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STOICHIOMETRY OF THE HUMAN LYSOSOMAL CARBOXYPEPTIDASE-ß-GALACTOSIDASE COMPLEX

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The understanding of the lysosomal ß-galactosidase-carboxypeptidase-neuraminidase multienzymatic complex structure and function requires an efficient system for dissociation and association of its isolated protein components under controlled conditions. In this paper such a system was used to determine the stoichiometry of the two main components of this complex - ß-galactosidase and carboxypeptidase. The complex, after affinity purification from human placenta, was dissociated at pH 7.5 and ß-galactosidase and carboxypeptidase were separated and purified to homogeneity by FPLC anion-exchange chromatography. The 680 kD complex of ß-galactosidase and carboxypeptidase was reconstituted *in vitro* by mixing the isolated enzymes in a 1:2 molar ratio at pH 7.5 and then progressively acidifying the medium towards the intra-lysosomal pH value of 4.75. Under the same conditions, ß-galactosidase and carboxypeptidase independently existed as 306 kDa tetramer and 98 kDa dimer, respectively. Reconstitution experiments with various ratios of purified ß-galactosidase and carboxypeptidase allowed us to conclude that the whole complex is made of 4 ß-galactosidase and 8 carboxypeptidase monomers. Cross-linking of the native and reconstituted complexes with dimethylsuberimidate or glutaric dialdehyde suggested that the native and the reconstituted complexes have the same supramolecular structure.

The lysosomal carboxypeptidase (CP) also named "protective protein" (EC 3.4.16.1) is a part of a large multi-enzymatic complex with ß-galactosidase (GAL) (EC 3.2.1.23) and neuraminidase (NEUR) (EC 3.2.1.18) [1-4]. The association of CP with GAL and NEUR in lysosome is essential for the stabilization of GAL and for the expression of NEUR activity [1,3,5]. The primary defect in CP results in galactosialidosis [6,7] characterized by combined deficiency of GAL and NEUR activities and the accumulation of gangliosides and sialyl-oligosaccharides in tissues. Galactosialidosis like many lysosomal storage diseases shows considerable clinical heterogeneity with infantile, juvenile and adult forms of the disease [7,8]

Abbreviations used: ß-galactosidase - GAL, neuraminidase - NEUR, carboxypeptidase - CP, Concanavalin A - ConA, *p*-aminophenylthio-ß-D-galactopyranoside - PATGAL, Muf-ß-Gal and Muf-α-Neur are 4-methylumbelliferyl derivatives of corresponding glycopyranoside. CBZ-PheLeu - N-CBZ-L-phenylalanine-L-leucine.

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but most patients have reduced level of CP activity in cultured skin fibroblasts, leucocytes and lymphoblasts [4,8].

A complete understanding of the NEUR-GAL-CP complex structure and function requires precise determination of the stoichiometry and relationship between its protomeric components. This is best studied by using a dissociation-reassociation system in which the conditions and the relative quantities of each component can be varied experimentally. It was previously shown that the complex is in equilibrium with its individual components [9] and that GAL exists in different oligomeric forms depending on pH and ionic strength (monomers, dimers and tetramers) [2,5,10-12]. An active NEUR-GAL-CP complex was produced *in vitro* by concentration of the glycoprotein fractions of human placenta isolated by concanavalin A (ConA) - Sepharose chromatography [13]. These results suggest that reconstitution of the complex can be accomplished *in vitro* at a relatively high protein concentrations. In this paper, we focused on the reconstitution and determination of the stoichiometry of GAL-CP complex because NEUR is only a minor component of the complex.

EXPERIMENTAL PROCEDURES

Purification of GAL-CP complex. The GAL-CP complex was purified from human placenta by the method of Verheijen at al. [5] using affinity chromatography on ConA - Sepharose and *p*-aminophenylthio-ß-D-galactopyranoside (PATGAL) - agarose. After dialysis against 20 mM sodium acetate buffer, pH 4.75, 0.15 M NaCl, 0.02% (w/v) NaN_3 (buffer A), the preparation of purified complex was concentrated to 2 mg of protein/ml and applied to a FPLC Superose^R 6 HR column eluted with buffer A. Fractions corresponding to a 680 kDa species and containing both GAL and CP activities were pooled, concentrated to 0.84 mg/ml and stored at 4° C up to 30 days without any loss of GAL and CP activities.

