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GLUCOCEREBROSIDASE: STOICHIOMETRY OF ASSOCIATION BETWEEN EFFECTOR AND CATALYTIC PROTEINS

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Summary

1. The effector and catalytic proteins of glucocerebrosidase associated in the presence of acidic phospholipid to give active enzyme.

2. At optimum concentrations of acidic phospholipid (about 0.15 mM), the association reached equilibrium instantaneously.

3. From the experimental data, a tentative model of the association was deduced. This involved a two-step complex formation. When the effector concentration was limiting, a simple binary complex was formed between one molecule each of effector and catalytic proteins; the reaction proceeded rapidly to completion. When the effector was in excess, a ternary complex was formed by the addition of another molecule of effector; this reaction did not go to completion and was characterised by a finite equilibrium constant.

4. The experimental data were curve fitted to an equation derived from the model.

Introduction

The glucocerebrosidase system [1—5] consists of three components: the catalytic protein, normally membrane bound; the lipid membrane and/or lipid cofactor; and the effector protein, normally soluble in the cytosol.

The catalytic protein, formerly referred to as factor C, is heat labile, of large molecular weight (excluded from Sephadex G-200) [1—3] and associated with the membrane of the 'crude mitochondrial-lysosomal' fraction [2]. On solubilisation from the membrane, it becomes highly unstable unless various membrane lipids are solubilised with it [4]. In the absence of the effector, the catalytic protein is inactive but may be alternatively activated by sodium taurocholate [2].

The effector protein, formerly called factor P, is an extremely heat-stable glycoprotein of low molecular weight (<20 000) and occurs predominantly in

the cytosol [1]. It is also without enzyme activity [1,2].

The association of the effector protein with membrane-bound catalytic protein exhibited a heat and time dependence [1] probably due to the restricted availability of acidic phospholipid [5] on which the association is absolutely dependent [4].

As the optimum conditions of association between the catalytic and effector proteins have been established [4], it is now possible to examine the stoichiometry of the association. This system represents a well-defined example of protein-protein and protein-lipid interactions essential for catalytic activity. A study of its characteristics contributes to an understanding of similar interactions essential for biologic activity in cellular membranes [5].

Materials

Human spleens were provided by Dr A.D. Patrick, Institute of Child Health, University of London, U.K.

Radioactive glucocerebrosidase, made by N-acylation of glucosylsphingosine with [^{14}C]stearic acid [6], was a gift from Dr Norman Radin, University of Michigan, Ann Arbor, Mich., U.S.A. The following materials were from commercial sources as indicated: Triton X-100 (B grade) and sodium taurocholate (A grade) from Calbiochem, Los Angeles, Calif., U.S.A.; 4-methylumbelliferyl-β-D-glucopyranoside, 2,5-diphenyloxazole, 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, phosphatidylserine (ex ox brain), from Koch-Light Laboratories Ltd, Colnbrook, Bucks., U.K.; 1,4-dioxan and naphthalene from B.D.H. Chemicals Ltd, Poole, Dorset, U.K.; Sephadex G-150 from Pharmacia, Uppsala, Sweden; silica gel-precoated plates from E. Merck A-G., Darmstadt, Germany.

Methods

Preparation of the catalytic protein (factor C)

Preparation of the catalytic protein (factor C) was described previously [4]. This involved solubilisation from a total membrane fraction of normal spleen followed by gel filtration and $(NH_4)_2SO_4$ precipitation (35 g/100 ml). The product had a specific activity of 808 and represented a purification of 40-fold over the total homogenate.

Purification of the effector protein (factor P)

The effector protein was purified from the spleen of a patient with adult Gaucher's disease in which its soluble concentration was increased 10-fold [1]. A 10% (w/v) homogenate in distilled water was centrifuged (100 000 × *g*, 1 h). The supernatant was heated at 100°C for 4 min; the precipitated protein was removed by centrifugation (1000 × *g*, 15 min). The clear supernatant was concentrated 5-fold by ultrafiltration [7], then added to 9 volumes of absolute ethanol at 0°C. The precipitate was collected by centrifugation (1000 × *g*, 15 min) redissolved in water and dialysed overnight against two changes of 200 volumes of distilled water. The precipitate was removed by centrifugation (1000 × *g*, 15 min). To the clear supernatant at 0°C was added dropwise with

mixing 12% (w/v) trichloroacetic acid to a final concentration of 2% (w/v). The precipitate was removed by centrifugation (1000 × *g*, 15 min) and the supernatant dialysed overnight against water. The product ran as a single diffuse periodic acid-Schiff-positive and Coomassie blue-positive band on sodium dodecyl sulphate gel electrophoresis [8] (average molecular weight 15 000 ± 2000). The specific activity (see below) was 10 050.

