Evidence for a Particulate Location of Ubiquitin Conjugates and Ubiquitin-conjugating Enzymes in Rabbit Brain*

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Conjugate ubiquitin was previously found in the nucleus, cytoplasm, and membranes of eukaryotic cells while the enzymes of the ubiquitin-conjugating system appear to be cytoplasmic. We have prepared the mitochondrial fraction from rabbit brain by discontinuous density gradient ultracentrifugation and by Western blotting, using a specific antibody against conjugate ubiquitin, showing that it contains ubiquitin conjugates in a very wide molecular weight range. Electron microscopy and measurement of specific enzyme markers show that this fraction not only contains mitochondria but also some endoplasmic reticulum vesicles. Immunostaining with anti-ubiquitin IgG followed by immunodecoration with colloidal gold particles provides evidence for the presence of conjugate ubiquitin both in mitochondria and in the endoplasmic reticulum. Furthermore, this "mitochondrial fraction" shows a pronounced ATP-dependent ability to conjugate 125I-ubiquitin into a number of endogenous proteins as evidenced by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Addition of $E_1$, $E_2$, and $E_3$, the enzymes of the ubiquitin conjugating system purified from rabbit reticulocytes, does not further increase this ubiquitination nor incorporate 125I-ubiquitin into additional protein bands. The same mitochondrial fraction is not able to carry out any ATP-dependent degradation of 125I-albumin; however, it contains an isopeptidase activity able to release the covalently incorporated 125I-ubiquitin and is also able to conjugate 125I-ubiquitin to exogenous proteins as oxidized RNase. By affinity chromatography on ubiquitin-agarose of fraction II of a crude Triton X-100 extract of the mitochondrial fraction, several proteins corresponding in Mr to the $E_1$ and $E_2$ enzymes were obtained. These proteins were also able to form specific ubiquitin-thiol ester bounds on sodium dodecyl sulfate-polyacrylamide gels and to support 125I-ubiquitin conjugation to oxidized RNase. Detergent fractionation of the mitochondrial fraction provided evidence for a possible localization of the ubiquitin conjugating activity in the mitochondrial external membrane and endoplasmic reticulum. The presence of an active ubiquitin protein conjugating system in mitochondria and endoplasmic reticulum may be related to the turnover of organelle proteins as well as to specific cell functions such as import of proteins into mitochondria.

Ubiquitin is a small polypeptide of Mr. 8565 with an amino acid sequence extremely conserved and involved in several basic cellular functions (for a review, see Ref. 1). In 1977 (2, 3) ubiquitin was found in the nucleus, bound to histone H2A, and a few years later it was found to mediate the cytosolic protein breakdown (4, 5) and also bound to cell surface proteins (6–8). More recently ubiquitin was also identified as a component of neurofibrillary tangles in Alzheimer's disease and other neurodegenerative diseases (9–11). In other words, ubiquitin was found to mediate gene transcription (12), DNA repair (13), and cell cycle progression (14, 15) as well as selective protein degradation (16), stress responses (17), and modulation of immune response (6). All these different processes involve the conjugation of ubiquitin to specific target proteins through the action of an ATP-dependent ligation system (16). This conjugation is mediated by an ubiquitin activating enzyme $E_1$ (18–20), a family of ubiquitin carrier proteins $E_2$ (21–23), and a ubiquitin-protein ligase $E_3$ with several different substrate specificities (24, 25). Although there are ubiquitin conjugates in the nucleus, at the cell surface, and in the cytoplasm, the enzymes of the ubiquitin conjugating system appear to be cytoplasmic (26), with few exceptions (27).

In this paper we present evidence for the presence of ubiquitin conjugates in a particulate fraction of rabbit brain that contains mitochondria and endoplasmic reticulum. Furthermore, we also surprisingly found that this fraction, although not able to carry out protein degradation, is able to covalently conjugate ubiquitin in an ATP-dependent manner to endogenous and exogenous substrate proteins and also possesses an isopeptidase activity responsible for ubiquitin release from the conjugates. These data seem to be of interest in understanding the intracellular localization of the pathway(s) responsible in eukaryotic cells for ubiquitin conjugation, and to our knowledge they represent the first evidence for an active ubiquitin-protein conjugating system in a particulate fraction of the brain.

