Consequences of Ligand Bivalency in Interactions Involving Particulate Receptors: Equilibrium and Kinetic Studies with Sephadex–Concanavalin A, Butylagarose–Phosphorylase *b*, and Fc Receptor–IgG Dimer Interactions as Model Systems†

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ABSTRACT: Theoretical consideration is given to the interaction of a bivalent ligand with particulate receptor sites, not only from the viewpoint of quantitatively describing the binding behavior but also from that of the kinetics of ligand release upon infinite dilution of a receptor–ligand mixture. In the latter regard, a general expression is derived that describes the time dependence of the amount of ligand bound as a function of two rate constants for the stepwise dissociation of cross-linked ligand–receptor complex and a thermodynamic parameter expressing the initial ratio of singly linked to doubly linked ligand–receptor complexes. An experimental study of the interaction between Sephadex and concanavalin A is then used to illustrate application of this recommended theoretical approach for characterizing the binding behavior and dissociation kinetics of a bivalent ligand for a system in which all ligand–receptor interactions may be described by a single intrinsic association constant. Published results on the interaction of phosphorylase *b* with butylagarose are also shown to comply with this simplest model of the bivalent ligand hypothesis; but those for the interaction between immunoglobulin G (IgG) dimers and Fc receptors require modification of the model by incorporation of different intrinsic association constants for the successive binding of receptor sites to the bivalent ligand. These results emphasize the need to consider ligand bivalency as a potential phenomenon in studies of interactions between protein ligands and particulate receptors and illustrate procedures by which the effects of ligand bivalency may be identified and characterized.

Despite the existence of several theoretical considerations of the interactions between particulate receptors and bivalent ligands [e.g., Crothers and Metzger (1972), Reynolds (1979), DeLisi (1979), Minton (1981), and Dwyer and Broomfield (1981)], there seems to be a reluctance by experimenters [e.g., De Meyts (1980) and Keefer et al. (1981a,b)] to consider the consequent possibility that such bivalency could well account, at least partly, for biological responses elicited by hormones such as insulin at the cellular level (DeLisi, 1979; Minton, 1981, 1982). In the past, the curvilinearity of Scatchard (1949) plots for the binding of insulin to cell membranes has been attributed either to heterogeneity (Gavin et al., 1973) or to negative cooperativity (De Meyts et al., 1973) of cell receptor sites. Although there are still some adherents (Herzberg et al., 1980) to the explanation in terms of receptor-site heterogeneity, such a concept as the sole explanation is precluded on the grounds that it disregards completely the well-documented evidence (De Meyts et al., 1973, 1976, 1978; De Meyts, 1976, 1980; Kahn et al., 1978; Keefer et al., 1981a,b) of an enhanced dissociation rate of bound tracer hormone (^{125}I-insulin) in the presence of an excess of unlabeled insulin. Indeed, such kinetic evidence was at one stage considered to be diagnostic of occupancy-dependent binding of ligands to receptor systems (Rodbard, 1979). However, as noted by DeLisi (1979), ligand bivalency also gives rise to enhanced dissociation rates in the presence of unlabeled ligand, and accordingly, the distinction between receptor-site cooperativity and ligand multivalency relies upon the kinetic order of the dissociation. For the situation based on the classical concept of negative cooperativity (Levitsky & Koshland, 1967), first-order kinetics describe the release of this small amount of ligand from receptor sites in the high-affinity state. For a system involving the release of a bivalent ligand, the first-order plot is of the curvilinear form found for the insulin–lymphocyte system (De Meyts et al., 1973, 1976). A possible reason for this observation (DeLisi, 1979) continuing to be ignored [e.g., De Meyts (1980) and Keefer et al. (1981a,b)] is the need for experimental verification of the theoretical predictions with a system for which the bivalent ligand hypothesis is not a matter of dispute.

The present investigation considers further the physicochemical characterization of the interaction between a bivalent ligand and sites on the surface of a particulate phase. After theoretical relationships that describe the equilibrium binding of ligand and the kinetics of ligand release are presented, their use is illustrated by application to an experimental study of the Sephadex–concanavalin A system, to published results (Jennissen, 1979; Jennissen & Botzet, 1979) on the interaction of phosphorylase *b* with butylagarose, and to published results (Dower et al., 1981b) on the interaction between Fc receptors and cross-linked immunoglobulin G (IgG) dimers.

Theory

Thermodynamic Considerations. Consider a series of partition experiments in which the interactions of a bivalent ligand, A, with sites X on a solid matrix (e.g., cell surface) are governed by a single intrinsic association constant, k_{AX}. From the viewpoint of quantitatively characterizing the system, this equilibrium constant is related to the liquid-phase (m_A) and total ($\bar{m}_A$) ligand concentrations by the relationship (Nichol et al., 1981)

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$$k_{AX} = \frac{1 - (m_A/\bar{m}_A)^{1/2}}{(m_A/\bar{m}_A)^{1/2}\{\bar{m}_X - 2\bar{m}_A[1 - (m_A/\bar{m}_A)^{1/2}]\}} \quad (1a)$$

where $\bar{m}_X$, the total concentration of matrix sites, is a parameter that (in addition to k_{AX}) requires evaluation from measurements of the ratio $m_A/\bar{m}_A$ as a function of $\bar{m}_A$. Simultaneous evaluation of these two parameters is conveniently accomplished (Hogg & Winzor, 1984) via a linear transform of eq 1a, namely

$$[1 - (m_A/\bar{m}_A)^{1/2}]/(m_A/\bar{m}_A)^{1/2} = k_{AX}\bar{m}_X - 2k_{AX}\bar{m}_A[1 - (m_A/\bar{m}_A)^{1/2}] \quad (1b)$$

Thus a plot of $[1 - (m_A/\bar{m}_A)^{1/2}]/(m_A/\bar{m}_A)^{1/2}$ vs. $\bar{m}_A[1 - (m_A/\bar{m}_A)^{1/2}]$ has a slope of $-2k_{AX}$ and an ordinate intercept of $k_{AX}\bar{m}_X$.

