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Purification of a HeLa Cell High Molecular Weight Actin Binding Protein and Its Identification in HeLa Cell Plasma Membrane Ghosts and Intact HeLa Cells†

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ABSTRACT: The high molecular weight protein (HMWP) which was previously observed to be a major component of the actin based gels formed by incubating cytoplasmic extracts of HeLa cells at 25 °C [Weihing, R. R. (1977) *J. Cell Biol. 75*, 95–103] has now been purified by gel filtration of 0.6 M KCl extracts of precipitated gels. A few hundred micrograms of HMWP, which is about 90% pure, can be isolated from 4 × 10^9 cells. HMWP can gel muscle actin and cross-link it into filament bundles. Its subunit molecular weight is 250 000, its Stokes radius is 125 Å, and its sedimentation coefficient is 9 S. A native molecular weight of 480 000 was calculated by using the latter two parameters, and therefore the native molecule is a dimer. Its amino acid analysis is nearly indistinguishable from that of macrophage actin binding protein and of mammalian and avian filamins. All of these findings indicate that HMWP is homologous to the latter proteins. However, HeLa cell HMWP and avian filamin must differ in their primary sequences because their partial peptide maps are distinct and because an antiserum against HMWP reacts only weakly with filamin. For studies on the intracellular location of HMWP, a goat antiserum against purified HMWP was prepared and characterized and then used to localize HMWP in suspension grown cells. The technique of immunoblotting revealed that the antiserum reacted virtually exclusively with the high molecular weight polypeptide that comigrates with HMWP in cell lysates and in $ZnCl_2$-stabilized plasma membrane ghosts prepared from HeLa cells [Gruenstein, E., Rich, A., & Weihing, R. R. (1975) *J. Cell Biol. 64*, 223–234] and that it did not react with rabbit myosin heavy chain, microtubule proteins (MAPS and tubulin) from HeLa cells and calf brain, or the proteins of human erythrocyte ghosts including spectrin. Suspension-grown cells which were stained with the antiserum by the technique of indirect immunofluorescence showed bright fluorescence at the rim of the cells and less intense generalized fluorescence. If preimmune serum or immune serum treated with HMWP was substituted for the immune serum, then staining at the rim was not observed, but the generalized fluorescence was only slightly reduced; unpermeabilized cells were not stained. These results indicate that HMWP is a component of the cortical cytoplasm of HeLa cells. Possible functions of cortical HMWP are discussed briefly.

Eukaryotic cells contain a variety of actin binding proteins that are believed to influence the organization and function of the actin-based microfilament system [reviewed in Schliwa (1981) and Weeds (1982)]. This laboratory has investigated an actin binding protein of HeLa cells through studies of the actin-based gelation of cytoplasmic extracts of HeLa cells. These studies produced three lines of evidence suggesting that gelation could be explained, at least in part, by cross-linking of actin into a three-dimensional network by a protein designated HMWP.[1] First, electrophoretic analysis showed that actin and HMWP are prominent polypeptide components of isolated gels (Weihing, 1976a,b, 1977). Second, dilution of cell extract (Weihing, 1977) or treatment of cell extract with micromolar concentrations of cytochalasin B (Weihing, 1976b) inhibits gelation and concomitantly prevents the increase in sedimentability of actin and HMWP which always accompanies gelation. Finally, heavy meromyosin inhibits gelation and concomitantly prevents the increase in sedimentability of HMWP (Weihing, 1977), possibly by competing with HMWP for sites on actin. I now report the purification and partial characterization of HMWP from HeLa cells, its identification in plasma membrane ghosts, and its localization in situ in cells grown in suspension.

Experimental Procedures

General Methods and Reagents. Growth and harvesting of cells, protein analysis, testing of gelation, gel electrophoresis, and preparation of myosin were done as described previously (Weihing, 1977). Muscle actin was purified from an acetone

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[1] Abbreviations: HMWP, high molecular weight protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; NaDodSO$_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; TLCK, tosyllysine chloromethyl ketone; DTE, dithioerythritol; DE-52, microgranular diethylaminoethylcellulose, manufactured by Whatman; Tris, tris(hydroxymethyl)aminomethane.

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powder of rabbit skeletal muscle by using the method of Spudich & Watt (1971) through the steps of collecting the actin after it was incubated in 0.6 M KCl, suspended in their buffer A, and dialyzed overnight against buffer A. The actin was used within 1 week of preparation. α-Actinin from pig muscle was kindly provided by Dr. D. E. Goll. HMWP was purified from HeLa cells as described under Results. Filamin was prepared from chicken gizzards (provided by Dr. Harriet Robinson) by using the method of Feramisco & Burridge (1980). Bovine serum albumin, α-chymotrypsinogen, bovine fibrinogen, bovine thyroglobulin, apoferritin, and phosphorylase *a* were all purchased from Boehringer-Mannheim. Triton X-100 was the product of Rohm and Haas. Trasylol was purchased from Mobay Chemical Corp., New York, NY. Bio-Gel A15m was purchased from Bio-Rad Laboratories, Richmond, CA. Blue Dextran D-2000 was from Pharmacia.

Negative-Staining Electron Microscopy. HMWP eluted from Bio-Gel (Results) was first concentrated by using an Amicon (Lexington, MA) Diaflo XM100A membrane. Then a small amount (5–10% of the total final volume) of F-actin (polymerized with 0.1 M KCl and 2 mM $MgCl_2$) was added to the HMWP and incubated at 25 °C for 10 min. For the control sample, standard buffer[2] was substituted for HMWP. Incubated samples were then fixed with an equal volume of 1% glutaraldehyde in water, adsorbed for 2 min to 400 mesh, carbon-coated grids (Ladd Research Industries, Burlington, VT) which had been subjected to glow discharge for 3–5 min, rinsed with water, and treated for 1 min with 1% uranyl acetate in water (Weatherbee et al., 1980).

Stokes Radius. Stokes radius was measured by gel filtration on Bio-Gel A15m. Data were analyzed by using the correlation of Laurent & Killander (1964) as transformed by Siegel & Monty (1966). The parameters required for this analysis were determined as follows. The elution volume of Blue Dextran D-2000 was taken as the void volume, and the elution volume of the KCl present in the sample (located by conductivity measurements) was taken as the total column volume. The following proteins (and their Stokes radii) were used for calibration: bovine fibrinogen (107 Å); bovine thyroglobulin (85 Å); apoferritin (61 Å); aldolase (45 Å); ovalbumin (27.3 Å). Proteins were located initially by their positions on the trace produced by an Isco (Lincoln, NE) column monitor, and location was confirmed by protein and gel electrophoretic analysis. Calibration samples were prepared as follows. Mixtures of calibration proteins (about 2 mg/mL) and Blue Dextran D-2000 (about 1 mg/mL) prepared in standard buffer[2] plus 0.6 M KCl were incubated at 0–4 °C for 15 min and clarified by centrifugation in a Beckman 50 Ti rotor at 35000 rpm for 30 min at 0–4 °C. One milliliter of calibration solution was applied to a 50 × 2.6 cm column of Bio-Gel A15m and eluted with standard buffer at 0–4 °C. The Stokes radius was determined by extrapolating the least-squares line calculated for the plot of $(-\log K_{av})^{1/2}$ against R_s.

