Use of Affinity Chromatography for the Quantitative Study of Acceptor–Ligand Interactions: The Lactose Synthetase System

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From the effects of N-acetyl-D-glucosamine and D-glucose on the elution of the A protein of human lactose synthetase from a column of Sepharose–α-lactalbumin, values of $200\,\text{M}^{-1}$ and $0.57\,\text{M}^{-1}$ are deduced for the association constants describing the interaction between the enzyme and the respective monosaccharides.

The unique advantages of affinity chromatography as a preparative procedure in enzymology are widely recognized (Cuatrecasas & Anfinsen, 1971). However, its potential as a method for obtaining quantitative information on the interaction between an enzyme and the specific solute on which its elution is dependent remains unexplored. In the present communication we introduce this aspect of affinity chromatography by examining the effects of N-acetyl-D-glucosamine and D-glucose on the elution of the A protein of human lactose synthetase from a column of Sepharose–α-lactalbumin.

Experimental

The A protein of lactose synthetase was prepared from human milk by the method described previously (Andrews, 1970), and had a specific activity when freshly prepared of 6–7 units/mg of protein when assayed spectrophotometrically (Brodbeck & Ebner, 1966) at 25°C. Standard assays for lactose synthetase activity contained 50mM-Tris–HCl (pH7.5), 4mM-MnCl₂, 1mM-phosphoenolpyruvate, 0.1mM-ATP, 0.2mM-NADH, 0.2mM-UDP-galactose, 0.5mg of crude pyruvate kinase [type I; Sigma (London) Chemical Co., London S.W.6, U.K.]/ml, 80mM-D-glucose and 0.15mg of purified human α-lactalbumin/ml; the last-named was prepared as described by Barman (1970) for the corresponding bovine protein. In assays of N-acetyl-lactosamine synthetase activity, 6mM-N-acetylglucosamine replaced the glucose and α-lactalbumin in the above procedure.

Sepharose–α-lactalbumin was prepared by coupling bovine α-lactalbumin to activated Sepharose 6B in 0.1 M-sodium citrate buffer, pH 5.0 [see Andrews (1970) for further experimental details], and contained approx. 1.5mg of α-lactalbumin/ml of settled gel. Affinity chromatography was performed on a column (1cm × 10cm) of the modified gel, pre-

equilibrated with 0.01 M-Tris–HCl, pH 7.5, containing 0.04M-KCl and the desired concentration of monosaccharide (N-acetylglucosamine or D-glucose). In each experiment, conducted in a cold-room (5–10°C) with an effluent flow rate of 1–2ml/min, 50–100μg of A protein in 0.5ml of the pre-equilibrating medium was applied to the column and the eluate collected in 2ml fractions, which were then assayed for the appropriate activity (N-acetyl-lactosamine synthetase or lactose synthetase).

Results and discussion

Elution profiles from three affinity-chromatography experiments are shown in Fig. 1, from which it is evident that the elution volume of the A protein is strongly dependent on the concentration of N-acetylglucosamine included in the column eluent. Fig. 1 thus confirms and extends the earlier qualitative observations (Andrews, 1970; Mawal et al., 1971) that this monosaccharide retards the elution of galactosyltransferase (A protein) from columns of Sepharose–α-lactalbumin. Results obtained with glucose were similar in the sense that retardation of the A protein was again observed, but the range of glucose concentrations required to produce the effect was much higher (0.1–1.0M); this observation also finds parallel in earlier studies (Andrews, 1970; Trayer et al., 1970; Mawal et al., 1971; Trayer & Hill, 1971). Our aim is to relate the variation of elution volume with monosaccharide concentration to the association equilibrium constants of the interactions responsible for the retardation.

The theoretical treatment presented below takes into account the following features of the lactose synthetase system. (i) The presence of glucose or N-acetylglucosamine is required for pronounced interaction between α-lactalbumin and A protein: in the absence of monosaccharide, binding is not detected (Klee & Klee, 1972). (ii) From equilibrium-dialysis
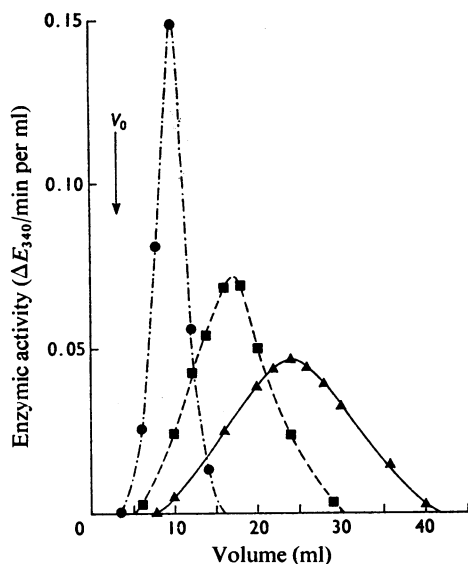
Fig. 1. *Effect of N-acetylglucosamine concentration on the elution of human galactosyltransferase from Sepharose-α-lactalbumin*