Dissociation Kinetics. Under consideration here is a system in which a mixture of ligand and matrix sites at equilibrium is suddenly diluted with sufficient buffer for the new equilibrium position, when reached, to reflect effectively complete dissociation of all ligand–matrix complexes (De Meyts, 1976). The breakdown of complexes between bivalent ligand, A, and matrix sites, X, may be represented by

$$\text{X–A–X} \xrightarrow{k} \text{X–A–} (+\text{X}) \xrightarrow{k'} \text{A} + \text{X} \quad (2)$$

where X–A–X denotes complexes with both A sites linked to matrix and X–A– those with only a single ligand–matrix link. The assumed irreversibility of the dissociation phenomenon is consistent with the design of the experimental conditions in that the concentration of free ligand at the new equilibrium position is too low for any significant extent of reassociation to occur. Determination of the rate of appearance of free ligand, A, is a standard textbook problem [see, e.g. Moore (1963)]. After inclusion of the initial $t = 0$ condition that $(m_{X\text{-}A\text{-}})_0 = \alpha(m_{X\text{-}A\text{-}X})_0$, the solution becomes

$$(m_A)_t = (m_{X\text{-}A\text{-}X})_0(1 + \alpha)\left[1 - \frac{\exp(-kt)}{1 + \alpha} - \frac{\exp(-k't)\{k\exp[(k' - k)t] + \alpha k' - k(1 + \alpha)\}}{(k' - k)(1 + \alpha)}\right] \quad (3)$$

For our purposes it is more convenient to express the time dependence of complex dissociation in terms of the relative decrease in the amount of ligand bound [equivalent to r_t/r_0, where r denotes the Klotz (1946) binding function, $r = (\bar{m}_A - m_A)/\bar{m}_X$]. Thus

$$\frac{r_t}{r_0} = 1 - \frac{(m_A)_t}{(m_{X\text{-}A\text{-}X})_0(1 + \alpha)} = \frac{k'\exp(-kt) + \{\alpha k' - k(1 + \alpha)\}\exp(-k't)}{(k' - k)(1 + \alpha)} \quad (4)$$

In addition to being dependent on the two rate constants k and k', r_t/r_0 is also seen to be a function of α, the ratio of X–A– to X–A–X initially present.

Figure 1a summarizes, in standard first-order kinetic format, the results of sample calculations with values of 10^{-4} s^{-1} and 10^{-2} s^{-1} for k and k', respectively, over the range of α values that could be encountered in practice. Only in the two extreme situations (α very small or very large) do these plots assume the first-order kinetic form that characterizes systems in which matrix sites are negatively cooperative: otherwise, the plots are curvilinear, this being a characteristic that at once distinguishes ligand bivalency from negative cooperativity of receptor sites (DeLisi, 1979).

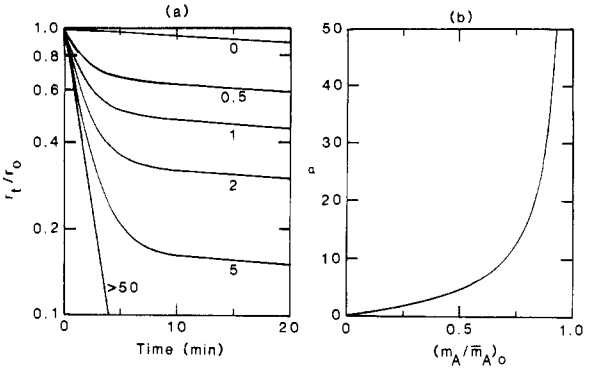

FIGURE 1: Effect of ligand bivalency on dissociation kinetics for a system (eq 2) with $k = 10^{-4}$ s^{-1} and $k' = 10^{-2}$ s^{-1}. (a) Simulated first-order kinetic plots calculated on the basis of eq 4 and the designated values of α, the ratio of X–A– to X–A–X initially present. (b) Dependence of α upon the initial distribution of ligand, $(m_A/\bar{m}_A)_0$.

From thermodynamic considerations, the ratio of initial concentrations of the two ligand–receptor complexes is given by

$$\alpha = (m_{X\text{-}A\text{-}})_0/(m_{X\text{-}A\text{-}X})_0 = 2k_{AX}m_Am_X/(k_{AX}^2m_Am_X^2) = 2/(k_{AX}m_X) \quad (5a)$$

where, from eq 2 and 5d of Nichol et al. (1981)