Sedimentation Coefficient. Bio-Gel purified HMWP was concentrated to 0.5 mg/mL by using an Amicon CS-15 miniconcentrator (Amicon Corp., Lexington, MA). Two hundred microliter samples containing 100 μg of HMWP alone or a mixture of 100 μg of HMWP plus 100 μg each of catalase ($s_{20,w}$ = 11.2 S) and aldolase ($s_{20,w}$ = 8.0 S) and 66 μg of phosphorylase *a* ($s_{20,w}$ = 13.7 S) or a mixture of the same amount of each of the three calibration proteins were applied to separate 5–20% sucrose gradients and centrifuged at 35000 rpm for 14.6 h at 0–4 °C in an SW56 rotor. All samples and gradients were in standard buffer. At the end of the centrifugation about 35 fractions were collected by pumping from the bottom of the tube, and identical aliquots of each fraction were analyzed for protein content and composition by protein and gel electrophoretic analysis. The sucrose concentration of the fractions was measured with a Bausch & Lomb refractometer. The sedimentation coefficient of HMWP was estimated by interpolating the position of HMWP on the gradient onto the graph of $s_{20,w}$ vs. gradient fraction for the standard proteins (Martin & Ames, 1961).

Amino Acid Analysis. Bio-Gel purified HMWP was mixed with an equal volume of cold, 100% trichloroacetic acid and incubated at 0–4 °C overnight. The resultant precipitate was collected by centrifugation at 10000 rpm in a Sorvall SS-34 rotor at 0–4 °C, washed 4 times with 95% ethanol, and dried. A 50-μg sample and an 85-μg sample were hydrolyzed for 22 h at 105 °C in 6 N HCl in a sealed, evacuated tube. The 50-μg sample was analyzed for total amino acid composition on a standard single column amino acid analyzer. The 85-μg sample was analyzed for content of basic amino acids including methylated lysines and histidines by using procedures similar to those described previously (Huszar & Elzinga, 1969; Huszar, 1972).

Peptide Maps. Freshly isolated HMWP and filamin were subjected to partial proteolytic digestion according to Cleveland et al. (1977). To prepare for digestion, the proteins were first concentrated with a Minicon CS-15 concentrator to a measured concentration of 0.5 mg/mL, and then 15-μg samples were mixed with 0.25 volume of a solution containing enough Tris and $NaDodSO_4$ to give final concentrations of 0.5% $NaDodSO_4$ and 0.125 M Tris-HCl, pH 6.8. Small volumes ($^1/_{20}$ the volume of the $NaDodSO_4$–protein mixtures) of α-chymotrypsin or papain (activated as described in The Worthington Manual) were immediately added to the final concentrations listed in the legend to Figure 4, the mixtures were incubated at 37 °C for 10 min, and the reaction was stopped by adding an equal volume of 2-fold concentrated electrophoresis sample buffer (made without Tris and substituting 0.8 M sucrose for the glycerol of the Laemmli formulation) and boiling for 2 min. Peptides were separated on 15% acrylamide slab gels prepared according to Laemmli (1970) and fixed and stained according to Fairbanks et al. (1971).

Isolation of Plasma Membrane Ghosts. Plasma membrane ghosts were isolated by the method of Brunette & Till (1971), modified as follows. PMSF (1 mM) and TLCK (0.1 mM) were included in the 0.15 M NaCl used to wash the cells and the 1 mM $ZnCl_2$ used to lyse the cells. The crude ghost fraction was then collected by centrifugation at 1400 rpm followed by a second centrifugation at 2800 rpm as previously described (Gruenstein et al., 1975). The ghosts were found in a distinct layer at the top of the pellet and could be suspended in upper phase (Brunette & Till, 1971) by careful scraping with a Pasteur pipet. Ghosts derived from 500 × 10^6 cells were suspended in 10 mL of upper phase, transferred to a 40-mL centrifuge tube, mixed by inverting 10 times with an equal volume of lower phase, and centrifuged in a Sorvall HB-4 rotor at 8500 rpm for 10 min at 0–4 °C. The ghosts were found in a pad at the interface of the upper and lower phases and could be removed in a minimal volume of upper and lower phases by using a 5-mL pipet controlled with a propipet. They were then diluted with 5 volumes of water or suitable buffer and collected by centrifugation at 2000 rpm for 20 min in an International 269 rotor at 0–4 °C.

[2] Standard buffer: 1 mM each ATP, DTE, and EGTA and 10 mM imidazole hydrochloride, pH 7.0.

Preparation of Antiserum against HMWP. Bio-Gel purified HMWP to be used as an immunogen was further purified on DE-52 (see Results). The fractions eluted at the leading edge of the DE-52 peak were combined and subjected to a final purification by $NaDodSO_4$ gel electrophoresis on either 5 or 6% slab gels. After fixation, staining, and destaining (Fairbanks et al., 1971), the band of gel containing HMWP was cut from the gel, minced with a razor blade, homogenized in water with a Dounce homogenizer, mixed with an equal volume of Freund's adjuvant (Difco Laboratories, Detroit, MI), and sonicated for 1 min. Freund's complete adjuvant was used for the initial injection and Freund's incomplete adjuvant for four subsequent injections. A nominal 150–200 μg of antigen was first injected at 4–5 subcutaneous sites on the back (total of two sets of initial injections) and then at 100–200 multiple intradermal sites in the inguinal region of a female goat (total of three more sets of injections). The preimmune serum and the sera collected 3–4 weeks after each injection were tested for reactivity against HMWP by immunodiffusion, a solid phase radioimmunoassay kindly described by Dr. Edward Berger, or by immunoblotting (see next section). Reactivity was never detected by immunodiffusion, and reactivity by solid phase radioimmunoassay was only detected in the sera from the fourth and fifth bleedings. All results reported are for experiments done with the antiserum from the fifth bleeding.