**EXPERIMENTAL PROCEDURES**

*Materials—Ubiquitin, chloramine T, Protein A, anti-ubiquitin antiserum, and anti-rabbit IgG gold conjugate (10 nm) were obtained from Sigma. Ubiquitin and Protein A were iodinated by Iodo-Beads (N-chlorobenzenesulfonylamine-derivatized polystyrene beads from Pierce Chemical Co.) as suggested by the manufacturer. The specific activity obtained was 2–2.5 $10^5$ cpm/μg. Ficoll was from Pharmacia LKB Biotechnology Inc.

*Preparation of the "Mitochondrial Fraction"—The mitochondrial fraction from rabbit brain was prepared by a modification of the procedure described in Ref. 28. Briefly, two rabbit brains were
chopped with scissors and homogenized in a Potter-Elvehjem homogenizer in 10 volumes of isolation medium (0.32 M sucrose, 0.5 M EDTA, 10 mM Tris-HCl, pH 7.4) with a Teflon pestle (clearance 0.1 mm). The homogenate was centrifuged for 3 min at 1,500 × g, the supernatant carefully decanted, and then centrifuged at 12,500 × g for 10 min to obtain the crude mitochondrial pellet. This pellet was resuspended in 7.5 M sucrose on glass paper as described above. The pellet of this last centrifugation was resuspended in 4 ml of isolation medium and layered onto a discontinuous Ficoll density gradient. The gradient consisted of 4 ml each of 15, 12, 9, and 7.5% Ficoll (w/w) in isolation medium. Each tube received 1.5 ml of sample and 1.0 ml of 100,000 × g supernatant. A SW28 swing-out rotor was used in a Beckman L8 70 ultracentrifuge. At the end of the centrifugation, the myelin was at the interface between the isolation medium and the 7.5% Ficoll solution, while the mitochondria were collected at the interface between the 9 and 12% Ficoll solution. The mitochondria were then resuspended in 40 ml of isolation medium and pelleted at 12,500 × g for 10 min. This procedure was repeated twice to wash out the Ficoll present. The final pellet was resuspended in 2 ml of isolation medium and used in further studies as outlined below.

Enzymes Assay and Protein Determination—The activities of hexokinase (EC 2.7.1.1), lactate dehydrogenase (EC 1.1.1.27), glutamate dehydrogenase (EC 1.4.1.3), and NADH cytochrome c reductase, and rotenone insensitive, (EC 1.6.2.3) were determined as described in Refs. 29-32, respectively. Protein was determined by the Bradford method (33) using bovine serum albumin as a standard.

Electron Microscopy—The mitochondrial fraction was fixed on 10% polyacrylamide slab gels essentially according to Laemmli (36). Gels were electrophoresed according to Towbin et al. (37). The buffer was 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.02% (w/v) sodium dodecyl sulfate. Blotting was performed at 0-4 °C for 20 h at 40 V. After blotting the nitrocellulose sheets were washed for 10 min in 20 mM Tris, 500 mM NaCl, pH 7.5 (TBS) and then agitated for 1 h in a 3% (w/v) gelatin solution in TBS. After washing with TBS membranes were incubated in 100 mM Tris, 0.5% (v/v) Tween-20, pH 7.5 (TTBS) the blots were then incubated for 5 h with 1:500 rabbit anti-ubiquitin IgGs (kindly provided by A. Haas, Milwaukee, WI) and diluted in 1% (w/v) gelatin in TTBS, which detect ubiquitin-protein conjugates or a Sigma anti-ubiquitin antiserum which detect preferentially soluble ubiquitin. Membranes were washed in TTBS and incubated for 1 h in 50 ml of 2.10^6 cpm/mI of 35S protein A. After washing first in TTBS and then in TBS, the membranes were dried and autoradiographed.

