|---------------|
|                       | Low molecular weight EGF | HMW-EGF |
| ____________________ |  | ____________________ |
| 7                     | 2.7 | 20 |
| 8                     | 1.3 | 5-10 |
| 9                     | 0.67 | 2.5 |
| 12                    | 0 | 0 |

* Data reported by Cohen (1).
+ Animals injected were littermates, with two animals used for each dose level.

TABLE V
Effect of high molecular weight epidermal growth factor on ornithine decarboxylase activity in epidermis cultured in vitro

| HMW-EGF | concentration | Ornithine de- | Low molecular weight EGF | Ornithine de- |
|---------|--------------|---------------|--------------------------|---------------|
| ug/ml   | ug/g body weight | activity | ug/ml | activity |
| 0       | 0.1          | 0.1           | 0.1          | 0.1           |
| 0.1     | 0.3          | 0.01          | 0.01         | 0.3           |
| 1.0     | 1.4          | 1.1           | 1.1           | 1.1           |

* A concentration of 5.0 µg per ml of EGF-binding protein showed no increase over the control value for enzyme activity induced.
+ A concentration of 5.0 ug per ml of EGF-binding protein reported no increase in enzyme activity.

The properties of the high molecular weight complex of EGF and its binding protein have been examined. The complex as a physical entity has a limited stability. It will dissociate into its components when desorbed to an ion exchange column (10) and whenever the solution pH exceeds the range of pH 5 to 8. The behavior of the complex during isoelectric focusing (under allowable pH conditions) and gel filtration on Sephadex columns suggests that the complex may be in a slow equilibrium with its subunits. A similar equilibrium state has also been observed for the high molecular weight nerve growth factor complex (16). Understanding of the physical state of HMW-EGF is complicated by the observation that the complex itself appears to undergo a concentration-dependent association. This situation is indicated by the low speed sedimentation equilibrium data and also by the gel filtration behavior. The sedimentation velocity-diffusion and high speed equilibrium studies suggest that the aggregate form has a plateau limit at approximately 72,000 to 74,000 molecular weight.

These data suggest that the aggregated form of the complex is composed of 2 molecules of low molecular weight EGF and 2 molecules of EGF-binding protein. This form of the complex is that which is isolated from the submaxillary glands. However, the complex in vivo may actually be composed of only one EGF and one binding protein.

When the biological activity of the HMW-EGF was examined by the eyelid-opening assay of Cohen (1), HMW-EGF was observed to have approximately one-sixth the biological activity of an equal weight of the low molecular weight EGF. Furthermore, HMW-EGF showed a dose-response effect in this assay similar to that of the low molecular weight EGF. These observations agree with the physical data which indicate that the low molecular weight EGF is one-sixth, by weight, of the HMW-EGF complex. The results of the tissue culture bioassay were consistent with these findings. No evidence was found in either assay system which indicated that the EGF-binding protein might affect the biological activity of the low molecular weight EGF. It is not likely, therefore, that the EGF-binding protein is involved in a direct regulation of the activity of the biologically active low molecular weight EGF.

The EGF-binding protein has been shown to be an arginine esterase (10,14). The observation that the low molecular weight EGF possesses a COOH-terminal arginine suggests that EGF may be generated from a precursor protein via the possible proteolytic action of the EGF-binding esterase. It seems reasonable to suggest, therefore, that the HMW-EGF structure is an enzyme (binding protein)-substrate (low molecular weight EGF) complex that has been isolated because of sufficiently strong, noncovalent peptide chain interactions. It could be postulated that the substrate, in vivo, is an inactive precursor of EGF. A specific arginine esterase "recognizes" the substrate at a specific amino acid sequence signal point and performs a restricted proteolytic cleavage at an arginine residue. In the case of EGF product, it remains loosely associated with the esterase (binding protein) such that it can be isolated from tissue homogenates as a high molecular weight complex. Such a restricted proteolytic cleavage may represent a post-translational regulatory event. A similar sequence of events might also explain the existence of the high molecular weight nerve growth factor complex; common antigenic determinants have been demonstrated recently between the nerve growth factor and EGF-associated arginine esterases (14).

REFERENCES
1. Cohen, S. (1962) J. Biol. Chem. 237, 1555-1560
2. Cohen, S. (1964) Nat. Cancer Inst. Monogr. 13, 13
3. Hoobler, J. K., and Cohen, S. (1967) Biochem. Biophys. Acta 158, 347-356
4. Cohen, S., and Stastny, M. (1968) Biochem. Biophys. Acta 166, 427-437
5. Stastny, M., and Cohen, S. (1970) Biochem. Biophys. Acta 204, 378-389
6. Cohen, S., and Taylor, J. M. (1972) Soc. Develop. Biol. Symp., in press
7. Taylor, J. M., Mitchell, W. M., and Cohen, S. (1972) J. Biol. Chem. 247, 5028-5034
8. Savage, C. R., Jr., Inagami, T., and Cohen, S. (1972) J. Biol. Chem. 247, 7612-7621
9. SAVAGE, C. R., JR., HASH, J. H., AND COHEN, S. (1973) *J. Biol. Chem.* 248, 7669–7672
10. TAYLOR, J. M., COHEN, S., AND MITCHELL, W. M. (1970) *Proc. Natl. Acad. Sci. U. S. A.* 67, 164–171
11. WHITAKER, J. R. (1963) *Anal. Chem.* 35, 1950
12. CHERVENKA, C. H. (1969) *A Manual of Methods for the Analytical Ultracentrifuge*, Beckman Instruments, Inc. Palo Alto
13. YPHANTIS, D. A. (1964) *Biochemistry* 3, 297
14. TAYLOR, J. M., MITCHELL, W. M., AND COHEN, S. (1974) *J. Biol. Chem.* in press
15. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951) *J. Biol. Chem.* 193, 265–275
16. GREENE, L. A., SCHOOTER, E. M., AND VARON, S. (1969) *Biochemistry* 8, 3735–3741
Characterization of the High Molecular Weight Form of Epidermal Growth Factor
John M. Taylor, William M. Mitchell and Stanley Cohen

*J. Biol. Chem.* 1974, 249:3198-3203.

Access the most updated version of this article at [http://www.jbc.org/content/249/10/3198](http://www.jbc.org/content/249/10/3198)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/249/10/3198.full.html#ref-list-1](http://www.jbc.org/content/249/10/3198.full.html#ref-list-1)