Immunoblotting. Proteins to be tested for reactivity with antisera were first separated by $NaDodSO_4$ gel electrophoresis and then transferred electrophoretically to nitrocellulose paper (Schleicher & Schuell, Keene, NH) by the basic method of Towbin et al. (1979) as worked out by Dr. Harriet Robinson. The transfer buffer used (0.0125 M Tris, 0.096 M glycine, 0.01% $NaDodSO_4$, and 0.01% 2-mercaptoethanol, pH 8.5) was devised by Drs. Richard Vallee and George Bloom. Transfer was effected by using a destaining apparatus assembled as described in Towbin et al. (1979) and run at 36 V for 14.5 h at room temperature. Nitrocellulose papers containing transferred proteins were first fixed and then stained with antibodies by using a technique worked out by Drs. Richard Vallee and George Bloom. All staining and washing steps were carried out at room temperature on a rocking agitator ("Labquake", Labindustries, Berkeley, CA). Proteins were fixed by immersing the paper in methanol–H_2O–acetic acid (5:5:1) for 15 min. The paper was then washed for 30 min in 0.15 M NaCl, 0.05 M Tris-HCl, 0.05% NP-40, and 0.25% BSA, pH 7.3 (BSA–saline–NP-40). Following this initial wash, the paper was treated for 3 h with antiserum appropriately diluted with BSA–saline–NP-40. Following aspiration of the antiserum, the papers were washed 4 times with BSA–saline–NP-40 (6 min for each wash); during washing steps the paper were turned over halfway through each wash to ensure complete rinsing away of antiserum. The papers were then treated for 2 h with rhodamine-conjugated rabbit anti-goat IgG (Cappel Laboratories, Cochranville, PA) appropriately diluted with BSA–saline–NP-40 and rinsed 4 times with BSA–saline–NP-40 as before. Following a final rinse with distilled water, the papers were sandwiched between several layers of Whatman No. 3M chromatographic paper and dried under weights in a 37 °C room. Stained proteins were located by observing the fluorescence elicited by a long wavelength ultraviolet light; staining patterns were photographed through a No. 8 Wratten filter on type 55 P/N film (Polaroid, Cambridge, MA).

Staining of Cells by Indirect Immunofluorescence. The staining protocol was adapted from Osborne et al. (1980).

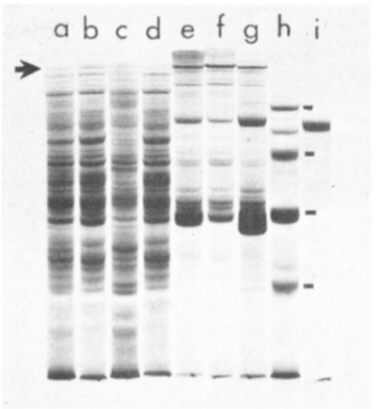

FIGURE 1: $NaDodSO_4$ gel analysis of the stages in purification of HMWP. The purification is described under Results. (a) Homogenate; (b) the 100000*g* supernatant fraction (cell extract) and (c) the 100000*g* pellet fraction of the homogenate; (d) the 10000 rpm supernatant and (e) the 10000 rpm pellet fraction (precipitated gel) of cell extract; (f) the 100000*g* supernatant fraction (35K-sup 3) and (g) the 100000*g* pellet fraction prepared by extracting precipitated gel with 0.6 M KCl in standard buffer; (h) lines point to standard proteins; from top to bottom these are β-galactosidase (M_r 130000), bovine serum albumin (M_r 68000), rabbit skeletal muscle actin (M_r 42000), and α-chymotrypsinogen (M_r 25700); (i) α-actinin from pig muscle. The arrow to the left of lane a indicates the position of HMWP.

About 10^7 suspension grown cells were collected by gentle centrifugation (International 269 rotor, 1500 rpm, 3 min), washed with 4 mL of Dulbecco's phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954), and then fixed and permeabilized by incubating them in 4 mL of 100% methanol at −20 °C for 6 min. After being washed twice with 4 mL of PBS, the cells were suspended in 0.8 mL of antiserum diluted $^1/_{50}$ with PBS and incubated at 37 °C for 30 min. After being washed twice with 4 mL of PBS, cells were then suspended in 0.7 mL of FITC-conjugated rabbit anti-goat IgG (0.4 mg/mL) (Cappel Laboratories, Cochranville, PA) and incubated at 37 °C for 30 min. After being washed twice with 4 mL of PBS, the cells were suspended in 0.1–0.2 mL of PBS, and a 5–10-μL sample was examined by phase and fluorescence microscopy with a Leitz microscope equipped with a 63× planapochromatic lens and photographed on Ilford XPI-400 film developed according to the manufacturer's directions.

The following control experiments were also performed: PBS at 4 °C was substituted for methanol at −20 °C; $^1/_{50}$ dilutions of preimmune serum or immune serum treated overnight at 0–4 °C with 0.1 mg/mL HMWP was substituted for the first antibody; PBS was substituted for the second antibody; PBS was substituted for both antibodies.

Results

Purification of HMWP. HMWP is routinely purified from 8 L of HeLa cells grown in suspension to a density of about 0.5×10^6 cells/mL. The 100000*g* supernatant fraction of HeLa cells (cell extract) is prepared as previously described (Weihing, 1977) except that Trasylol (diluted $^1/_{100}$) is included in the 0.1 M NaCl wash solution and the homogenization buffer and PMSF (1 mM) is included in the two solutions just mentioned plus the medium used to resuspend cells during harvest. The extract is then rapidly stirred at 25 °C for 1 h and then at 0 °C for 1 h. These steps produce a precipitated gel which is collected by centrifugation at 10000 rpm for 10 min at 0–4 °C in a Sorvall SS-34 rotor. The resultant pellet (Figure 1e) is designated precipitated gel. HMWP accounts for about 3% of this fraction; other major polypeptides have

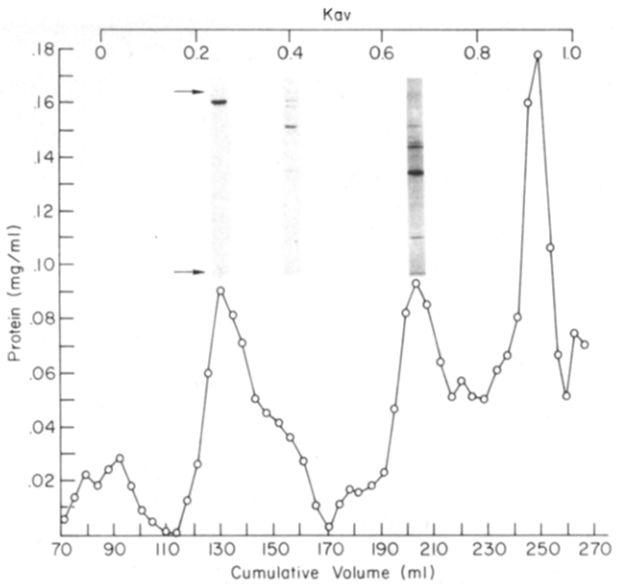

FIGURE 2: Purification of HeLa cell HMWP by gel filtration on Bio-Gel A15m. Fraction 35K-sup 3 (defined under Results and illustrated in Figure 1, lane f) was chromatographed as described under Experimental Procedures. The polypeptide composition as determined by $NaDodSO_4$ gel electrophoresis is shown for the major peaks.

the mobility of actin and α-actinin. The precipitated gel is extracted with 0.6 M KCl in standard buffer by suspending it with a tight-fitting Dounce homogenizer in a volume of buffer equal to $^1/_8$ the volume from which it was centrifuged and incubating at 0–4 °C for 1 h. Centrifugation at 35 000 rpm in a Beckman 50Ti rotor for 1 h at 0–4 °C produces a supernatant fraction (designated 35K-sup-3) which is enriched further for HMWP (Figure 1f), containing about 9% HMWP.