Separation of GAL and CP by ion-exchange chromatography. The purified GAL-CP complex preparation was dialysed overnight against 10 mM Tris-HCl buffer, pH 7.5 (buffer B), centrifuged at 14,000xg for 15 min and applied to a FPLC Mono Q HR column (Pharmacia) equilibrated with buffer B. Enzymes were eluted with 40 ml of a linear gradient of NaCl (0 - 0.4 M) in buffer B with plateaus at 0.090, 0.160 and 0.225 M NaCl in order to improve the separation. The elution rate was set at 0.5 ml/min.

SDS-polyacrylamide gel electrophoresis. The electrophoretic analysis of proteins was performed on SDS-polyacrylamide gel (11% (w/v) or with a 5-15% (w/v) acrylamide gradient) under reducing conditions according to Laemmli [14]. The proteins were stained with Coomassie blue followed by silver staining using the Bio-Rad Fast Silver Staining kit. Stained gels were scanned using an Ultroscan XL Laser Densitometer (LKB).

Reconstitution of GAL-CP complex in vitro. Purified preparations of CP and GAL, separated by anion exchange chromatography of the complex at pH 7.5, were mixed with increasing CP:GAL activity ratios (1:1, 2:1 and 2.5:1), dialysed against buffer A and concentrated 25-fold (up to 0.1-0.25 mg of protein/ml) in Minicon concentration cells (Amicon). The concentrated preparation was then incubated at 37° C for 60 min, centrifuged 15 min at 14,000xg and analyzed by gel-filtration (Superose 6 HR column) to determine the amount of complex formed .

Counter-migration electrophoresis of proteins on agarose gel. Counter-migration electrophoresis of GAL and CP was performed in 0.75% (w/v) agarose gel (Bethesda Research Laboratories) using 100 mM sodium acetate buffer, pH 5.3, 20 mM EDTA in a Pharmacia GNA

100 cell for 27 h at 50 V and 4° C. The tank buffer was circulated using a peristaltic pump. Samples of 25-50 µl of the enzyme solutions (1.5 mg/ml for CP, 1.0 mg/ml for GAL and 1.7 mg/ml for lysosomal GAL-CP complex) in 20 mM acetate buffer, pH 5.3, 10% (v/v) glycerol, 20 mM EDTA and 0.02% (w/v) bromophenol blue as a marker, were loaded on the cathodic side of gel for GAL and GAL-CP lysosomal complex preparations, and on anodic side for CP preparation. In such conditions, CP and GAL migrate towards each other.

To visualize GAL activity, the gel was overlaid with a Wathman 3 mm paper, soaked in 0.5 mM resorufin-ß-D-galactopyranoside (Sigma) in 20 mM acetate buffer, pH 4.75, and incubated at 37° C for 30 min. The paper was washed in 0.4 M glycine buffer, pH 10.5 and photographed under UV light. To visualize CP activity, the gel was overlaid with a Watman paper soaked in 0.75 mM CBZ-Phe-Leu and incubated at 37° for 40 min. After incubation, the paper was quickly dried with a hair dryer and soaked in 0.2 M phosphate buffer, pH 7.3, containing horseradish peroxidase (10 µg/ml), L-aminoacid oxidase (150 µg/ml) and *o*-dianizidine hydrochloride (300 µg/ml). The paper was incubated for 1 h at 37° C until brown bands appeared on white background. Proteins were stained with Coomassie Blue R 250 (Sigma).

