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Interaction of a Bivalent Ligand with IgM Anti-Lactose Antibody†

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ABSTRACT: A model system has been developed for the study of the interaction between bivalent ligands and multivalent antibody. This system utilizes modified β_2-microglobulin as the carrier of the reactive lactosyl groups and equine anti-lactose IgM antibody. Chemical modification of the carrier allows conjugation of two such groups to cysteinyl residues at positions 25 and 81 with a potential separation of 20 nm. Each lactosyl group contains a 2,4-dinitrophenyl moiety which serves as a sensor of the binding of the group to an antibody site and permits binding measurements by fluorescence quenching. Examination by analytical ultracentrifugation has demonstrated no significant cross-linking by the bivalent ligand of either the IgM antibody or the monomer (IgMs) derived from it. Binding constants for complex formation between the bivalent and monovalent ligands and IgM, IgMs, and Fabμ have been determined by fluorescence titrations. These measurements also established that the bivalent ligand was bound to IgM and IgMs primarily as a cyclic complex. The small enhancement factor (ca. threefold) for the binding of the bivalent ligand compared with the monovalent one was attributed to the loss of configurational entropy associated with the formation of the cyclic complex. The complex of bivalent ligand and IgM antibody was effective in the depletion of complement in contrast to the complex with the small monovalent ligand. It is suggested that this antigen-mediated effector activity is the consequence of a distortion of the normal planar structure of the IgM molecule. The structural perturbation would consist of a bending of some of the $F(ab')_2$ regions out of the plane containing the $(Fc\mu)_5$ core due to the cross-linking of adjacent $F(ab')_2$ regions by the bivalent ligand.

The interaction of soluble multivalent antibody with a multivalent particle containing identical antigenic determinants is a characteristic feature of the expression of biological function by humoral antibody. This feature is found in the interaction of IgG and IgM antibodies, for example, with viruses, bacteria, and malignant cells. It is in this context that the full potential of multivalent antibody to form complexes ("functional affinity") is evident (Karush, 1976) and in which conformational changes associated with effector functions would be revealed.

The thermodynamic and structural characterization of multivalent complex formation can, probably, be achieved most readily with relatively simple, soluble systems involving a bivalent ligand capable of forming a 1:1 cyclic complex. This capability depends on appropriate spacing between the two reactive groups of the ligand because of the apparent restriction on the distance of closest approach between the combining sites of a bivalent antibody (Werner et al., 1972). This separation is probably limited to a minimum value of ~9 nm.[1] The failure of several bivalent ligands unable to extend to this minimum distance to exhibit bivalent interaction with 7S anti-lactose antibody has pointed to the significance of the restriction (Gopalakrishnan & Karush, 1974).

The major published study of the formation of monomeric and higher cyclic bivalent complexes was carried out by Archer & Krakauer (1977) with equine anti-2,4-dinitrophenyl (Dnp[2]) antibodies of the IgG and IgG(T) classes. The bivalent ligands were linear poly(ethylene glycol) polymers, with Dnp groups attached to their ends, covering a molecular weight (number average) range of 25 000 to 75 000. This elegant and thorough study revealed that, with random coil ligands in this range, there was a large loss of configurational entropy associated with ring closure. This entropic term gave rise to a closure factor of the order of magnitude of 10^{-5} for the conversion of the linear 1:1 complex, for example, to the cyclic form. Nevertheless, it was found by measurement of light scattering that the predominant species in an equimolar mixture (5×10^{-6} M) of IgG antibody and ligand (58 000) was the cyclic monomeric form. Its stability was derived from the relatively

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[1] A recent study by R. Luedtke, C. Owen, and F. Karush (manuscript in preparation) has confirmed and extended the findings of Werner et al. (1972). In both studies, the nanosecond monophoton technique was used to detect energy transfer between a fluorescent donor bound at one antibody site and an acceptor group bound to the other site of the same molecule. In the earlier study, however, the single inter-heavy-chain disulfide of the rabbit IgG antibody had been reduced and alkylated. This cleavage introduced the possibility that the separation observed with the modified antibody was not characteristic of the native antibody. In the recent experiments, this ambiguity was eliminated by the re-formation of the interchain disulfide in the course of preparation of the hybrid IgG antibody.

[2] Abbreviations used: Dnp, 2,4-dinitrophenyl; EK, *N*-(N^{α}-acetyl-N^{ϵ}-Dnp-L-lysyl)-*p*-aminophenyl β-lactoside; iodo-EK, *N*-(N^{α}-iodoacetyl-N^{ϵ}-Dnp-L-lysyl)-*p*-aminophenyl β-lactoside; PBS, 0.15 M NaCl, 0.02 M phosphate, pH 7.4; TLC, thin-layer chromatography; BSA, bovine serum albumin; bis(EK), bivalent ligand in which EK groups are attached through the S atoms of the cysteine residues at positions 25 and 81 of β_2-microglobulin; $NaDodSO_4$, sodium dodecyl sulfate.

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large value of the intrinsic association constant (2×10^6 M^{-1}).

In another study, with rabbit IgG antibody to the loop peptide of lysozyme, measurements were made of circular polarization of luminescence of the antibody with monovalent and bivalent loop-containing ligands (Pecht et al., 1977). Differences in the circular polarized fluorescence spectra coupled with the ability of the antibody–bivalent ligand complex to fix complement led to the inference that this complex existed largely in the cyclic form.