$$k_{AX}m_X = [1 - (m_A/\bar{m}_A)_0^{1/2}]/(m_A/\bar{m}_A)_0^{1/2} \quad (5b)$$

From Figure 1b, which summarizes the dependence of α upon the initial distribution of ligand, $(m_A/\bar{m}_A)_0$, it is evident that small values of α are associated with small values of the ratio $(m_A/\bar{m}_A)_0$, i.e., with situations where most of the ligand is bound to the matrix: this in turn implies that $(\bar{m}_A)_0 \ll \bar{m}_X$ and that r_0 is also small. High values of α, on the other hand, pertain when $(m_A/\bar{m}_A)_0 \rightarrow 1$, i.e., when the concentration of ligand bound is miniscule in comparison with its free concentration: this implies that $(\bar{m}_A)_0 \gg \bar{m}_X$ and that $r_0 \rightarrow 2$. Thus, in relation to Figure 1a, a small magnitude of α signifies a small extent of receptor-site occupancy, whereas a value of infinity would refer to an experiment in which all receptor sites were saturated during preincubation with labeled ligand. Under the latter circumstances, all ligand–matrix complexes are of the form X–A– (Sawyer & Winzor, 1975; Nichol & Winzor, 1976; Calvert et al., 1979), and accordingly, the first-order kinetic behavior (Figure 1a) defines k'. When doubly linked ligand–receptor complexes contribute significantly to the concentration of bound ligand ($\alpha < 10$), the first-order kinetic plots still exhibit an initial rapid decrease in relative amount of labeled ligand bound—reflecting dissociation of the X–A– species initially present. Thereafter, the rate of dissociation slows noticeably to a constant value governed by the rate-limiting step—the breakdown of X–A–X to X–A–.

Experimental Procedures

Materials. Concanavalin A (type III) and bovine serum albumin were obtained from Sigma Chemical Co., who also supplied the methyl glucoside (methyl α-D-glucopyranoside) used in this study. Excess NaCl was removed from the concanavalin A by dialysis for 24 h at 4 °C against phosphate–chloride buffer (0.05 M NaH_2PO_4–0.2 mM $CaCl_2$–0.2 mM $MnCl_2$–0.442 M NaCl), pH 5.5 (3 × 500 mL), after which undissolved material was removed by filtration: in some experiments methyl glucoside (80 μM) was included in the buffer used for dialysis. Concentrations of the dialyzed solutions were determined on the bases of an absorption coefficient ($A^{1\%}_{1cm}$) of 11.4 at 280 nm (Agrawal & Goldstein, 1968) and a M_r of

51 000 for concanavalin A (Becker et al., 1975).

A study of the interaction of concanavalin A with immobilized carbohydrate sites employed a batch of Sephadex G-50 medium (Pharmacia) with a hydrated inner volume (V_i) of 6.04 ± 0.07 mL/g, a value obtained by the partition equilibrium technique described previously (Nichol et al., 1974; Tellam & Winzor, 1980) but with bovine serum albumin as the excluded solute.

Thermodynamic Studies. Quantitative affinity chromatography (Nichol et al., 1974, 1981; Hogg & Winzor, 1984) has been used to obtain intrinsic association constants for the interactions of concanavalin A with methyl glucoside (k_{AS}) and the polysaccharide matrix of Sephadex (k_{AX}) by subjecting appropriately dialyzed solutions of concanavalin A (5–40 μM) to frontal chromatography (Winzor & Scheraga, 1963) on a column (1.4 × 2.9 cm) of Sephadex G-50 preequilibrated with either the above phosphate–chloride buffer, pH 5.5, or the same buffer supplemented with 80 μM methyl glucoside. The column, thermostatically maintained at 5 °C, was operated at a flow-rate of 40 mL/h, the column effluent being monitored continuously at 280 nm. $\bar{V}_A$, the elution volume of concanavalin A under the particular conditions, was obtained from the median bisector of the advancing elution profile, and V_A^*, the elution volume in the absence of any interaction with matrix, was taken as the elution volume of bovine serum albumin, a protein that is also excluded from the gel phase of Sephadex G-50. Results were analyzed in accordance with the expression (Hogg & Winzor, 1984)

$$[1 - (V_A^*/\bar{V}_A)^{1/2}]/(V_A^*/\bar{V}_A)^{1/2} = k_{AX}\bar{m}_X - 2k_{AX}(\bar{V}_A/V_A^*)m_A[1 - (V_A^*/\bar{V}_A)^{1/2}] \quad (6)$$

in which m_A refers to the concentration of concanavalin A in the plateau region of the elution profile. Allowance for competition between matrix sites and methyl glucoside, present at concentration m_S, for concanavalin A sites was accomplished (Kuter et al., 1983; Harris & Winzor, 1985) by substituting for the ligand–matrix association constant a constituent parameter, $\bar{k}_{AX} = k_{AX}/(1 + k_{AS}m_S)$, which takes into account the effects of the ligand–methyl glucoside interactions governed by intrinsic binding constant k_{AS}.

Dissociation Kinetics. Sephadex G-50 (0.5 g) was placed in the glass column (4.5 × 6 cm) of the recycling partition assembly described previously (Ford & Winzor, 1981) and allowed to equilibrate with 20 mL of concanavalin A (50.6 or 25.7 μM) in the phosphate–chloride buffer, pH 5.5, for 3 h at 5 °C. Effective mixing of the gel slurry was ensured by use of an overhead stirrer and also by the relatively rapid flow rate (6.5 mL/min) maintained by a peristaltic pump in the recycling system, which returned the column effluent, via a flow-cell (5-mm path length), to the slurry. Displacement of the partition equilibrium was commenced by the rapid addition of 10 mM methyl glucoside in phosphate–chloride buffer (20 mL), and the progress of the consequent dissociation of concanavalin A from the Sephadex G-50 was monitored at 280 nm for the next 15–20 min (Figure 2). Control experiments in which potassium chromate (20 mL) was added to Sephadex G-50 (0.5 g) equilibrated with buffer (20 mL) signified the requirement of a 2–3-min period for the liquid phase to attain equilibrium in a noninteracting situation; and accordingly, absorbance readings were disregarded during the first 5 min after addition of methyl glucoside to initiate dissociation of concanavalin A from the Sephadex.