Cross-linking experiments. To 50 µl of the native GAL-CP complex, reconstituted GAL-CP complex, or isolated CP preparations (all in buffer A, pH 4.75, 0.5 mg of protein/ml) was added 12.5 µl of 1 and 10 mM dimethylsuberimidate (Sigma) in 0.2 M sodium carbonate buffer, pH 9.0 or 0.2 and 2 mM glutaric dialdehyde (Aldrich) in distilled water and the mixture was incubated at 37° C for 3 h. The reaction was stopped by addition of 25 µl of 0.4 M glycine buffer, pH 10.5, and the samples were analyzed by SDS-PAGE.

Enzyme assays. The neuraminidase and ß-galactosidase activities were determined using the corresponding fluorogenic 4-methylumbelliferyl-glycoside derivatives as substrates according to published procedures [15,16]. The carboxypeptidase activity was determined according to Tranchemontagne et al. [4] with CBZ-Phe-Leu as substrate at 37° C and pH 4.75. One unit of enzyme activity (U) is defined as the amount of enzyme that converts 1 µmole of substrate/min. Proteins were assayed according to Bradford [18] with bovine serum albumin as standard.

RESULTS

Separation of GAL and CP.

The GAL-CP complex was purified about 6000-fold relative to GAL activity in placenta homogenate by ConA-Sepharose and PATGAL-agarose affinity chromatography. Specific activities of GAL and CP in the purified preparation of the complex were 19 and 83 U/mg of protein, respectively. Fig 1 shows the elution profile of the purified preparation on a FPLC Superose^{R} 6 HR column. Almost 100% of CP and more than 70% of GAL activities were eluted together from the column as a high molecular mass complex of 680±10 kDa. In further experiments, only the fractions (elution volume 12 and 13 ml) containing the 680 kDa GAL-CP complex were used. Due to SDS-PAGE analysis (Fig.2 inset, panel a) the preparation contained only a 64 kDa protein, the ß-galactosidase protomer; and a 32 kDa and a 17-20 kDa protein, the two chains of CP [1], as a major components (together more than 95% of the total protein). The ratio of CP to GAL enzymatic activities in the complex was around 5.3.

As reported earlier [18], the GAL-CP lysosomal complex is dissociated into its components at pH 7.5. By gel-filtration on a FPLC Superose 12 HR column at this pH (data not shown), the major components of the complex (GAL and CP) were eluted as proteins with apparent molecular

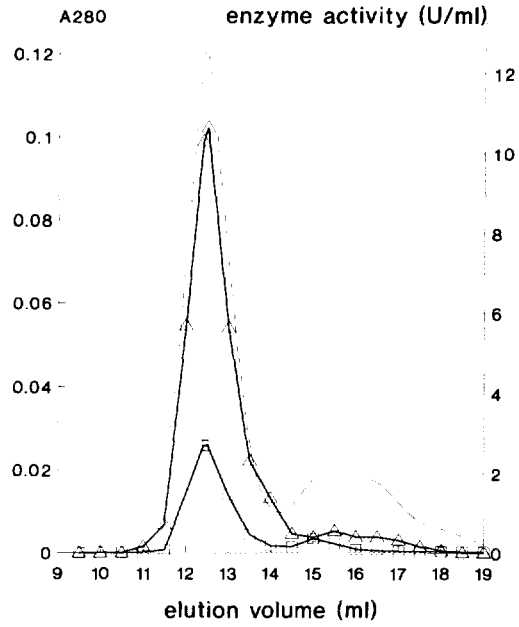

Fig.1. Gel-filtration of the preparation purified on a PATGAL-agarose column on FPLC Superose 6 HR column. (---) - absorbance at 280 nm; (-□-) - GAL activity; (-Δ-) - CP activity; (U/ml); Eluent, 20 mM sodium acetate buffer, pH 4.75, 0.15 M NaCl, 0.02% (w/v) NaN_3. Sample volume, 200 µl (protein concentration: 0.84 mg/ml). Flow rate, 0.4 ml/min. Fraction volume, 0.5 ml. CP and GAL activities in fractions were measured as described in EXPERIMENTAL PROCEDURES.