The column (1 cm × 10 cm) was pre-equilibrated with 0.01 M-Tris–HCl (pH 7.5) containing 0.04 M-KCl and the appropriate concentration of N-acetylglucos-amine: ●, none; ■, 1 mM; ▲, 2 mM.
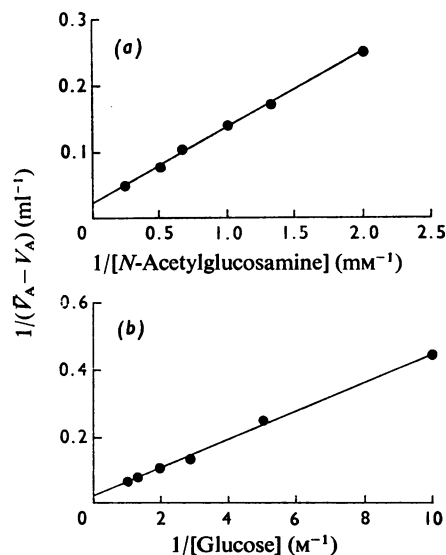

Fig. 2. *Double-reciprocal plots of the retardation of galactosyltransferase on a Sepharose-α-lactalbumin column as a function of monosaccharide concentration in the eluting buffer:* (a) N-acetylglucosamine; (b) glucose

Conditions were as in Fig. 1; for further details see the text.

(Hill *et al.*, 1969), kinetic (Morrison & Ebner, 1971*b*) and affinity-chromatography (Mawal *et al.*, 1971) studies, it seems likely that complex-formation be-tween monosaccharide and A protein precedes the interaction with α-lactalbumin. (iii) Since the double-reciprocal plot of kinetic data for the galactosyltrans-ferase reaction catalysed by the A protein alone is linear with either N-acetylglucosamine or glucose as substrate (Morrison & Ebner, 1971*a*), the interaction between monosaccharide and A protein is assigned a single equilibrium constant. Equivalence and inde-pendence of binding sites are thus assumed in the event that A protein should contain more than one binding site for carbohydrate. (iv) In the present study the concentration of galactosyltransferase (approx. 2–4 μM) is very small compared with the monosaccharide concentrations used for pre-equili-bration of the column (0.5–4 mM and 0.1–1.0 M for N-acetylglucosamine and glucose respectively), and hence the equilibrium concentration of free carbo-hydrate at all points within the column many be re-garded as indistinguishable experimentally from that used for pre-equilibration. This approximation is introduced to permit description of all parts of the migrating reaction zone in terms of a single velocity (partition coefficient).

Consider a chromatographic experiment in which a zone of galactosyltransferase (designated as acceptor A) is applied to a column of Sepharose-α-lactalbumin equilibrated with buffer containing no monosaccha-ride. Since the A protein is not eluted at the void volume (V_0) of the column, there is clearly an inter-action between A and the stationary phase G, which could be represented as $A + G \rightleftharpoons AG$. However, since the position of this equilibrium is governed largely by factors such as liquid–liquid partition of acceptor A between mobile and gel phases, it is preferable to leave the nature of the interactions unspecified, and to defined the equilibrium in terms of a partition parameter R_A, the ratio of the amount of A in the stationary phase to that in the mobile phase. A similar approach was adopted by Nichol *et al.* (1967), who used a parameter μ_A to denote the fraction of acceptor A in the mobile phase of a given segment of column. This quantity is related to the elution volume of acceptor A, V_A, by the expression $V_A = V_0/\mu_A$. The relationship between V_A and R_A is obtained by noting that:

$$\mu_A = \{[A]_m \Delta V_0\}/\{[A]_m \Delta V_0 + R_A [A]_m \Delta V_0\}$$
$$= 1/(1 + R_A) \qquad (1)$$

where $[A]_m$ is the concentration of acceptor in the mobile phase, and ΔV_0 is the volume of mobile phase in the segment of column being considered.