Electron Microscopy—The mitochondrial fraction was fixed in 2% (w/v) paraformaldehyde containing 0.5% (v/v) glutaraldehyde for 1.5 h at 4 °C. The fixative solution was chosen to block the natural degeneration of cellular component but not to interfere with the immunolabeling procedure. The fixed samples were then rinsed with Sorenson phosphate buffer, pH 7.4, and then kept in the same buffer overnight at 4 °C. The samples were then postfixed at 4 °C in 1% (w/v) osmium tetroxide for 45 min against the dialysis buffer, rinsed in phosphate buffered saline, pH 7.4, at room temperature, gradually dehydrated in mixtures of bidistilled water and acetone in a series of increasing acetone concentration, and finally embedded in Epon resin (the ultrstructural preservation is allowed by this resin and the protein antigenicity is retained). After polymerization, the samples were cut with a diamond knife on an ultramicrotome and the ultrathin sections (about 80 nm in thickness) were collected on nickel grids. Section staining was performed using floating grids, sections down, on droplets of the immunolabeling and washing solutions placed on strips of Parafilm, taking care not to wet the reverse side of the grid or to let the sections dry. Each incubation was washed on fresh droplets of buffer, and the excess reagent or buffer was removed with pieces of filter paper. All steps of the immunolabeling procedure were carried out at room temperature as follows. The sections were hydrated in a 60% acetone solution, then transferred in TBS for 10 min. Blocking was performed in 5% (w/v) gelatin in TTBS for 30 min followed by two washing steps of 5 min each in TTBS. The sections were then incubated with 1:50 rabbit anti-ubiquitin IgG (A. Haas) for 3 h at room temperature in the antibody buffer (1% gelatine in TTBS). The samples were then washed three times in TTBS and then incubated for 1.5 h with 1:16 dilution of anti-rabbit IgG gold conjugate in 50 mM sodium buffer. The final immunolabeling step was followed by two washes of 5 min each of TTBS followed by two further washes in TBS. At the end, grids were washed in distilled water, air dried, stained with lead citrate, and observed under the E. M. Philips CM 10.

Treatment with Digitonin—The mitochondrial fraction was incubated with increasing concentrations of digitonin in isotonic media at 1.5 mg of protein/ml. Incubations were at 4 °C for 30 min with constant stirring. The suspensions were immediately centrifuged at 14,000 rpm in an Eppendorf microcentrifuge for 10 min at 4 °C and the supernatant collected for the measurements of specific enzyme markers as outlined under "Results."
these difficulties are the heterogeneity of the nervous tissue, the large amounts of lipid and membranous materials, and the heterogeneity of mitochondria in their contents. Nevertheless, appropriate methods have been devised and relatively pure mitochondrial fractions may be isolated (28). The procedure we employed to isolate brain mitochondria involves the use of density gradient centrifugation and provides a particulate fraction which contains significant amounts of hexokinase. In fact the specific activity of this enzyme (810 ± 50 milliunits/mg protein) is higher than those previously reported for other mitochondria preparations as was the specific activity of another mitochondrial enzyme (glutamate dehydrogenase 913 ± 40 milliunits/mg) (38, 39). Furthermore, the contamination by cytoplasmic proteins evaluated by measuring the lactate dehydrogenase activity of this fraction was less than 3–4% of the total activity of the homogenate. In contrast, the activity of an endoplasmic reticulum marker (NADPH cytochrome c reductase) was 25% of the total activity. Several different conditions (i.e. homogenizing conditions and solutions, density gradients, temperature, etc.) were modified to further improve the purification of this mitochondrial fraction but without significant differences in the results obtained. This contamination of brain mitochondria by endoplasmic reticulum has also been observed by others (39).

The particulate association of a ubiquitin conjugating activity within the mitochondrial fraction was investigated in the fractions obtained by discontinuous density gradient ultracentrifugation of the crude mitochondrial pellet (see "Experimental Procedures" for details). As shown in Table I, the lactate dehydrogenase activity (a cytoplasmic marker) is highest in the fraction containing mainly myelin while the 125I-ubiquitin conjugating activity is mainly present in the mitochondrial fraction. The increased 125I-ubiquitin conjugating activity to lactate dehydrogenase ratio in the mitochondrial fraction suggests that the former could not have arisen from contamination with cytosol.