masses of around 60 kD, corresponding GAL and CP monomers. These results indicate that the conditions are adequate for dissociation of the complex. GAL and CP were then separated by FPLC anion-exchange chromatography on a Mono Q HR column eluted with a discontinuous NaCl gradient at pH 7.5 (Fig.2). CP was eluted from the column with 0.090 M NaCl, and GAL with 0.160 M NaCl followed by the peak of undissociated complex (usually 10-30% of total amount) eluted with 0.225 M NaCl. The SDS-PAGE analysis of protein components in the eluted fractions (Fig.2, inset, panel b) confirmed the results of the enzyme activity measurements. The 64 kDa GAL protomer was found in the fractions corresponding to the GAL activity peak, and the 20 and 32 kDa proteins were associated with CP activity peak. GAL (elution volume 20 to 27 ml) and CP (elution volume 12 to 17 ml) preparations collected were suitable for the reconstitution experiments since they were more than 98% pure by microdensitometric analysis of the SDS-PAGE gels.

Specific activities of the isolated preparations of CP and GAL were 131±5 and 41±3 U/mg of protein, respectively. Based on these values and on the 5.3 ratio of CP to GAL activities in the complex, we calculated that the stoichiometric molar ratio of CP:GAL in the lysosomal complex is 2:1. In preliminary experiments, we tested that specific activities of GAL and CP were