Results

Interaction of Concanavalin A with Sephadex. The first experimental system selected to illustrate application of the above theoretical expressions entails the interaction between the polysaccharide matrix of Sephadex G-50 and concanavalin A, a dimeric lectin with two equivalent and independent carbohydrate-binding sites (So & Goldstein, 1968). The interaction was investigated at 5 °C in a phosphate–chloride buffer (pH 5.5, I = 0.5), conditions selected to render negligible the extent of concanavalin A self-association (Senear & Teller, 1981). In these studies advantage is taken of the fact that effectively complete dissociation of matrix–ligand complexes may also be achieved by including a sufficiently high concentration of a univalent saccharide (methyl glucoside), which competes with matrix for ligand sites. Under such circumstances the kinetics of release continue to be governed by eq 2–4 inclusive and are much more easily followed inasmuch as less sensitive assay procedures may be used. Thus, whereas resort to dilution as a means of effecting dissociation of ligand–matrix complexes requires the use of radiolabeled ligand to allow its detection in the highly dilute environment, the same end result (effectively complete dissociation of all Sephadex–concanavalin A complexes) is accomplished by a dilution (less than 2-fold) that still allows the appearance of concanavalin A in the liquid phase to be followed spectrophotometrically. However, in such experiments it is clearly important to establish the extent to which the concentration of free ligand is depleted by methyl glucoside addition, and accordingly, the thermodynamic characterization should include evaluation of the ligand–methyl glucoside association constant (k_{AS}) as well as that for the ligand–matrix interaction (k_{AX}).

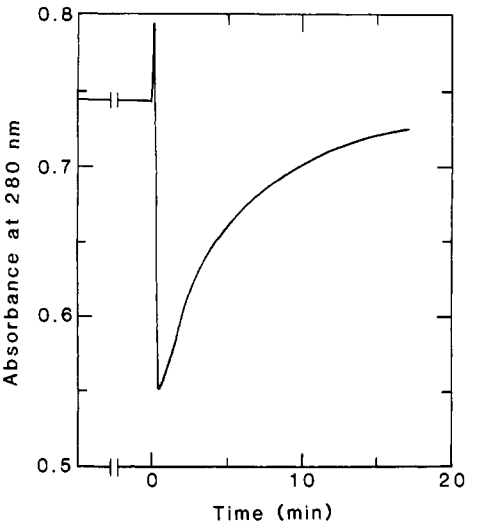

FIGURE 2: Experimental record from a recycling partition study of the kinetics of lectin dissociation at 5 °C effected by the addition of methyl glucoside (20 mL, 10 mM) to a 20-mL slurry of Sephadex G-50 (0.5 g) and concanavalin A (50.6 μM) in phosphate–chloride buffer, pH 5.5.

Figure 3 summarizes results of frontal affinity chromatography experiments with concanavalin A (5–40 μM) in the absence (□) and presence (■) of methyl glucoside (80 μM), the format of presentation being in accordance with eq 6. For each set of results the essential linearity of the plot signifies conformity of the system with eq 1 and hence with the concept that a single intrinsic association constant, k_{AX}, suffices to describe the interactions between Sephadex and the bivalent concanavalin A molecules. Least-squares calculations on the results obtained in the absence of methyl glucoside (□) yield a value of 13 000 ± 3000 M^{-1} for the ligand–matrix intrinsic association constant and an effective matrix site concentration ($\bar{m}_X$) of 46.3 μM. The value of 5000 M^{-1} for k_{AS} that is obtained from the ratio of either the slopes or the ordinate

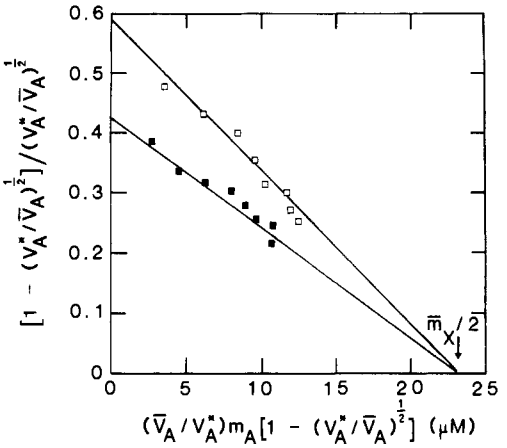

FIGURE 3: Thermodynamic characterization of the interactions of concanavalin A with Sephadex and methyl glucoside by quantitative affinity chromatography. (□) Results (plotted in accordance with eq 6) from frontal experiments on a column (1.4 × 2.9 cm) of Sephadex G-50 equilibrated at 5 °C with phosphate–chloride buffer, pH 5.5. (■) Corresponding results from experiments using buffer supplemented with 80 μM methyl glucoside.