Purified HMWP is prepared by gel filtration of 35K-sup-3 on a column of Bio-Gel A15m equilibrated and eluted with standard buffer (Figure 2). A few hundred micrograms of HMWP of high purity (average of 90% in three recent experiments) is eluted at a column volume corresponding to a Stokes radius of 125 Å. In experiments not shown, DE-52 chromatography of HMWP purified by gel filtration inconsistently provided further purification, small amounts of HMWP being eluted at the leading edge of the main protein peak eluted from DE-52 with a convex 0–0.4 M KCl gradient. Because use of DE-52 did not consistently provide further purification, and because its use caused further loss of the small amount of HMWP which could be isolated with Bio-Gel, only experiments with Bio-Gel purified HMWP are reported below.

Interactions of HMWP with Actin. HMWP purified by gel filtration (or by gel filtration followed by DE-52 chromatography) at a concentration of 0.3–0.4 mg/mL will cause a solution of muscle actin (2 mg/mL actin polymerized with 2 mM $MgCl_2$) to form a gel which retains its shape when the tube in which it is formed is tilted. This result suggests that HMWP can cross-link actin to isotropic networks (Hartwig & Stossel, 1979).

HMWP can also cross-link actin into filament bundles. This has been observed in three experiments in which mixtures of HMWP (final concentrations of 0.075–0.16 mg/mL) and F-actin (final concentration of 0.2 or 0.4 mg/mL) were incubated at 25 °C for 10 min and then studied by negative-staining electron microscopy. Bundles of actin filaments were easily found in the mixtures of HMWP and actin (Figure 3a), but they were never observed in pure actin (Figure 3b).

Hydrodynamic Parameters. The subunit molecular weight of HMWP was measured by the usual method of plotting log M_r against the R_f observed in $NaDodSO_4$ gel electrophoresis

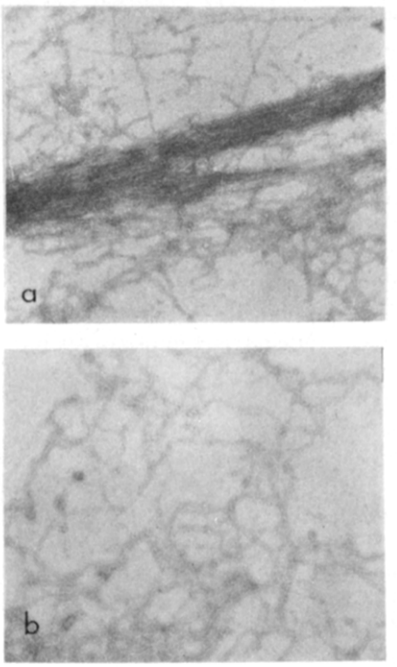

FIGURE 3: Negative-staining electron microscopy of HMWP mixed with actin and of actin alone. Mixtures of HMWP and actin (a) or of actin alone (b) prepared as described under Results were examined by negative-staining electron microscopy as described under Experimental Procedures. Bundles of actin filaments were found only in mixtures of actin and HMWP. The magnification is 23800×.

and found to be 250 000. It was also observed that HMWP and chicken gizzard filamin do not separate when they are mixed and subjected to prolonged $NaDodSO_4$ gel electrophoresis on the same gel (Figure 5), showing that their subunit molecular weights are identical.

For measurement of the Stokes radius, 35K-sup-3 was subjected to gel filtration on a calibrated column of Bio-Gel A15m. HMWP was eluted at a position corresponding to a Stokes radius of 125 ± 16 Å (mean ± SD) (n = 10).

For measurement of the sedimentation coefficient, The HMWP which was eluted from Biogel at the column volume corresponding to a Stokes radius of 125 A was subjected to ultracentrifugation at 0–4 °C on 5–20% sucrose gradients. The protein sedimented as a single symmetrical peak with a sedimentation coefficient of 9 S (average of two experiments).

The Stokes radius and the sedimentation coefficient were used to calculate the native molecular weight (Siegel & Monty, 1966). For this calculation, the partial specific volume was assumed to be 0.734 mL/g as previously calculated for filamin from chicken gizzard (Shizuta et al., 1976) because the amino acid composition of HMWP and filamin is very close (Table II). The native molecular weight was calculated to be 480 000, which is very nearly twice the subunit molecular weight. Native HMWP therefore appears to be a dimer. The data were also used to calculate a frictional ratio, f/f_0, of 2.4, suggesting that HMWP is asymmetrical. The hydrodynamic parameters of HMWP, actin binding protein, and filamin are summarized in Table I, which shows that all these proteins are very similar when compared in this way.

Amino Acid Analysis. The amino acid composition of HeLa cell HMWP closely resembles that previously reported for actin binding protein from rabbit alveolar macrophages (Stossel & Hartwig, 1976), filamin from chicken gizzard (Wang, 1977) and from guinea pig vas deferens (Wallach et al., 1978), and HMWP from BHK-21 cells (Schloss & Goldman, 1979) (Table II). No unusual amino acids such as N^{τ}-methylhistidine or N^{ϵ}-methylated lysines were detected.

Table I: Hydrodynamic Parameters for HMWP and Similar Proteins

parameter	HMWP	actin binding protein	filamin	
		macrophage[a]	chicken[b] gizzard	chicken[c] gizzard
subunit molecular weight	250 000	270 000	240 000	250 000–255 000
Stokes radius (Å)	125	135		120
sedimentation coefficient (S)	9	9.36	8.86	10
native molecular weight	480 000	540 000	498 000	480 000–520 000
frictional ratio	2.4	2.56		2.2–2.3

[a] Hartwig & Stossel (1981). [b] Shizuta et al. (1976). [c] Wang (1977).