Enzyme assays

Enzyme assays for acid β-glucosidase activity were performed with both the synthetic substrate, 4-methylumbelliferyl-β-D-glucopyranoside, and the natural substrate, glucocerebroside radioactively labelled in the N-acyl moiety, as already described in detail [2,3]. In the analysis of stoichiometry (see below) a limited number of data points were obtained using the natural substrate in order to confirm the general pattern of the results obtained using the synthetic substrate. No attempt was made to subject the former to detailed analysis on account of the large variation in the substrate blanks (±20%) which made it impossible to determine low enzyme activities accurately, or to pool data obtained in different experiments. Studies on stoichiometry were carried out using the synthetic substrate only. The ease of the assay procedure enabled numerous necessary determinations to be carried out at the same time.

One unit of enzyme activity is defined as that equivalent to 1 nmol substrate hydrolysed per h. The effector and catalytic protein activities are expressed in terms of units of enzyme activity. One unit of effector activity gives one unit of enzyme activity in the presence of optimum concentrations (or excess) of the catalytic protein, and vice versa [4].

Analysis of the association between effector and catalytic proteins

The basic assumptions inherent in this analysis are (a) enzyme activity is a direct measure of the concentrations of active enzyme species, and (b) active enzyme species is a simple function of the association between catalytic and effector proteins.

The justification for (a) is that the enzyme reaction follows zero-order kinetics under the assay conditions used [2]. Assumption (b) is inferred from the following observations. (i) When the availability of acidic phospholipid was limiting (e.g. when a membrane-bound preparation of the catalytic protein was incubated with the soluble effector protein in the presence of little or no detergent) the incorporation of effector into the membranous fraction (i.e. association) coincided in quantity and in time with the appearance of enzyme activity in the membranous fraction [1]. (ii) When the incubation was carried out in the presence of higher concentrations of detergents [2] or in the presence of optimum concentrations of acidic phospholipids [4] the generation of active enzyme did not exhibit the same heat and time dependence as in (i). Under these conditions, the association to form active enzyme species is assumed to reach equilibrium instantaneously. The results to be presented in this paper support this conclusion.

Analysis of stoichiometry

The effector and catalytic proteins were mixed in various proportions and

assayed for enzyme activity. The data were plotted to show the dependence of activity on the effector or catalytic protein concentration. From the qualitative form of these results, a probable model of the association was deduced, as represented in Eqns 1a and 1b, where P and C represent the effector and catalytic proteins, respectively:

$$P + C \rightarrow PC \tag{1a}$$

$$PC + P \rightleftharpoons PCP \tag{1b}$$

The association is supposed to occur in two steps. The first is the formation of a 1 : 1 complex PC going essentially to completion. The equilibrium constant K_{eq1} is thus very large. In the presence of more effector, a 2 : 1 complex PCP is formed. The equilibrium constant K_{eq2} given by Eqn 2, is believed to be small relative to K_{eq1}.

$$K_{eq2} = \frac{[PCP]}{[PC][P]} \tag{2}$$

The total activity observed is due to a combination of activities contributed by the two active enzyme species PC and PCP as follows:

$$F = c_1 [PC] + c_2 [PCP] \tag{3}$$

where F is the total activity, c_1 and c_2 are the specific activities of the active enzyme species PC and PCP, respectively.
If the initial concentrations of P and C are x and y, respectively, and $x > y$, the concentrations of PCP and PC are given by:

$$[PCP] = \tfrac{1}{2}\{x + 1/K_{eq2} - \sqrt{(x + 1/K_{eq2})^2 - 4y(x - y)}\} \tag{4}$$

$$[PC] = y - [PCP] \tag{5}$$

Optimised values for the molar ratios of the starting solutions of P and C, $[P]_0/[C]_0$; K_{eq2}, c_1 and c_2 could be obtained by a least squares fit to eqn 3 using a Fortran Computer program [9].
These calculations were based on the specific activity of the purified effector protein which was apparently homogeneous on sodium dodecyl sulphate gel electrophoresis. The preparation may, however, contain contaminants which comigrate on electrophoresis. The values of K_{eq2}, c_1 and c_2 thus represent least estimates*.