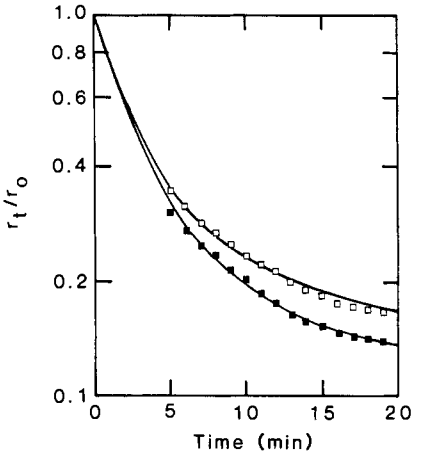

FIGURE 4: First-order kinetic plot of the dissociation of concanavalin A from Sephadex G-50 effected by introduction (at zero time) of methyl glucoside. (■) Results inferred from the experimental record shown in Figure 2 for an initial system comprising 50.6 μM lectin ($\bar{m}_A$) in a 20-mL slurry containing 0.5 g of Sephadex. (□) Corresponding results in a recycling partition experiment with an initial concanavalin A concentration ($\bar{m}_A$) of 27.5 μM. The solid lines are theoretical plots predicted by eq 4 for a system with $k = 4 \times 10^{-4}$ s^{-1} and $k' = 6 \times 10^{-3}$ s^{-1}, together with values of α determined from $(m_A/\bar{m}_A)_0$ and eq 5.

intercepts of the plots in Figure 3 is in excellent agreement with that of 4900 M^{-1} reported in Table II of So and Goldstein (1968) for the interaction of methyl glucoside with two equivalent and independent carbohydrate-binding sites on concanavalin A. In this regard it should be noted that the value of k_{AS} alone has absolute physical significance, since k_{AX} and $\bar{m}_X$ are defined, for convenience, on the basis of a system in which the matrix sites are distributed uniformly in the volume of liquid phase accessible to ligand. Although thermodynamically valid as a means of expressing the concentrations of matrix–ligand complexes in terms of the concentration of free A in the experimental system studied, these values of k_{AX} and $\bar{m}_X$ do not necessarily apply to other experiments in which the amounts of liquid and gel phases vary from those used for the characterization of $\bar{m}_X$ and k_{AX}. Of greatest importance from the present viewpoint, therefore, is that fact that Figure 3 provides further support for the bivalency of concanavalin A in its interaction with Sephadex, and hence for the suitability of this system for exploring the dissociation kinetics of a bivalent ligand.

Figure 4 summarizes results obtained in two recycling partition experiments designed to evaluate the dissociation kinetics of concanavalin A from Sephadex G-50. Closed symbols refer to an experiment in which dissociation was effected by adding an equal volume (20 mL) of 10 mM methyl glucoside to Sephadex G-50 (0.5 g) equilibrated with 50.6 μM concanavalin A and open symbols to a corresponding experiment with approximately half that lectin concentration (27.5 μM concanavalin A). Several points are noted.

(i) Combination of a partial specific volume of 0.60 for Sephadex G-50 (Tellam & Winzor, 1980) with the measured stationary phase volume (6.04 mL/g) yields a value of 17.3 mL for the volume of liquid phase in these experiments.

(ii) On the basis of a k_{AS} of 5000 M^{-1} (Figure 3) and a final concentration of 5 mM methyl glucoside in the diluted environment, the extent by which the concentration of free ligand is depleted, namely, $2(1 + k_{AS}m_S)^2$, is approximately 1400-fold.

(iii) From the initial concentration of concanavalin A in the liquid phase (Figure 2), the value of $(m_A/\bar{m}_A)_0$ in the experiment with higher lectin concentration (■) is calculated to be 0.438, which in turn (via eq 5) signifies a magnitude of 3.9 for α. This value of α was then employed in eq 4 to obtain best fit estimates of k and k', the curve in Figure 4 corresponding to $k = 4 \times 10^{-4}$ s^{-1} and $k' = 6 \times 10^{-3}$ s^{-1}.

(iv) Combination of these estimates of k and k' with the value of 3.1 inferred for α from the initial distribution of concanavalin A in the experiment with lower lectin concentration (□) leads to the other theoretical curve shown in Figure 4: clearly, the agreement between experiment and prediction is eminently reasonable.

(v) Thus, there is not only experimental verification of the qualitative features of Figure 1a for this model system but also quantitative agreement with the predictions of eq 4 and 5.

(vi) Since eq 4 and 5 have kinetic and thermodynamic bases, respectively, the present approach to testing for possible ligand bivalency seemingly overcomes an earlier criticism (De Meyts, 1979) that the concept lacks credibility in the absence of any demonstrated correspondence between analyses of binding data and dilution kinetics.

Interaction of Phosphorylase b with Butylagarose. Attention is now turned to the analysis of a second model system, the interaction of phosphorylase *b* with hydrophobic sites on butylagarose, for which the nonrectangular hyperbolic form of the binding curve has also been recognized to be due to multivalency of this dimeric enzyme (Jennissen, 1976, 1979). Moreover, although not so interpreted, the kinetic behavior in dilution experiments (Jennissen & Botzet, 1979) is qualitatively similar to that observed for the Sephadex–concanavalin A system. Subject to the binding data being described adequately by eq 1, this system should provide a second, independent illustration of the potential of eq 4 and 5 for the identification and characterization of ligand bivalency as the source of deviations in the binding response of immobilized receptors from rectangular hyperbolic form.