Evidence for the Presence of Bound Ubiquitin—The mitochondrial fraction prepared as above was solubilized with 0.25% (v/v) Triton X-100 in 0.1 M Tris-HCl, pH 7.5, for 30 min, submitted to SDS-polyacrylamide gel electrophoresis and Western blotting. Immunostaining of bound ubiquitin with an affinity purified rabbit-anti ubiquitin antibody provided evidence for the presence of ubiquitin conjugates to a number of proteins with different M, (Fig. 1). This result was confirmed using several different preparations of the mitochondrial fraction and its specificity was proved by the absence of detectable protein bands when using a normal rabbit IgG instead of the anti-ubiquitin IgG, or an antiserum prepared against non-denatured ubiquitin that is much more specific for the unconjugate polypeptide (Sigma U-5379). Further evidence for the presence of conjugate ubiquitin in this mitochondrial fraction was obtained by electron microscopy and immunodecoration with anti-ubiquitin IgG followed by a goat anti-rabbit IgG colloidal gold conjugate. As shown in Fig. 2 this mitochondrial fraction, as expected from the assay of enzyme markers of the different cellular organelles, not only contains mitochondria but also a number of endoplasmic reticulum vesicles.

Furthermore, both mitochondria and endoplasmic reticulum contain ubiquitin conjugates in similar proportions and specifically bound to the membranes of these organelles. The addition of only the second antibody or of normal rabbit IgG does not show any specific gold bead conjugation (not shown).

Ubiquitin Conjugation—The presence of conjugate ubiquitin in mitochondria and endoplasmic reticulum prompted us to investigate the capability of the rabbit brain mitochondrial fraction to conjugate 125I-ubiquitin to endogenous protein substrates. The mitochondrial fraction was incubated with 125I-ubiquitin, in the presence and absence of ATP and an ATP-generating system. Samples were removed at different time intervals from zero to 60 min, loaded onto 0.3 M sucrose solution, and centrifuged for 15 min in an Eppendorf microcentrifuge to separate the protein pellet from unconjugate 125I-ubiquitin. The mitochondrial pellet was then solubilized with 0.25% Triton X-100 in 0.1 M Tris-HCl, pH 7.5, and the solubilized protein separated by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Fig. 3 shows that the rabbit brain mitochondrial fraction contains both the enzymes of the ubiquitin-conjugating system as well as proteins that can be ubiquitinated. The most prominent 125I-ubiquitin conjugates in the autoradiograms show molecular mass of 91, 87, 67, 50, 34, and 33 kDa. In addition a band of >200 kDa is also evident. Ubiquitination of proteins present in the mitochondrial fraction seems to be limited by substrate availability since addition of the exogenous (from rabbit re-
ubiquitin-protein conjugates followed by goat anti-rabbit IgG colloidal gold conjugate as a second antibody. Left and right panels show the results of two different experiments.

**Estimation of Conjugation Activity and Conjugate Turnover**—In order to have a careful estimate of both the rates of ubiquitin conjugation to and ubiquitin release from the mitochondrial fraction the amount of \(^{125}\text{I}-\text{ubiquitin}\) that become conjugated through isopeptide and thiol ester linkages were determined. \(^{125}\text{I}-\text{Ubiquitin}\) conjugation assays were found to be linear for at least 30 min at 37 °C, to be strictly ATP-dependent, and to proceed at a rate of \(0.67 \pm 0.1 \text{ nmol/min/100 } \mu\text{g protein}\) (not shown). After 15 min of incubation, the \(^{125}\text{I}-\text{ubiquitin}\) bound through thiol esters was 40 ± 5% of the total \(^{125}\text{I}-\text{ubiquitin}\) incorporated (as determined by \(^{125}\text{I}-\text{ubiquitin}\) released by boiling in the presence of an excess of 2-mercaptoethanol). This percentage decreases while increasing the incubation time. After 30 min of incubation, a typical figure for the \(^{125}\text{I}-\text{ubiquitin}\)-thiol esters was 5 ± 1% of total covalently bound \(^{125}\text{I}-\text{ubiquitin}\). In order to estimate the rate of ubiquitin release from the mitochondrial fraction, unreacted \(^{125}\text{I}-\text{ubiquitin}\) and the soluble components of the ubiquitin conjugating system (ATP, creatine phosphokinase, creatine phosphate, etc.) were separated from the mitochondrial fraction by centrifugation at 14,000 × g over a 0.35 M sucrose cushion. The mitochondrial pellet was then resuspended in a similar volume of isolation medium containing unlabeled ubiquitin and the incubation continued for 30 min at 37 °C. Evaluation of \(^{125}\text{I}-\text{ubiquitin}\) release was linear with time and with the amount of protein and provided values of \(0.15 \pm 0.03 \text{ nmol/min/100 } \mu\text{g protein}\).