Results

Time-course of phospholipid activation

The time-course of enzyme activity was linear for different mixtures of effector and catalytic proteins in the presence of 0.15 mM (optimum concen-

*A further 10-fold purification of the effector has been achieved (ref. 12) since the completion of this work.

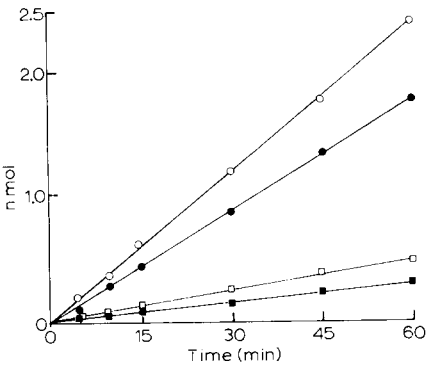

Fig. 1. Time course of enzyme activity in the presence of acidic phospholipid. The effector and catalytic proteins were mixed in two different proportions and assayed for glucocerebrosidase (solid symbols) and 4-methylumbelliferyl- β-glucosidase (open symbols) activities in the presence of 5 μg phosphatidylserine per assay volume of 40 μl (see Methods). The mixtures contained 2.2 units of C plus 5.4 units of P (circles); and 2.2 units of C plus 0.5 unit of P (squares). Each point is an average of duplicate determinations agreeing within 5%.

tration) phosphatidylserine (Fig. 1). Pre-incubation of the mixtures for various time intervals at 37°C before adding to substrate did not affect the time-course of activity nor the amount of enzyme activity observed. This indicated that in the presence of acidic phospholipid, the association reached equilibrium more rapidly than can be measured by the experimental methods used.

Stoichiometry of association

The experimental data were plotted to show the dependence of enzyme activity on initial concentrations of effector or catalytic protein (Figs 2a and 2b).

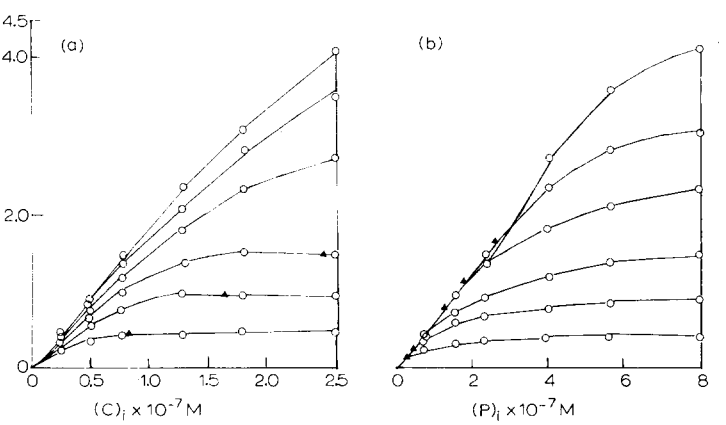

Fig. 2. Analysis of stoichiometry. Starting solutions of P ($[P]_0 = 8 \cdot 10^{-7}$ M) and C ($[C]_0 = 2.55 \cdot 10^{-7}$ M from curve fitting) were diluted and mixed in various proportions. These were assayed for enzyme activity as in Fig. 1. Enzyme activity in nmol per h was plotted against (a) initial concentration of C, $[C]_i$ and (b) initial concentration of P, $[P]_i$. Curves from the bottom upwards represent fixed initial concentrations of 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 of $[P]_0$ or $[C]_0$. Solid lines represent theoretical curves generated by the computer based on the model. The experimental data in open circles represent the average of duplicate determinations which agreed within 5%. Solid triangles represent points of theoretical equivalence of $[P]_i$ and $[C]_i$.