Figure 5a presents the reappraisal, in accordance with eq 1b, of a binding study reported in Figure 3 of Jennissen (1979) for the interaction of phosphorylase *b* with butylagarose. Least-squares calculations signify values of 16000 (±3000) M^{-1} and 0.19 (±0.07) mM for k_{AX} and $\bar{m}_X$, respectively, there being no obvious systematic departure of these results from such a description (Figure 5a). This finding allows progression to Figure 2c of Jennissen and Botzet (1979), which presents the time dependence of phosphorylase *b* dissociation following a 1000-fold dilution of an enzyme–butylagarose mixture with

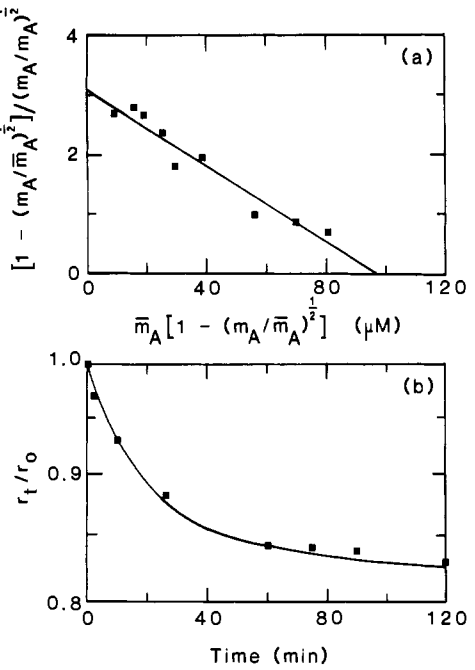

FIGURE 5: Analyses of published results for the butylagarose–phosphorylase *b* system in terms of the bivalent ligand hypothesis. (a) Plot of binding data, inferred from Figure 3 of Jennissen (1979), in accordance with eq 1b. (b) Reassessment of results on the kinetics of dissociation, together with the theoretical curve predicted (eq 4 and 5) for a system with $k = 5 \times 10^{-6}$ s^{-1}, $k' = 10^{-3}$ s^{-1}, and $(m_A/\bar{m}_A)_0 = 0.0068$: the experimental points are recalculated from those presented in Figure 2c of Jennissen and Botzet (1979).

total $[(\bar{m}_A)_0]$ and free $[(m_A)_0]$ phosphorylase *b* concentrations of 9.6 and 0.065 mg/mL packed gel, respectively: a value of 0.18 for α is thus obtained from eq 5. Figure 5b presents the dilution kinetics results, together with the theoretical behavior of a system with $\alpha = 0.18$, $k = 5 \times 10^{-6}$ s^{-1}, and $k' = 10^{-3}$ s^{-1}, a description that is certainly acceptable. Thus, whereas the second phase of the kinetic experiment ($t > 60$ min) was originally considered (Jennissen, 1978; Jennissen & Botzet, 1979) to signify a hysteretic effect whereby the desorption isotherm did not retrace its adsorption counterpart, Figure 5b suggests nonattainment of equilibrium in the desorption experiments as a likely source of the observed hysteresis. In that regard, the present interpretation is strengthened by the fact that α is not merely a parameter whose magnitude is gleaned from curve fitting of results to eq 4. Predetermination of the magnitude of α from thermodynamic considerations (eq 5) clearly adds weight to the possibility that results during the second hour of the dilution experiment signify a slow but systematic decline in r_t/r_0 rather than attainment of a desorption equilibrium state that differs from that predicted on the basis of the adsorption isotherm.

Interaction of IgG Dimer with Fc Receptors. The Sephadex–concanavalin A and butylagarose–phosphorylase *b* interactions have both served as satisfactory model systems for testing the theoretical procedures developed to analyze the binding of a bivalent ligand to cell surface receptors. By so doing they also serve to indicate the possibility that thermodynamic description of ligand–receptor interactions in terms of a single intrinsic binding constant is not necessarily a gross oversimplification of the experimental situation: in the event that the two intrinsic binding constants were to differ slightly, the determined k_{AX} would be a mean value. However, as noted by Dower et al. (1981a,b), there are certainly systems for which such description is totally inappropriate and for which, therefore, the present approach requires modification. In the

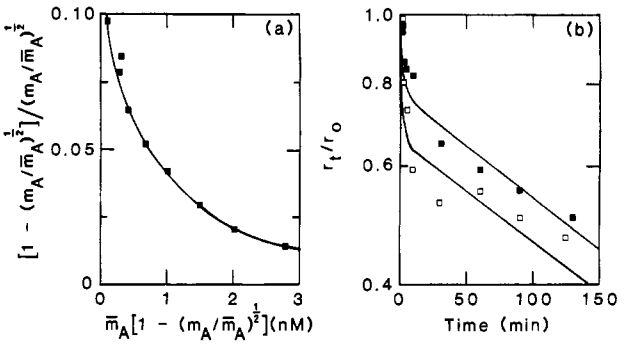

FIGURE 6: Analysis of published results for the interaction of IgG dimers with P338$_1$ cells. (a) Replot of binding data, taken from Figure 4 of Dower et al. (1981b), in accordance with eq 1b, the theoretical plot having been calculated on the basis of eq 7 with $k_{AX} = 1.6 \times 10^6$ M^{-1}, $\bar{m}_X = 5.8 \times 10^{-9}$ M, and $B = 5.7 \times 10^{-4}$. (b) Replot of results, obtained from Figure 3 of Dower et al. (1981b), on the kinetics of dissociation from experiments with antibody concentrations of 81 (□) and 11 μM (■). The lines are theoretical plots calculated (eq 4) on the basis of values of 6×10^{-5} s^{-1} and 10^{-2} s^{-1} for k and k', respectively, and magnitudes of α calculated from the thermodynamic analysis (panel a and eq 8).

event that eq 1 should fail to describe adequately the binding data, the plot of results in accordance with its linear transform (eq 1b) would exhibit curvature. The use of this bivalent equivalent of the Scatchard analysis (Hogg & Winzor, 1985) to diagnose the experimental requirement of a different intrinsic binding constant for the second matrix–ligand interaction is illustrated in Figure 6a, which presents reanalysis of published results (Dower et al., 1981b) for the binding of dimeric (bivalent) anti-2,4-dinitrophenol antibodies to P338D$_1$ cells.