Table II: Amino Acid Composition of HMWP from HeLa Cells: Comparison with Similar Mammalian and Chicken Proteins

amino acid	HMWP (mol %)		actin binding protein (mol %)	filamin (mol %)	
	HeLa[a]	BHK-21[b]	macrophages[c]	guinea[d] pig	chicken[e] gizzard
Lys	6.3	6.0	6.0	6.4	4.8
His	2.4	2.1	2.2	2.1	2.3
Arg	3.8	3.3	4.1	3.5	5.5
Asp	8.4	8.9	8.7	9.2	7.6
Thr	5.8	6.6	6.2	6.4	5.3
Ser	6.0	7.5	6.8	6.8	6.8
Glu	11.3	11.0	11.4	10.8	9.5
Pro	8.4	8.0	7.1	8.7	8.0
Gly	12.5	10.7	11.8	12.0	12.5
Ala	7.3	6.9	7.4	7.7	9.3
½-Cys	1.4		0.46		2.0
Val	9.9	9.6	8.5	9.8	10.0
Met	1.3	1.3	1.3	1.1	0.8
Ile	4.3	4.4	4.4	4.1	3.3
Leu	6.0	5.8	6.2	6.0	6.0
Tyr	2.7	2.8	3.1	2.7	2.6
Phe	2.9	2.8	3.2	2.2	2.9

[a] 24-h hydrolysis. [b] 24-h hydrolysis, Schloss & Goldman (1979). [c] 24-h hydrolysis, Hartwig & Stossel (1975), recalculated by Wang (1977). [d] 24- and 48-h hydrolyses, Wallach et al. (1978). [e] 24-, 48-, and 72-h hydrolyses, Wang (1977).

These amino acids were also not detected in filamin from chicken gizzard (Wang, 1977).

Peptide Maps. The above observations provide strong evidence that the proteins designated actin binding protein, filamin, and HMWP are homologous. The extent of this homology was explored further by comparing the partial peptide maps (Cleveland et al., 1977) of HMWP to those of filamin. Peptides were prepared by digesting identical amounts of the two proteins with increasing concentrations of chymotrypsin or papain and separating the peptides by $NaDodSO_4$ gel electrophoresis. It was found that HeLa cell HMWP and chicken gizzard filamin produce distinct patterns of peptides [e.g., compare lanes i (HMWP) and j (filamin) in Figure 4A (chymotryptic digestion) and lanes i′ (HMWP) and j′ (filamin) in Figure 4B (papain digestion)]. Evidently the homology between HeLa cell HMWP and chicken gizzard filamin does not extend to the details of their primary structures.

HMWP and Plasma Membrane Ghosts. Previously, this laboratory reported that plasma membrane ghosts prepared from HeLa cells grown in suspension contain several prominent high molecular weight polypeptides (Gruenstein et al., 1975).

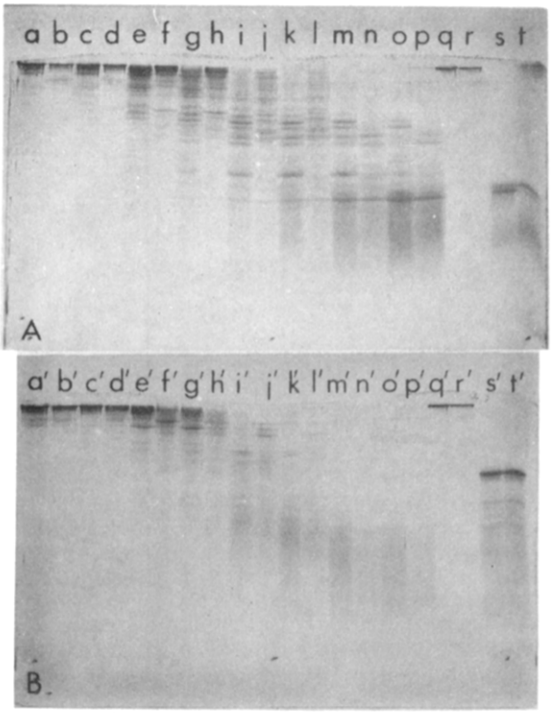

FIGURE 4: Partial peptide maps of HeLa cell HMWP and chicken gizzard filamin. Conditions of digestion and electrophoresis are described under Experimental Procedures. (Panel A) Chymotryptic digests. HMWP plus chymotrypsin is in lanes a, c, e, g, i, k, m, and o; filamin plus chymotrypsin is in lanes b, d, f, h, j, l, n, and p. Concentrations of chymotrypsin used for digestion were 0.5, 1.0, 5.0, 10, 25, 50, 100, and 200 μg/mL for pairs ab, cd, ef, gh, ij, kl, mn, and op, respectively. (Panel B) Papain digests. HMWP plus papain is in lanes a′, c′, e′, g′, i′, k′, m′, and o′; filamin plus papain is in lanes b′, d′, f′, h′, j′, l′, n′, and p′. Concentrations of papain used for digestion were 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.25, and 2.5 μg/mL for pairs a′b′, c′d′, e′f′, g′h′, i′j′, k′l′, m′n′, and o′p′, respectively. For panels A and B, pure HMWP in lanes q and q′ and pure filamin in lanes r and r′. For panel A, lanes s and t, pure chymotrypsin. For panel B, lanes s′ and t′, pure papain.

Two techniques, coelectrophoresis and immunoblotting, indicate that one of these polypeptides is HMWP. Pure HMWP mixed with ghosts comigrates (Figure 5) with one of the high molecular weight polypeptides of the ghosts (lanes f and i) and did not separate from it even when the duration of electrophoresis was 3 times longer than usual (lane i). Comparison of these patterns with those previously published shows that pure HMWP is comigrating with the membrane polypeptide that was previously found to migrate to the same position as the HMWP in isolated cytoplasmic gels when membranes and isolated cytoplasmic gels were subjected to electrophoresis in parallel (Weihing, 1976a). In addition, HMWP and filamin also comigrate when subjected to electrophoresis in the same gel (Figure 5, lane k; Figure 6, lane b), and therefore this polypeptide must also correspond to the polypeptide previously identified as filamin (Burridge & McCullough, 1980). Confirmation that myosin heavy chain comigrates with another of the high molecular weight polypeptides of the ghosts (Burridge & McCullough, 1980) was also obtained (lane e).