The two families of curves were generally biphasic; there was an initial rapid and linear rise in enzyme activity up to the points of theoretical molar equivalence between P and C; followed by no increase in enzyme activity when C was in excess (Fig. 2a, bottom 3 curves) but a slow increase with excess of P (Fig. 2b, all curves). With an excess of C, the amount of enzyme activity was strictly proportional to the concentration of P (Fig. 2a, maximum enzyme activities attained for bottom 3 curves were in multiples of that in the bottom curve. This appeared in Fig. 2b as an apparently constant initial slope of all curves).

From these observations, it may be surmised that the association was a two-step process (the first step exhibiting a 1 : 1 stoichiometry giving rise to the complex PC); that the hypothetical complex CPC was not formed but the complex PCP was formed.

These considerations gave the model presented (see Methods). The theoretical set of curves generated from optimised parameters based on the model

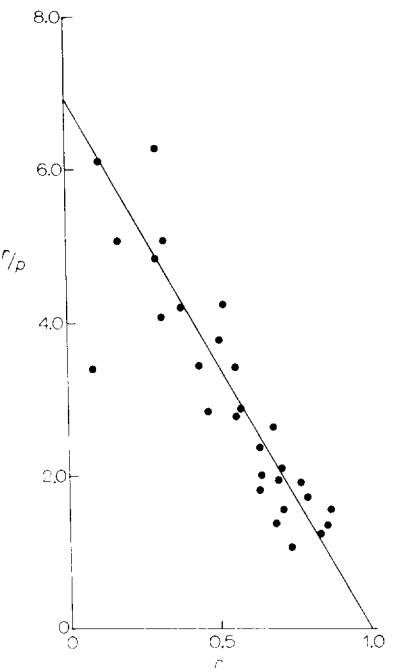

Fig. 3. Scatchard plot of second association. Data from Fig. 2 (where applicable) were transformed using Eqn 3 to calculate concentrations of PCP, PC and P in the equilibrium mixtures where $[P]_i$ was in excess.

Since $F = c_1[PC] + c_2[PCP]$ (eqn 3)

and $[PC] = [C]_i - [PCP]$, when $[P]_i$ is in excess. [PCP] and [PC] may be calculated from observed activity F using c_1 and c_2 obtained from curve fitting. The number of mol P bound per mol total PC

is, $r = \dfrac{[PCP]}{[PC] + [PCP]} = \dfrac{[PCP]}{[C]}$

Mol free P is $p = [P]_i - ([PC] + 2[PCP])$

where $[P]_i$ is the initial concentration of P in the mixture. The solid line is the theoretical-predicted curve from the model. The data points are in closed circles.

was in close agreement with the observed data points (Figs 2a and 2b). The optimised values (with standard deviations) for $K_{eq\,2}$, c_1 and c_2 were $6.9 \cdot 10^6 \pm 2.1 \cdot 10^6$ l · M^{-1}; 10.05 ± 0.5 μmol/h per mg and 35.3 ± 6.7 μmol/h per mg, respectively.

Two other sets of data using different starting concentrations of P and C were analysed and gave consistent results for c_1 and c_2 but $K_{eq\,2}$ was less well defined. Depending on initial estimates the optimised $K_{eq\,2}$ gave values between $4.5 \cdot 10^6$ and $7.0 \cdot 10^6 \cdot M^{-1}$.

None of the sets of data fitted a simple one-step model or a two-step model without the assumption of a large $K_{eq\,1}$.

The concentrations of PC, and PCP and P may be calculated from specific activities of PC and PCP according to Eqn 3. This gives a Scatchard plot of the second association (Eqn 1b). Fig. 3 shows the scatter of the actual data points around the theoretical straight line calculated from predicted values based on the model.

Discussion

The glucocerebrosidase model system may be quite general for other lipid hydrolases. Li et al. [10] have discovered that a heat-stable non-dialyzable factor from liver is required in the hydrolysis of GM_2-ganglioside by purified β-hexosaminidase A isoenzyme. This system in turn bears some resemblance to the cerebroside sulphatase system discovered some years ago, but only recently described in detail [11].

The question remains of specificity of effector proteins. Is there a single effector for each enzyme or are all these effects due to a single molecule species? Recent work to be described elsewhere [12] indicates that the first supposition may be true. The glucocerebrosidase system thus represents a particular example of perhaps a class of protein-protein and protein-lipid interactions essential to the living system.

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