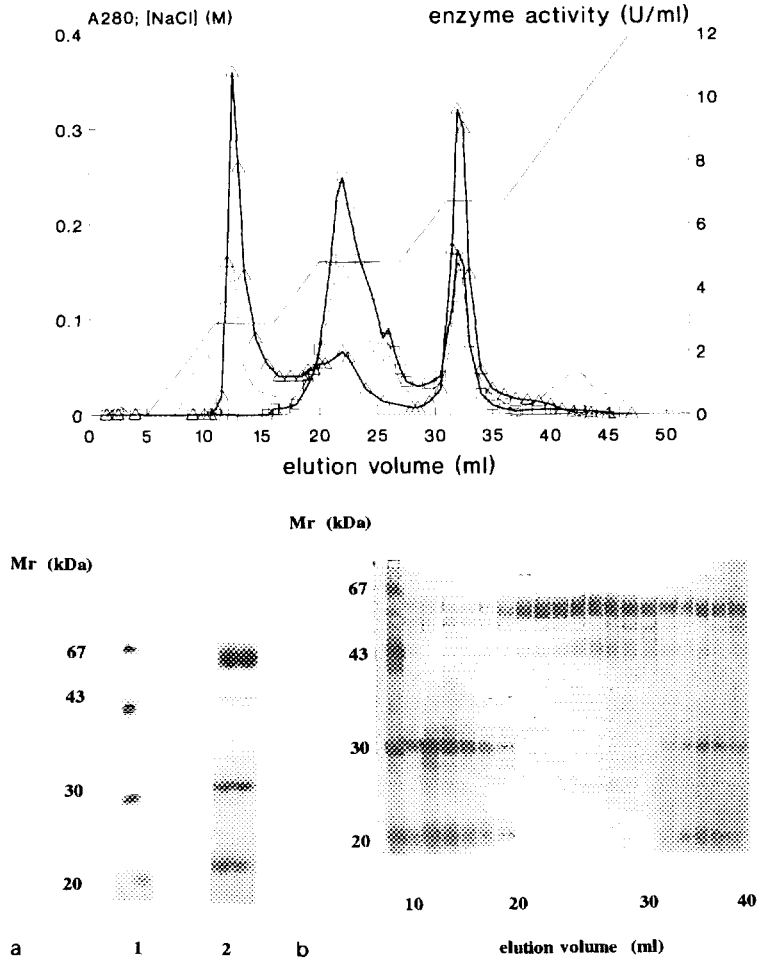

Fig.2. Separation of GAL and CP by FPLC anion-exchange chromatography on Mono Q column (---) - absorbance at 280 nm; (-□-) - GAL activity; (-Δ-) - CP activity (U/ml); (—) - NaCl concentration (M). Sample - 1 ml of the GAL-CP complex preparation (0.72 mg of protein/ml). The column was equilibrated with 10 mM Tris-HCl buffer, pH 7.5, and eluted with 40 ml of a 0 to 0.4 M NaCl gradient in the same buffer. Flow rate, 0.5 ml/min. Fraction size, 0.5 ml. Inset: SDS-PAGE of 680 kD GAL-CP complex preparation (a) and of 50 µl of each second eluted eluted from Mono Q column fraction (b). a - Lane 1, molecular mass markers; lane 2 - GAL-CP complex 10 µg. b - Lane 1, molecular mass markers; lanes 2-20 followed the elution volume. The gel concentration was 11% (w/v) and the protein bands were revealed by a combined Coomassie blue and silver staining procedure.

unaffected whether the two enzymes are separated or bound together in the complex. A molar CP:GAL ratio of 2:1 was also found by microdensitometric analysis of CP and GAL bands after SDS-PAGE of the purified 680 kDa GAL-CP complex (Fig.2, inset panel a). A calibration curve of increasing amounts of isolated CP and GAL versus microdensitometer response was first established to calculate molar quantities of CP and GAL in the complex.

Reconstitution of GAL-CP complex.

The purified preparations of CP and GAL separated by chromatography on a Mono Q HR column were used for the reconstitution experiments. They were mixed together in different molar ratios of CP and GAL of 1:1, 2:1 and 2.5:1, dialysed against buffer A (pH 4.75) and concentrated 25-fold (up to a concentration of 0.1 to 0.25 mg of protein/ml) in a Minicon concentration cell. The concentrated preparations were analyzed by gel-filtration (Superose 6 HR column) to detect the formation of the GAL-CP complex (M=680±20 kDa) (Fig.3a). Isolated GAL and CP preparations, concentrated at pH 4.75 and applied to the column independently, each eluted as a single peak with apparent M of 306±10 (GAL tetramers) and 98±10 kDa (CP dimers), respectively (data not shown). By decreasing the amount of GAL while the amount of CP in the reconstitution mixture was kept constant, approximately the same quantity of complex was formed while the amount of free GAL tetramers decreased. At a CP:GAL molar ratio of 2:1 (Fig.3a), 88% of CP and 94% of GAL activity was eluted as GAL-CP complex thus confirming the 2:1 molar ratio of CP:GAL in the complex. A GAL-CP complex with the same molecular mass and CP:GAL activity ratio was also reconstituted from GAL tetramers and CP dimers which have been treated separately at pH 4.75 and then mixed together. These results suggest that CP dimers and GAL tetramers are the basic structural elements of the 680 kDa GAL-CP complex.

GAL-CP complex formation was also studied using a counter-migration electrophoresis system[1] of these two enzymes in agarose gel. The gel pH was 5.3, in between the pI values of GAL and CP, to ensure counter-migration of the two enzymes. GAL isolated tetramers (Fig.3b, lanes 1 and 2) migrated towards the anode, CP isolated dimers (lanes 5 and 6) migrated towards the cathode and the CAL-CP complex which was used as a control (lanes 7 and 8) showed very low electrophoretic mobility. When GAL and CP preparations were loaded on the opposite ends of the same lane, an additional protein band corresponding to the merger point of GAL and CP was found (lanes 2 and 3). Staining of the gel for CP and GAL activities showed that the merger band contained both CP and GAL activities (data not shown). CP and GAL preparations were applied on the gel in CP:GAL molar ratios of 4:1 and 2:1. At the higher CP:GAL ratio (Fig. 3b), a protein band corresponding to the excess of CP (40-45% of total by microdensitometric analysis) appeared on the gel. In the second case only the merger band was found (data not shown). This result also confirms the 2:1 molar ratio value of CP:GAL in the complex and together with determined molecular mass of the complex of 680±10 kDa [9] suggest that the GAL-CP complex is composed of 8 protomers of CP and 4 of GAL.