An antiserum raised against purified HMWP reacts with the membrane polypeptide that comigrates with pure HMWP. When tested by immunoblotting (Figure 5), the antiserum reacts strongly with pure HMWP, the membrane band that comigrates with HMWP, the mixture of these two (lanes q, o, and p), and a homogenate band with the same mobility as HMWP (lane n). It also reacts weakly with filamin from chicken gizzard (Figure 5, lane s, and Figure 6, lane c). It did not react with other high molecular weight polypeptides

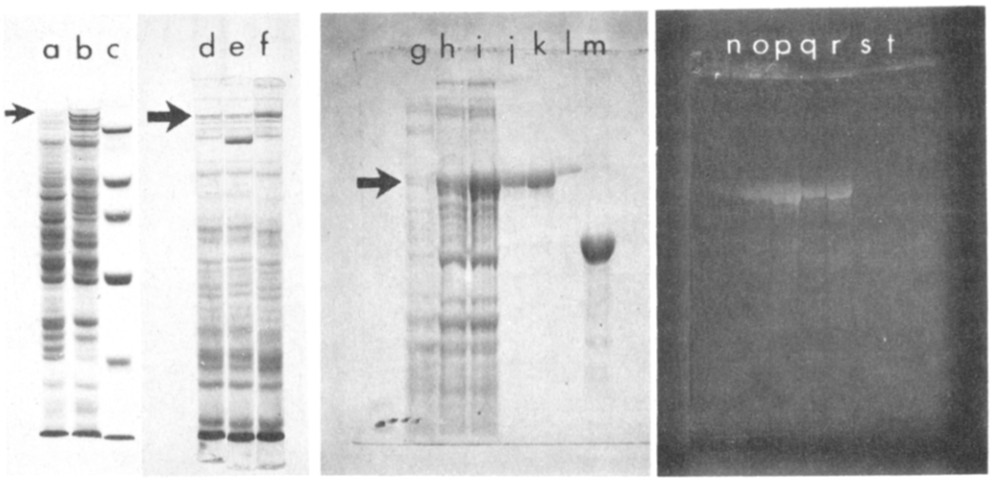

FIGURE 5: Identification of HMWP in $ZnCl_2$-stabilized plasma membrane ghosts from HeLa cells by coelectrophoresis and immunoblotting. Fifty-microgram samples of protein mixtures and 2-μg samples of pure proteins were subjected to $NaDodSO_4$ gel electrophoresis on 8% tube gels (lanes a–c), 5% tube gels (lanes d–f), or 5% slab gels (lanes g–m). Lanes n–t show the immunoblot of a gel identical with that shown in lanes g–m and run in parallel; the blot was stained first with a 250-fold dilution of goat antiserum against HMWP and then with a solution containing 0.22 mg/mL rabbit anti-goat IgG tagged with rhodamine. No staining was observed when the preimmune serum or antiserum absorbed with insoluble HMWP was substituted for the antiserum (not shown). (Lane a) Cell homogenate; (lane b) ghosts; (lane c) standards, from top to bottom, are myosin heavy chain (M_r 200 000), α-actinin (M_r 100 000), bovine serum albumin (M_r 68 000), rabbit muscle actin (M_r 42 000), and α-chymotrypsinogen (M_r 25 700). (Lane d) Ghosts; (lane e) ghosts plus myosin; (lane f) ghosts plus HMWP. (Lanes g and n) Cell homogenate; (lanes h and o) ghosts; (lanes i and p) ghosts plus HMWP; (lanes j and q) HMWP; (lanes k and r) mixture of HMWP and filamin; (lanes l and s) filamin; (lanes m and t) myosin heavy chain. The arrows point to HMWP. Tube gels (lanes a–f) were subjected to electrophoresis for 3 h; the slab gel (lanes g–m) was subjected to electrophoresis for 9 h.

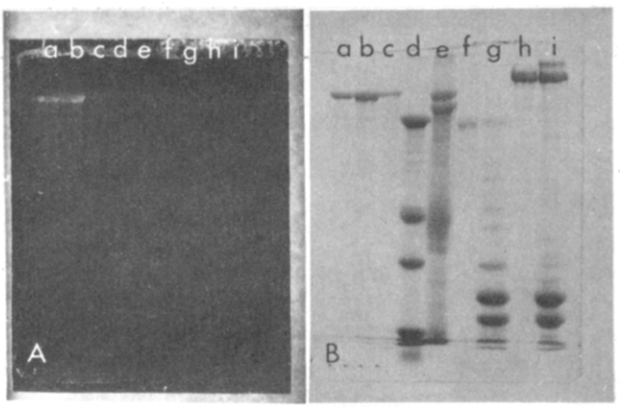

FIGURE 6: Investigation of the specificity of the goat antiserum to HeLa cell HMWP by immunoblotting. Various cytoskeletal proteins were subjected to $NaDodSO_4$ gel electrophoresis on parallel 5% slab gels. Panel A shows the immunoblot from one of the gels; panel B shows the parallel gel stained with Coomassie blue. The conditions of immunoblotting are described in the legend to Figure 5. The following samples were used: (a) 2 μg of HMWP; (b) 2 μg of HMWP plus 4 μg of filamin; (c) 4 μg of filamin; (d) four standard proteins, from top to bottom, are rabbit skeletal myosin heavy chain, pig muscle α-actinin, bovine serum albumin, and rabbit skeletal muscle actin; (e) 25 μg of human erythrocyte ghosts; (f) 2 μg of M_r 200 000 HeLa cell MAPs purified from microtubule protein by boiling and gel filtration (Weatherbee et al., 1982); (g) 25 μg of HeLa cell microtubule protein purified through two cycles of polymerization and depolymerization; (h) 2 μg of MAP-2 from calf brain microtubule protein; (i) 25 μg of calf brain microtubule protein. Sample e was kindly provided by Dr. V. Patel, samples f, g, and h were provided by Dr. J. A. Weatherbee, and sample i was provided by Dr. R. Vallee.

of the ghosts including myosin heavy chain (Figure 5, lane t, and Figure 6, lane d), and it also failed to react with other high molecular weight cytoskeletal proteins that are known to be present in HeLa cells including the 200K MAPs from HeLa cells, or MAP-2 from brain microtubules (Weatherbee et al., 1982) (Figure 6, lanes f–i). It also does not react with human erythrocyte spectrin (Figure 6, lane e). These results provide strong evidence that the antiserum is reacting exclusively with HMWP present in the ghosts.

We do observe faint staining of minor bands that migrate faster than HMWP, raising the possibility that the antiserum contains antibodies to proteins other than HMWP. Two arguments are offered against this interpretation. First, the HMWP used for immunization was subjected to two additional purification steps beyond Bio-Gel before injection into the goat, and great care was taken to cut out as narrow a gel band as possible for use as antigen. Second, the faintly staining bands which migrate faster than HMWP stain more brightly if PMSF and TLCK are omitted from the medium used to isolate the membranes. Thus these minor bands actually appear to be minor proteolytic digestion products of HMWP. We conclude, therefore, that the antiserum reacts specifically with HMWP and, therefore, that HMWP is a component of the ghosts.