**Evidence for the Enzymes of the Ubiquitin Conjugating System**—Although from the data reported above it is evident that the mitochondrial fraction contains the enzymes of the ubiquitin conjugating system, we performed more accurate analyses to identify the possible isozymic species involved. In fact, eukaryotic cells are known to contain five low Mₙ proteins (Eᵢ) able to accept ubiquitin from the Eᵢ-ubiquitin thiol ester and able to function in ubiquitin transfer to proteins. However, these isozymes have different functions and only some of these function in Eᵢ-dependent conjugation of ubiquitin to proteins (1). Several attempts performed on the crude Triton X-100 extract of the mitochondrial fraction to estimate Eᵢ and Eₒ contents were without success. Finally, we decided to prepare the fraction II of the soluble Triton X-100 extract and to purify these enzymes by ubiquitin-affinity chromatography according to previously published procedures (21). SDS-polyacrylamide gel electrophoresis and silver staining of the protein eluted from the affinity column with 2 mM AMP and 0.04 mM sodium pyrophosphate provided evidence for the presence of a protein with a molecular mass of 105 kDa, while the subsequent elution of the same column with 50 mM Tris-HCl, pH 9.0, and 2 mM dithiothreitol eluted several proteins with the prominent components showing molecular mass of 105 kDa, and 32, 24, and 17 kDa (Fig. 4). It is worth noting...
that similar M values have been previously reported for the Ei and E2 enzymes respectively (22, 40). In similar experiments the enzymes of the ubiquitin conjugating system were eluted together from the affinity column in a single step by 50 mM Tris-HCl, pH 9.0, and 10 mM dithiothreitol and their ability to form 125I-ubiquitin adducts determined in the absence of 2-mercaptoethanol. These results are shown in Fig. 4 and provide evidence for the presence of active ubiquitin conjugating enzymes in the mitochondrial fraction. Addition of E2 purified as above does not increase the number of E2s detectable by this system (not shown).

125I-Ubiquitin Conjugation to Exogenous Proteins—The evidence for an active ubiquitin conjugating system in the rabbit brain mitochondrial fraction prompted us to investigate the ability of this system to conjugate ubiquitin to exogenous proteins. As shown in Fig. 5, the ubiquitin conjugating enzymes of the mitochondrial fraction can form multiple 125I-ubiquitin conjugates with oxidized RNase. These results were obtained without any addition of detergents and under isotonic conditions thus indicating that the ubiquitin conjugating enzymes of this fraction can have access to soluble proteins as well. Finally, addition of low concentrations of detergents as 0.2 mg of digitonin/mg of proteins (Fig. 6) or 0.05% Triton X-100 (not shown) increase the conjugation of 125I-ubiquitin to the endogenous protein substrates. Since this conjugation involves additional protein bands it can be concluded that ubiquitin conjugation in the mitochondrial fraction is limited by substrate availability and that the addition of low concentrations of detergents or the addition of exogenous protein substrates increase the amount of ubiquitin-protein conjugates. Conjugation of 125I-ubiquitin to endogenous substrates decrease at high digitonine concentration due to a partial solubilization of the 125I-ubiquitin conjugating activity (see below) and of protein substrates.

Digitonin Fractionation of the Mitochondrial Fraction—The results reported above show that the ubiquitin conjugating activity does not distribute exclusively with mitochondria markers. To assess to what extent mitochondria serve as a source for the enzymes of this conjugating system, the mitochondrial fraction was fractionated with digitonin. The results obtained (Fig. 7) show that the release of lactate dehydrogenase (a cytoplasmic marker enzyme that is probably entrapped in the few synaptosomes present in the mitochondrial fraction) is not paralleled by loss of conjugating activity, thus excluding the cytoplasmic origin for the latter activity of this particulate fraction. Furthermore, the response to digitonin of the 125I-ubiquitin conjugating activity appears to correlate with the release of NADH-cytochrome c reductase, an enzyme marker for the mitochondrial external membrane (39).
for 2 h at 37 °C does not show any ATP- and/or ubiquitin-dependent degradation (not shown).