In view of the demonstrated nonconformity of these results with the concept of a single intrinsic association constant for all receptor–antibody interactions, the thermodynamic part of the present analysis is modified by writing the binding equation for the system as

$$r = \frac{2k_{AX}m_A m_X + Bk_{AX}^2 m_A m_X^2}{m_X + 2k_{AX}m_A m_X + 2Bk_{AX}^2 m_A m_X^2} \qquad (7)$$

in which the association constant for formation of doubly linked complex (X–A–X) is expressed as Bk_{AX}^2 (Sawyer & Winzor, 1976). Dower et al. (1981a,b) have also adopted the viewpoint that B should be coupled with k_{AX} and hence regarded as a steric strain factor (Schumaker et al., 1973) that modifies the intrinsic association constant for the second interaction of A with X. On the other hand, it is also reasonable to argue that the second interaction requires description in terms of a localized surface concentration of receptor sites (Reynolds, 1979), in which case the product Bm_X expresses this concentration in terms of the bulk receptor concentration m_X. Fortunately, it is immaterial which of these interpretations of B is adopted, since they merely represent different but thermodynamically equivalent mechanisms to account for the relative concentrations of X–A– and X–A–X. The curve drawn in Figure 6a corresponds to the system considered (Dower et al., 1981b) to provide the best description of the experimental data, namely, K_1 ($\equiv k_{AX}$) $= 1.6 \times 10^6$ M^{-1}, K_2R_0 ($\equiv Bk_{AX}^2\bar{m}_X$) $= 8.4$, and a receptor-site concentration of 4.1×10^6 cells/mL ($R_0 \equiv \bar{m}_X = 5.8 \times 10^{-9}$ M): on these bases B is 5.7×10^{-4}.

In order to proceed with analysis of results on the kinetics of dissociation, we clearly need an expression for α that is the equivalent of eq 5. From the numerator of eq 7, the analogue of eq 5a is simply

$$\alpha = 2k_{AX}m_A m_X / Bk_{AX}^2 m_A m_X^2 = 2/(Bk_{AX}m_X) \quad (8a)$$

but unfortunately there is no direct equivalent for eq 5b. This difficulty may be circumvented by noting that the total concentration of receptor sites ($\bar{m}_X$), given by the denominator of eq 7, is a quadratic in m_X for which the only phsically acceptable solution is

$$m_X = [-(1 + 2k_{AX}m_A) + \sqrt{\Delta}]/(4Bk_{AX}^2 m_A) \quad (8b)$$

$$\Delta = (1 + 2k_{AX}m_A)^2 + 8Bk_{AX}^2 m_A \bar{m}_X \quad (8c)$$

Although the required value of m_A, the free concentration of ligand, is directly measurable prior to the addition of buffer to effect dissociation, the values of m_A appropriate to the results presented in Figure 6b were not reported by Dower et al. (1981b): they did, however, provide the relevant values of $\bar{m}_A$ (81 nM and 11 nM for the experiments indicated by open and closed symbols, respectively). From considerations of mass conservation, the free and total ligand concentrations are related by the expression

$$m_A = \bar{m}_A/[1 + k_{AX}m_X(2 + Bk_{AX}m_X)] \quad (9)$$

and hence the values of m_A and m_X may be obtained by iterative application of eq 9 and 8 with $\bar{m}_X$ as the initial estimate of m_X in eq 9. Such iteration has yielded values of 0.30 and 0.52 for α in the experiments conducted with lower (■) and higher (□) ligand concentrations, respectively. The curves drawn in Figure 6b were constructed on the basis of these values of α and respective estimates of 6×10^{-5} s^{-1} and 10^{-2} s^{-1} for k and k', which seemingly provide reasonable descriptions of the dissociation kinetics. Further refinement of the magnitudes of k and k' does not seem warranted in view of the considerable scatter exhibited by the experimental points and of the uncertainty inherent in the magnitudes of α as the result of the tortuous pathway by which they were estimated. Of much greater interest from the present viewpoint is the fact that the thermodynamic characterization of the IgG dimer–receptor system (Figure 6a and eq 7) is entirely consistent with analysis of the dissociation kinetics (eq 4) in experiments conducted with different initial degrees of receptor site saturation. Such a finding obviously adds weight to the validity of considering the results (Figure 6) in terms of eqs 2, 4, and 7, which express quantitatively the bivalent ligand hypothesis for this particular ligand–receptor system.

Discussion

The aim of this investigation has been to provide and consider experimental results that reflect the interaction of bivalent ligands with particulate receptors and thereby to explore the importance of this concept as a plausible alternative to receptor site cooperativity as an explanation of the binding behavior and dissociation kinetics observed with some cell surface receptor–protein systems. Whereas results for two model systems (the Sephadex–concanavalin A and butylagarose–phosphorylase *b* interactions) comply with the simplest concept that all ligand–matrix interactions are describable by a single intrinsic association constant k_{AX}, those for the interaction of IgG dimers with Fc receptors do not. Decisions about the requirement of one or two intrinsic association constants are readily made by assessing binding results in accordance with the bivalent equivalent (Hogg & Winzor, 1985) of the Scatchard analysis, the linearity of Figures 3 and 5a signifying the sufficiency of a single k_{AX}, whereas the curvilinearity of Figure 6a signifies the requirement of a second constant to describe the interaction that forms the doubly linked X–A–X species from its singly linked counterpart, X–A–. Irrespective of which situation pertains, the dissociation kinetics may be considered to be described by eq 4, in which the above differences are manifested in the thermodynamic parameter α, which is given either by eq 5 or by eq 8. It is therefore a relatively simple matter to decide whether the curvilinear form of the first-order kinetic plot (e.g., Figures 4, 5b, and 6b) is adequately described by the thermodynamically determined α value and hence whether ligand bivalency suffices to account for the observed curvilinearity.