Staining HMWP in Situ. This laboratory previously reported that the polypeptide which has now been identified as HMWP accounts for nearly 3% of the stain bound to polypeptides of plasma membrane ghosts but only 0.14% of the stain bound to the polypeptides of total cell lysates (Weihing, 1976a). This result suggests that HMWP is a prominent component of ghosts because it is present in the cortical cytoplasm of intact cells. So that this could be tested, suspension-grown cells were studied by the technique of indirect immunofluorescence with the goat antiserum against HMWP as the first antibody and FITC-conjugated rabbit anti-goat IgG as the second.

A bright peripheral ring of fluorescence and less intense generalized fluorescence was observed when cells were treated with the antiserum to HMWP (Figure 7a,b). Several controls indicate that the ring is produced by staining of cortical HMWP. First, the ring is not observed if immune serum treated with HMWP (Figure 7c) or the preimmune serum (Figure 7d) is substituted for the immune serum. These controls plus the immunoblotting experiments discussed earlier indicate that it is HMWP that produces the ring stain. Second, no staining was observed if cells were not permeabilized with methanol (Figure 7e) or if the first antibody or if both antibodies were omitted (not shown), indicating that the ring

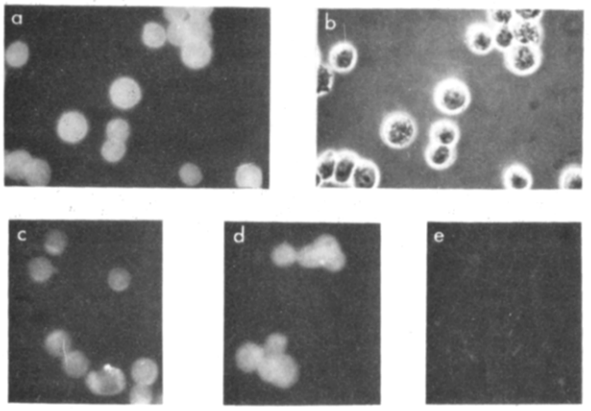

FIGURE 7: Localization of HMWP in HeLa cells by indirect immunofluorescence. (a) Epifluorescence image of cells treated with antiserum to HMWP; (b) phase image of the cells shown in panel a. (c–e) Epifluorescence images of cells treated with various control sera: (c) antiserum neutralized with HMWP; (d) cells treated with preimmune serum; (e) cells not permeabilized with methanol. The final magnification is 630×.

pattern is produced by reaction of the antibody against HMWP with an intracellular component. Third, the ring coincides with the periphery of the cells as seen by phase contrast. It is concluded, therefore, that the ring staining pattern indicates that HMWP is a component of the cortical cytoplasm in HeLa cells grown in suspension.

Discussion

HMWP from HeLa cells shares many properties with the high molecular weight actin binding proteins that have been designated actin binding protein (Hartwig & Stossel, 1975, 1981; Rosenberg et al., 1981), filamin (Shizuta et al., 1976; Wang, 1977; Wallach et al., 1978), or HMWP (Schloss & Goldman, 1979). All these proteins have a subunit molecular weight of about 250000, and all are able to gel actin. Filamin (Wang & Singer, 1977), HMWP (Schloss & Goldman, 1979; this report), and actin binding protein (Hartwig & Stossel, 1981) can also cross-link actin filaments into bundles. In addition, the hydrodynamic properties (Table I) and the amino acid compositions (Table II) of several of these proteins, including HMWP from HeLa cells, are very similar. Some of these proteins also contain related antigens as shown by observations that antibodies to mammalian filamin (from guinea pig vas deferens) (Wallach et al., 1978) react with avian filamin (from chicken gizzard) and with a M_r 250000 polypeptide in extracts of guinea pig vas deferens, rabbit alveolar macrophages (the original source of actin binding protein), and human platelets (another source of actin binding protein) (Rosenberg et al., 1981) and that antibody against avian filamin (from chicken gizzard) also reacts with mammalian filamin (from guinea pig vas deferens) (Wallach et al., 1978). All this evidence implies that these particular high molecular weight actin binding proteins are homologous (cf. Weeds, 1982). On the basis of these comparisons, it seems surprising that the antiserum to HMWP reacts only weakly with chicken gizzard filamin and that partial peptide maps of HMWP and chicken gizzard filamin are different. If HMWP and chicken gizzard filamin are actually homologues, as is strongly suggested by the similarities in their physical parameters and amino acid composition, then the differences in their peptide maps and in their reactivity to antiserum to HMWP indicate that these two proteins are related by the divergent evolution of a single homologous protein in mammals and birds and/or by the evolution of tissue-specific forms of these proteins. Additional comparative studies will be necessary to distinguish these possibilities and to rule out the less likely possibility that HMWP is not homologous to filamin and actin binding protein.

HMWP is clearly not homologous to spectrin, fodrin, or TW 260/240 (which are also asymmetrical, high molecular weight actin binding proteins) because their hydrodynamic and antigenic properties are different. Thus, native HMWP and its homologues are 9S dimers (Table I), but native fodrin and TW260/240 are 11S tetramers (Glenney et al., 1982a). In addition, spectrin can undergo a reversible dimer–tetramer transition (Branton et al., 1981) while filamin undergoes a spontaneous, *irreversible* transition from dimer to tetramer (Davies et al., 1980). Finally, antibodies to guinea pig vas deferens filamin and chicken gizzard filamin and our antiserum to HMWP do not react with spectrin (Wallach et al., 1978; Glenney et al., 1982a,b; this report). In addition, an antibody to filamin does not react with fodrin or TW 260/240 (Glenney et al., 1982a,b), nor do antibodies to TW 260/240 react with filamin (Glenney et al., 1982a,b).

Immunocytochemical investigation reveals that HMWP is a component of the cortical cytoplasm of HeLa cells grown in suspension. Its presence here can be explained most simply by its ability to bind to the cortical actin which has been identified in situ in HeLa cells by binding of heavy meromyosin (Schroeder, 1973; Miranda et al., 1974). Granting this, then its presence in plasma membrane ghosts is more or less to be expected because the ghosts are known to contain actin (Gruenstein et al., 1975). Its occurrence in cortical cytoplasm and in ghosts strengthens the argument for its homology with actin binding protein and filamin, which exhibit similar localization. The former protein has been identified by an immunologic test in phagosomes prepared from macrophages (Boxer et al., 1976), by coelectrophoresis in cortical fragments (podosomes) prepared from macrophages (Davies & Stossel, 1977), and by immunocytochemical techniques in the cortical cytoplasm of macrophages (Stendahl et al., 1980; Valerius et al., 1981) and in the cortical cytoplasm and the base of microvilli of cultured lymphoid cells (Thorstensson et al., 1982). Filamin has been identified by immunocytochemical techniques in the ruffled membrane and in the base of microvilli of NRK cells (Heggeness et al., 1977), in the ruffled membrane and the contractile ring of chick embryo cells (Nunnally et al., 1980), in the terminal web region of intestinal epithelial cells (Bretscher & Weber, 1979), and in the filopodia of activated platelets (Debus et al., 1981). In addition, a protein designated actin binding protein has also been identified by coelectrophoresis in $ZnCl_2$-stabilized ghosts prepared from sarcoma 180 cells (Moore et al., 1978), and an actin binding protein with subunit M_r 250000 has been purified from platelets (Rosenberg et al., 1981), which are cortical fragments of megakaryocytes. Thus HMWP and its apparent homologues have all been identified in cortical cytoplasm, but it is not yet known if protein homologous to HMWP is present in the cortex of all cells.