**Discussion**

Ubiquitin has been implicated in several basic cellular functions in the nucleolus, cytosol, and membranes in a number of cell types (reviewed in Ref. 1). All these functions require the conjugation of ubiquitin to specific target proteins (through its carboxyl terminal) catalyzed by $E_1$, $E_2$, and $E_3$, commonly known as the enzymes of the ubiquitin conjugating system. In some cases it has been shown that $E_3$ is not necessary for ubiquitin protein conjugation (22, 40, 41). The results reported in this paper show that a particulate fraction of the brain, enriched in mitochondria and with some endoplasmic reticulum, contains ubiquitin conjugated to a number of proteins as well as the enzymes of the ubiquitin conjugating system. This is somewhat unexpected since many reports have provided evidence for an intracellular distribution of ubiquitin that involves the nucleus and cytoplasm. However, specific roles for ubiquitin in mitochondria and endoplasmic reticulum have been suggested. In mitochondria ubiquitin has been implicated in the insertion of newly synthesized enzymes into the outer membrane (42) and in labeling this organelle for proteolytic degradation (43, 44). It has been suggested, though not demonstrated, that the endoplasmic reticulum may represent a place for ubiquitination of extracellular domains of cell surface proteins (45). Furthermore, it has been also shown that newly synthesized proteins in the endoplasmic reticulum that fail to fold correctly or assemble into appropriate oligomeric complex are retained in the endoplasmic reticulum and eventually degraded in an ATP-dependent manner, distinct from lysosomal degradation (46). To what extent our observation, namely that a particulate fraction of the brain is able to covalently conjugate ubiquitin to both endogenous and exogenous substrates, can contribute to clarify the points mentioned above is difficult to say. In fact, the mitochondrial fraction we have prepared contains both mitochondria and endoplasmic reticulum so that one or both cellular fractions can contribute to the activity observed. However some evidence suggests that the second hypothesis is likely to be correct. In fact, immunoelectron microscopy showed the presence of ubiquitin conjugates in both mitochondria and endoplasmic reticulum. Since these compartments would be normally inaccessible to cytoplasmic ubiquitin conjugating enzymes we may conclude that these conjugates are formed by local ubiquitin conjugating enzymes. As an alternative explanation these conjugates would have been formed in the cytosol and imported as ubiquitin conjugates in the compartments considered. However, this seems to be unlikely for the reasons discussed in Ref. 45. Furthermore, preliminary results from our laboratory show that both the endoplasmic reticulum and mitochondria prepared from rabbit liver (that is much more easy to fractionate than the brain) are able, although to a different extent, to conjugate ubiquitin. Finally, we have observed (not shown) that several mitochondrial fractions with different contents of endoplasmic reticulum contain almost similar specific activity of the ubiquitin conjugating system suggesting that this system is not a peculiar property of one or the other of these cell compartments.

The characterization of the ubiquitin conjugating system of the mitochondrial fraction reported in this paper provides further support to the idea of compartimentalization of the system. The evidence that low concentrations of detergents

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that release residual traces of cytoplasmic enzyme markers have no effect on ubiquitin conjugation excludes the possibility of a cytoplasmic origin for the ubiquitin conjugating enzymes of the mitochondrial fraction.

In conclusion, the present report provides evidence for a previously unrecognized cellular localization of the ubiquitin conjugating system, namely the mitochondria and endoplasmic reticulum of the rabbit brain. The biological significance of this localization is not at present clear. Although several reasonable hypotheses have been suggested and the presence for a ubiquitin conjugating system in these organelles could explain several important cellular functions as protein organelle turnover or endoplasmic reticulum degradation of newly synthesized proteins that fail to fold correctly, no experimental data are available. The results reported in this paper suggest that the cellular compartmentalization of the ubiquitin system will be a new area for investigating the role of ubiquitin in the physiology and pathology of the nervous system.

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