From the viewpoint of interpreting the results of dissociation kinetics experiments, a major advantage of the present approach is its reliance upon predetermination of α from equilibrium considerations, a factor that automatically guarantees the compatibility of analyses of the thermodynamic and kinetic results. Dower et al. (1981b) also recognized the advantage of such a combination of binding and kinetic data but chose to do so by incorporating the thermodynamic constants and concentrations directly into the kinetic expression: the latter was also more complicated because of its more rigorous consideration of the dissociation process in terms of forward and reverse rate constants. The present decisions (a) to incorporate the thermodynamic characterization into a single parameter α and (b) to simplify the kinetic analysis by disregarding reassociation were taken in the belief that many researchers in the cell surface receptor field may find the consequent analysis easier to interpret and perform. It must be stressed, however, that the present analysis and the one devised by Dower et al. (1981b) are based on comparable models of the bivalent ligand hypothesis.

Minton (1981, 1982), on the other hand, has considered a slightly different version of the bivalent ligand hypothesis in which the two ligand sites interact with different types of receptor site. Thus, whereas the curvilinearity of the multivalent Scatchard plot for the IgG dimer–Fc receptor system was attributed to nonidentity of the intrinsic association constants for the two interactions of IgG dimer with identical Fc receptor sites, Minton (1981, 1982) considers the possibility that different constants may pertain because of ligand interactions with different types of receptor sites: the same situation would arise, of course, if the two sites on the ligand were nonidentical. The important point to note is that eq 4 may be considered to describe the kinetics of dissociation for any of these variants of the cross-linking hypothesis, the only changes required being in the expression for the thermodynamic parameter α.

Finally, although this investigation has emphasized effects of bivalency of a protein ligand on its interaction with particulate receptor sites, the need to consider cross-linked ligand–receptor complexes clearly arises with any multivalent ligand, which should therefore exhibit qualitatively similar behavior to that illustrated for the three bivalent protein systems. This expectation is borne out by results (Cuatrecasas, 1973a,b) for the interaction of cholera toxin, then considered to be a tetramer, with liver membranes. Analysis of the binding results [Figure 5 of Cuatrecasas (1973a)] in terms of the tetravalent counterpart of the Scatchard plot (Figure 7a) signifies adequacy of a single intrinsic association constant [$k_{AX} = 1.1\ (\pm 0.4) \times 10^8$ M^{-1}] to describe the toxin–receptor interactions,[1] and the dissociation kinetics [Figure 7b; calculated from Figure 7 of Cuatrecasas (1973b)] are clearly of the same general form as those obtained for the three bivalent ligands. Unfortunately, the existence of many forms of cross-linked complexes precludes analysis of the kinetics in

[1] An even better description, viz., $k_{AX} = 8.4\ (\pm 0.8) \times 10^7$ M^{-1}, is obtained by considering cholera toxin to be pentavalent (Dwyer & Bloomfield, 1982) rather than tetravalent.

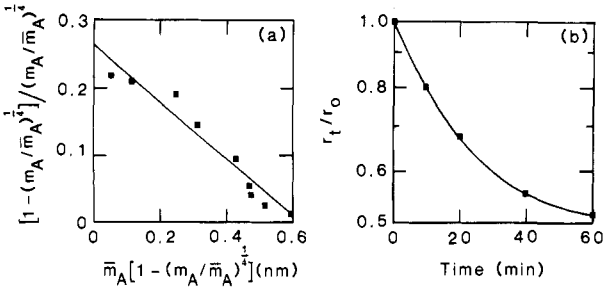

FIGURE 7: Analysis of published results on the interaction of cholera toxin with liver cells. (a) Test of binding data, taken from Figure 5 of Cuatrecasas (1973a), for conformity with the concept that the toxin (a tetramer) is a tetravalent ligand. (b) First-order kinetic plot of results presented in Figure 7 of Cuatrecasas (1973b) for the time dependence of dissociation effected by dilution.

accordance with eq 4, and accordingly, no quantitative interpretation is attempted. The results do, however, serve to emphasize that ligand multivalency is a possibility that cannot be ignored in studies of interactions between proteins and cell surface receptors. Furthermore, consideration of binding curves and dissociation kinetics for a system such as the insulin–receptor interaction to be a consequence of the ligand rather than of the receptor (DeLisi, 1979; Minton, 1981, 1982) has the undeniable attraction that it obviates the need to postulate the occurrence of similar insulin-dependent transitions within the diverse cell and membrane preparations for which the "negatively cooperative" behavior is observed [Table I of De Meyts (1976)]. It is hoped that this investigation may stimulate serious consideration of the possible consequences of ligand multivalency in future analyses of protein interactions with cell surface receptors.

Registry No. Concanavalin A, 11028-71-0; Sephadex G-50, 9048-71-9; butylagarose, 72980-05-3; phosphorylase *b*, 9012-69-5.

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