It follows that:

$$V_A = V_0(1 + R_A) \qquad (2)$$

If the above experiment is now repeated with a column pre-equilibrated with ligand S (either N-acetylglucosamine or glucose), the monosaccharide forms a complex with acceptor A and this complex C combines with the immobilized α-lactalbumin. The formation of complex C from A and S is assigned an association equilibrium constant K_A, but the interphasal interaction between complex C and α-lactalbumin is defined in terms of a partition parameter R_C, the ratio of the amount of C in the stationary phase to that in the mobile phase. In a segment of column containing a concentration [S] of ligand, the total amount of acceptor A (as free A or in complex C) in the mobile phase is $[A]_m \Delta V_0(1 + K_A[S])$. The corresponding amount in the stationary phase is $[A]_m \Delta V_0(R_A + R_C K_A[S])$, and an effective partition parameter for A in the presence of ligand ($\bar{R}_A$) may therefore be defined for that column segment. Clearly, a range of values of $\bar{R}_A$ will apply to a zone of acceptor within which [S] varies. However, in the present experiments approximate constancy of [S] [point (iv) above] and hence a single value of $\bar{R}_A$ may be assumed. In these circumstances we can derive an expression for $\bar{V}_A$, the elution volume at which A emerges from the column when retarded by complex-formation (eqn. 3):

$$\bar{V}_A = V_0(1 + \bar{R}_A)$$
$$= V_0\{1 + [(R_A + R_C K_A[S])/(1 + K_A[S])]\} \qquad (3)$$

On subtracting eqn. (2) from eqn. (3) we obtain eqn. (4):

$$(\bar{V}_A - V_A) = V_0(R_C - R_A)K_A[S]/(1 + K_A[S]) \qquad (4)$$

which clearly defines a rectangular hyperbola for the relationship between the extent of retardation of acceptor A due to the presence of ligand, $(\bar{V}_A - V_A)$, and the free ligand concentration [S]. Since a plot of $1/(\bar{V}_A - V_A)$ versus $1/[S]$ yields a straight line, the slope and ordinate intercept of which are $1/V_0(R_C - R_A)K_A$ and $1/V_0(R_C - R_A)$ respectively, the magnitudes of K_A and the parameter $V_0(R_C - R_A)$ may both be determined. As R_C is related to V_C, the hypothetical elution volume of pure galactosyltransferase–monosaccharide complex interacting fully with the bound α-lactalbumin, by $V_C = V_0(1 + R_C)$, and R_A is related to V_A in a similar manner (eqn. 2), the parameter $V_0(R_C - R_A)$ is simply the maximal retardation $(V_C - V_A)$ of the complex on the column.

The double-reciprocal plots of results obtained with N-acetylglucosamine and glucose are shown in Fig. 2. The linearity of both plots establishes confor-

mity of the results with the predicted relationship between retardation and [S] (eqn. 4). Assuming that eqn. (4) does describe the present system, least-squares calculations on the data of Fig. 2 yield an association constant K_A of $200 \pm 30 \, M^{-1}$ for the galactosyltransferase–N-acetylglucosamine interaction, and a maximal retardation $(V_C - V_A)$ of $43 \pm 5 \, ml$. The corresponding values with glucose as the monosaccharide are $0.57 \pm 0.05 \, M^{-1}$ and $42 \pm 4 \, ml$. It should be stressed that these conclusions rely on the relative unimportance of interaction between monosaccharide and α-lactalbumin, an assumption based on evidence presented above.

Support for the present interpretation in terms of monosaccharide association constants is provided by the results of various kinetic studies on galactosyltransferase with the two monosaccharides as substrates in the absence of α-lactalbumin. The reciprocals of Michaelis constants for the bovine enzyme give values of $280 \, M^{-1}$ (Klee & Klee, 1970) and $100 \, M^{-1}$ (Morrison & Ebner, 1971a) for the reaction involving N-acetylglucosamine, and $0.7 \, M^{-1}$ (Fitzgerald et al., 1970), approx. $1 \, M^{-1}$ (Klee & Klee, 1970) and $10 \, M^{-1}$ (Morrison & Ebner, 1971a) for the reaction involving glucose. The most recent studies on human enzyme (Kitchen & Andrews, 1973) give $1/K_m$ values of $330 \, M^{-1}$ and $1 \, M^{-1}$ for N-acetylglucosamine and glucose respectively. General similarity between these values and the above association constants is evident, but it should be noted that the K_m values may also reflect interactions of UDP-galactose and Mn^{2+} with the enzyme.

The fact that glucose and N-acetylglucosamine cause approximately the same maximal retardation of A protein on the column signifies a similar R_C value for the two galactosyltransferase–monosaccharide complexes. In terms of the present model this result implies approximate identity of the equilibrium constants for the interaction between the enzyme–carbohydrate complex and the immobilized α-lactalbumin.

The above treatment of results obtained with the lactose synthetase system illustrates a potential of affinity chromatography for studying sequential or interdependent acceptor–ligand interactions. As outlined, the method applies only to those systems in which the concentration of acceptor is negligible compared with that of ligand used for elution. In this respect, a major virtue of affinity chromatography is its ability to provide quantitative information on relatively weak interactions involving acceptors that are available only in μg quantities.

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