We tested whether the structure of the reconstituted GAL-CP complex is the same as that of the native GAL-CP complex using cross-linking of the complex protomers. Both native and

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[1] Ashmarina, L. I. , Pshezhetsky, A.V., Spivey, O. H., Potier, M., manuscript in preparation.

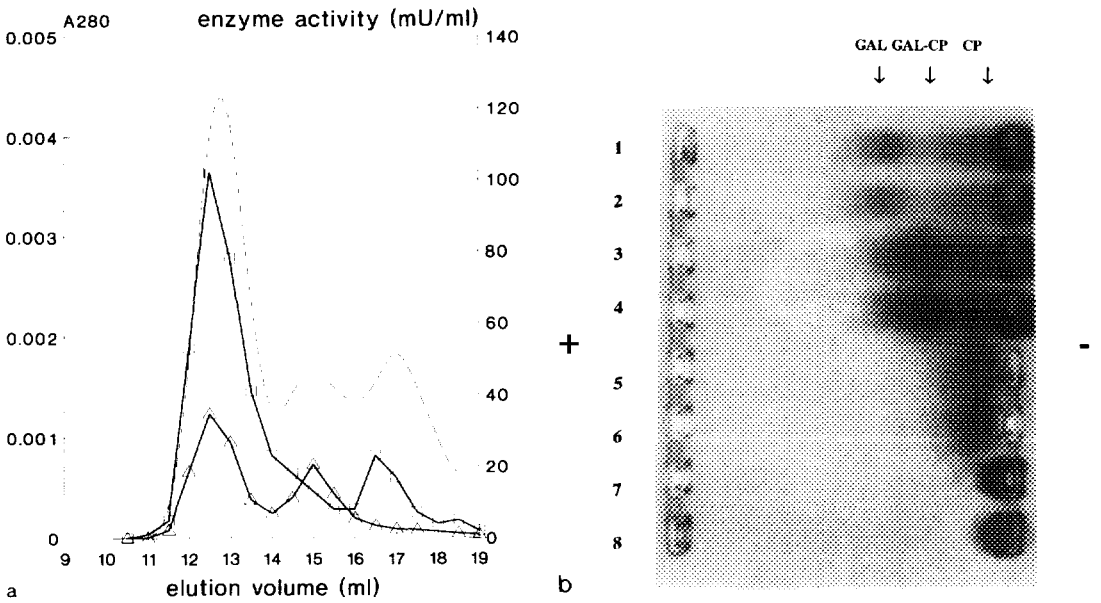

Fig 3. Reconstitution of GAL-CP complex *in vitro*. a - FPLC gel-filtration of mixture of CP and GAL individual preparations (molar ratio 2:1), dialysed against buffer A and concentrated to 200 µl in a Minicon cell. (---) - absorbance at 280 nm; (-□-) - GAL activity; (-Δ-) - CP activity (U/ml). Sample contained approximately 12 µg of CP and 7.3 µg of GAL. For the other experimental conditions, see legend to Fig.1. b - counter migration electrophoresis of GAL and CP individual preparations and lysosomal GAL-CP complex in 0.75% agarose gel. 25 µg of GAL preparation was applied to the cathodic region of lanes 1-4; 75 µg of CP preparation was applied to anodic region of lanes 3-6; 85 µg of lysosomal GAL-CP complex was applied on the cathodic region of lanes 7 and 8. Tank buffer: 0.1 M sodium acetate, pH 5.3, 0.02 M EDTA. Electrophoresis was performed at 4° C and 50 V for 27 h. Protein were stained by Coomassie blue.

reconstituted GAL-CP complexes after treatment with bifunctional reagents of different nature and length (dimethylsuberimidate and glutaric dialdehyde) yielded the same cross-linked proteins of 105, 130-140 and 680 kDa (Fig.4) which suggests that both native and reconstituted complexes have the same structural arrangement of CP and GAL protomers. The 105 kDa protein was also identified after the cross-linking of purified CP preparation (Fig.4), confirming that CP dimers are present in both native and reconstituted complexes. The glutaric dialdehyde reaction was performed at pH 4.75, a conditions which preserved the structure of the complex. However the same cross-linked species were also found after treatment with dimethylsuberimidate (Fig.4) suggesting that the dissociation of the complex at basic pH is a relatively slow process as compared to dimethylsuberimidate reaction .