It is likely that the general role of cortical HMWP is to help cross-link cortical actin filaments into supramolecular structures. Two such structures, cortical networks and stress fibers, have been tentatively identified so far. The localization observed in this study suggests that the HMWP of suspension-grown cells is bound to the thin cortical layer of actin filaments that has previously been identified in HeLa cells by binding of heavy meromyosin (Miranda et al., 1974). Here, by analogy to the function of actin binding protein in the extensive cortical actin networks of macrophages (Stossel et al., 1982), it may

help organize cortical actin into networks. A different localization has been observed in HeLa cells grown in monolayers, where HMWP is found in linear arrays resembling stress fibers (Weihing, 1982). This observation is consistent with previous reports that filamin is localized in stress fibers in mammalian fibroblasts (Heggeness et al., 1977) and chick embryo cells (Nunnally et al., 1980), and it suggests that HMWP might also help organize actin filament bundles in vivo.

The HeLa cell cortex contains two other structures that contain bundles of microfilaments—the contractile ring (Schroeder, 1973) and the microvilli (Porter et al., 1974). The microfilaments of the contractile ring bind heavy meromyosin (Schroeder, 1973), and therefore they are undoubtedly actin; the microfilaments at the core of the microvilli are in continuity with the thin cortical layer of microfilaments (Porter et al., 1974) that also bind heavy meromyosin (Miranda et al., 1974), and therefore they are almost certainly actin. HMWP has not yet been observed in either of these structures, but previous observations on other cells, already discussed, suggest that it may well be present at these sites. This is currently being investigated.

α-Actinin also appears to be a component of the cortical cytoplasm of HeLa cells because it has been identified by immunocytochemical techniques in the contractile ring of HeLa cells (Fujiwara et al., 1979) and in plasma membrane ghosts from HeLa cells (Burridge & McCullough, 1980). Because purified HeLa cell α-actinin is capable of cross-linking actin in vitro (Burridge & Feramisco, 1981), it may also help organize cortical actin filaments into supramolecular structures in vivo. In addition, two lines of indirect evidence suggests that it participates in gelation of cytoplasmic extracts of HeLa cells. First, a polypeptide with the electrophoretic mobility of α-actinin is a prominent component of the precipitated gel (Figure 1e). Second, 2 mM $CaCl_2$ inhibits gelation of cytoplasmic extract (Weihing, 1976a), an observation which may be related to the observation that Ca^{2+} inhibits gelation of muscle actin by HeLa cell α-actinin (Burridge & Feramisco, 1981).

Acknowledgments

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Spectral Properties of Cobalt Carboxypeptidase A. Interaction of the Metal Atom with Anions†

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ABSTRACT: At pH >7 the absorption and magnetic circular dichroic spectra of cobalt carboxypeptidase A are insensitive to anions [Latt, S. A., & Vallee, B. L. (1971) *Biochemistry 10*, 4263–4270], but at pH <6 chloride and other anions perturb them in a manner specific for each anion. Lowering of the pH apparently facilitates the entry of an anion into the metal coordination sphere, suggesting that an acidic group normally stabilizes a metal-coordinated water molecule against displacement. The lack of sensitivity to anions at pHs between 7 and 9—when the enzyme is maximally active—and its evident abolition upon protonation of an active-site group are consistent with this interpretation. Selective modification of cobalt carboxypeptidase at Glu-270 using a carbodiimide affinity reagent generates sensitivity to anions at pH 7 very similar to that of the unmodified enzyme at pH ~5. This suggests that the group stabilizing the metal-coordinated water is the catalytically essential carboxylate of Glu-270. These and related results provide evidence for a mechanistically important interaction of Glu-270 with a metal-bound water molecule.

Replacement of the zinc atom of carboxypeptidase A with cobalt(II) provides a powerful spectral probe of its environment at the active site while maintaining enzymatic activity (Coleman & Vallee, 1960; Vallee, 1981). The accompanying electronic absorption, CD,[1] and MCD spectra (Latt & Vallee, 1971; Vallee & Holmquist, 1980) together with the X-ray structure analysis of the crystalline zinc enzyme (Lipscomb et al., 1968) have delineated the nature of the coordination of the active-site metal atom. Jointly, they suggest that the imidazolyl groups of His-69 and -196 and the carboxylate of Glu-72, together with the oxygen atom of either a water molecule or a hydroxide ion, constitute an irregular, tetrahedral-like environment of the metal atom.

The γ-carboxylate group of Glu-270 is also located close to the metal atom (Lipscomb et al., 1968). The results of chemical modifications implicate this group in the mechanism of catalysis (Riordan & Hayashida, 1970; Hass & Neurath, 1971; Pêtra & Neurath, 1971; Nau & Riordan, 1975), but the exact nature of its function remains to be determined. Chemical and kinetic studies with oligopeptide substrates and their ester analogues have failed to substantiate claims that Glu-270 forms a covalent anhydride link with the substrate in the course of catalysis (Breslow & Wernick, 1976; Galdes et al., 1983), although covalent intermediates are thought to occur in the hydrolysis of (chlorocinnamoyl)-L-phenyllactate (Makinen et al., 1976). Alternatively, it has been proposed that a residue such as Glu-270 could serve as a general base to deliver water as the nucleophile which attacks the substrate (Vallee et al., 1963; Vallee, 1964; Breslow & Wernick, 1976), but this, too, awaits verification.

The available evidence suggests that ligand exchange in the inner coordination sphere of the metal atom is an essential step in the hydrolysis of substrates catalyzed by zinc proteases, including carboxypeptidase A (Van Wart & Vallee, 1978). Consistent with this, previous studies of this enzyme employing the probe properties of cobalt substituted for zinc in the active center have shown that pseudosubstrates and specific inhibitors alter the symmetry of metal binding to the enzyme (Latt & Vallee, 1971; Vallee & Holmquist, 1980). However, simple anions such as halides and others of the spectrochemical series (Phillips & Williams, 1966) either fail to perturb its spectra

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[1] Abbreviations: CD, circular dichroism; MCD, magnetic circular dichroism; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; CMC, 1-cyclohexyl-3-[2-(*N*-methylmorpholino)ethyl]carbodiimide.

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