DISCUSSION

Previous works have shown that GAL, CP and NEUR are not already associated in crude homogenate of human placenta but the complex can be reconstituted *in vitro* at acidic pH and at

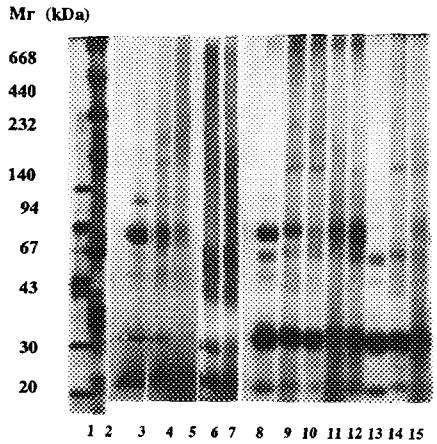

Fig.4. SDS-PAGE analysis of native GAL-CP complex, reconstituted GAL-CP complex and CP dimers, treated by cross-linking agents. Lane 1, high molecular mass markers; lane 2, low molecular mass markers; lanes 3-7, native GAL-CP complex; lanes 8-12, reconstituted GAL-CP complex; and lanes 13-15, CP dimers. The preparations (25 μg of protein) were applied without treatment (lanes 3, 8, 13) or treated by 12.5 μl of 10 mM dimethylsuberimidate (lanes 4, 9, 14); 1 mM dimethylsuberimidate (lanes 5, 10); 2 mM glutaric dialdehyde (lanes 6, 11, 15), or 0.2 mM glutaric dialdehyde (lanes 7, 12) as described in EXPERIMENTAL PROCEDURES.

relatively high protein concentrations [13]. Recently, the GAL-CP complex was also reconstituted using surfactant reversed micelles in non-polar organic solvent as microcontainers for protein assembly [18]. However, these experiments were performed with partially purified preparations of GAL and CP and therefore cannot be used to answer questions on the stoichiometry and relationship between protomeric components of the complex. In this paper, the purified complex was dissociated into its main components, CP and GAL, which were each purified to homogeneity and the 680 kDa complex was reconstituted *in vitro* by mixing the isolated enzymes in a 2:1 molar ratio at pH 4.75; the intra-lysosomal pH value [19]. The molar ratio of CP:GAL, 2:1 in the lysosomal complex, was confirmed by several independent experimental methods: 1) gel-filtration analysis of mixtures of various ratios of purified GAL and CP, 2) microdensitometric SDS-PAGE analysis of the purified complex, 3) calculations based on the specific activities of CP and GAL, and 4) counter-migration electrophoresis of the enzymes in agarose gel. The molecular mass of the GAL-CP complex was determined as 680±10 kDa by HPLC gel-filtration and sedimentation analysis [9]. These results, together with the 2:1 CP:GAL molar ratio, allow us to suggest that the complex is composed of 4 protomers of GAL (1 tetramer) and 8 of CP protomers (4 dimers).

The 680 kDa lysosomal complex of GAL and CP may play a role in the lysosome being a sort of matrix on which several lysosomal enzymes (proteins) can associate forming functional supramolecular organization or "methabolon" [20]. Two other lysosomal enzymes have been reported to be associated to this complex - NEUR [5] and NAc-α-GAL [21,22]. However, only

the NEUR association with GAL-CP complex seems to be functionally important because this enzyme was deficient, like GAL and CP, in the cells of patient affected with galactosialidosis whereas the NAc-α-GAL activity was normal [4,6-8]. Knowledge of the stoichiometry and tructure of the GAL-CP complex will allow further studies on structure-function relationships in this important lysosomal multienzymatic complex.

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