The investigation described in this paper is based on the use of β_2-microglobulin as the carrier for the reactive groups of the bivalent ligand. This protein was initially identified and characterized by Berggård & Bearn (1968) as a single-chain protein with 100 residues and one disulfide bond. Its amino acid sequence (Cunningham et al., 1973) revealed a close similarity to immunoglobulin domains and, in particular, located the half-cystines at positions 25 and 81. These features provided the possibility of attaching two reactive groups at specified positions of the protein with a potential maximum separation of approximately 20 nm. This separation is sufficient to span the distance between the combining sites of a bivalent antibody molecule. For example, in the crystal of the myeloma protein KOL (IgG1), the distance between the ends of the Fab arms is ~14.6 nm (Colman et al., 1976). A bivalent ligand was therefore prepared by reacting the sulfhydryl groups, generated by reduction of the protein, with a lactose-containing moiety, namely, *N*-(N^{α}-iodoacetyl-N^{ϵ}-Dnp-L-lysyl)-*p*-aminophenyl β-lactoside (iodo-EK compound). Complex formation of this ligand with equine anti-lactose IgM antibody, and with IgMs and Fabμ derived from it, was evaluated with respect to the affinity of the complex and possible structural changes associated with its ability to inactivate complement.

The extent of complex formation was measured by fluorescence titration utilizing the Dnp group as a sensor, since it had previously been shown that binding of *N*-(N^{α}-acetyl-N^{ϵ}-Dnp-L-lysyl)-*p*-aminophenyl β-lactoside (EK compound) to anti-lactose antibody gave rise to a substantial quenching (Q_{max} = 40%) of the protein fluorescence (Gopalakrishnan & Karush, 1975). The experiments summarized here were directed particularly to the demonstration of the formation of a cyclic 1:1 complex since the appropriate interpretation of the affinity measurements and complement fixation results was predicated on its existence.

Materials and Methods

Preparation of Purified Anti-Lactose IgM Antibody. Horses were immunized with a sterile, heat-killed suspension of *Streptococcus faecalis* (strain N) in PBS. The detailed schedule of immunization and bleeding has been described previously (Chua et al., 1975). Anti-lactose antibody (IgM) was purified from the antisera by affinity chromatography and gel filtration. The immunoadsorbent (Lac-Sepharose) was prepared by coupling *p*-aminophenyl β-lactoside to CNBr-activated Sepharose 4B by the method of Cuatrecasas et al. (1968) as described earlier (Mitchell et al., 1977). Antibody was eluted from a Lac-Sepharose column with 0.2 M lactose in PBS followed by extensive dialysis to remove lactose. The anti-lactose antibody was then separated into IgM and 7S fractions by gel filtration through Sephadex G-200. The IgM antibody usually constituted about 90% of the total.

Preparation of IgM Subunits (IgMs). The conversion of IgM to the monomeric form was carried out by reduction for 1 h under N_2 with 1 mM dithiothreitol in 0.2 M Tris-HCl, pH 8.0, followed by alkylation with 2.2 mM iodoacetamide. The product was purified by gel filtration (Sephadex G-200) and the active material isolated with the Lac-Sepharose column. Analysis of the product by $NaDodSO_4$–polyacrylamide gel electrophoresis revealed that most of the interchain disulfide bonds had been cleaved.

Preparation of the Fabμ Fragment. The fragment was obtained by peptic digestion of IgM. The antibody solution (5–10 mg/mL) was treated with pepsin (3× crystallized, Miles Lab., Inc., Kankakee, IL) at an enzyme-to-substrate ratio of 1:100 in 0.2 M sodium acetate buffer, pH 4.5 at 37 °C for 4 h. The reaction was terminated by raising the pH to 8.0 with saturated Tris solution. The reaction mixture was passed through a Sephadex G-200 column to obtain the 3.2S fraction and the active portion isolated by affinity chromatography with Lac-Sepharose.

Isolation and Purification of Human β_2-Microglobulin. The starting material for the isolation of β_2-microglobulin was a 48-h urine collection immediately following a kidney transplant.[3] The urine was concentrated with a Bio-Fiber Miniplant Dialyzer (Bio-Rad) and dialyzed against distilled water, and the concentrate was lyophilized. Gel filtration with Sephadex G-50 in 0.01 M NH_4HCO_3 was used for the initial fractionation. Individual tubes were tested for the presence of β_2-microglobulin by the Ouchterlony method by using rabbit antiserum to human β_2-microglobulin (Bio-Rad). The tubes yielding precipitin bands were pooled and lyophilized. The final stage of purification consisted of preparative isoelectric focusing in a liquid medium with a sucrose density gradient (Freedman & Painter, 1971). The density gradient was formed from 5% to 45% sucrose and the medium contained 1.6% ampholine (LKB) for the pH range 5 to 8. The sample (50–100 mg) was applied largely in the middle of the column and focused at 400 V for 2 h. The focusing was continued at 3 W overnight with the voltage rising to 800 V. The voltage was raised to 1000 V and the focusing continued for a total of 48 h.

The fraction containing the major peak, pH 5.6, was separated and passed through a column of Sephadex G-75 in 0.01 M NH_4HCO_3 to remove the components of low molecular weight. The protein fraction was lyophilized and stored in the cold. The purity of the β_2-microglobulin was established by analytical IEF in which only one major band was found. Its identity was further verified by amino acid analysis.

Tritiation Procedure. A solution containing 20–40 mg of protein in 1 mL of 0.5 M $NaHCO_3$ was cooled in an ice bath. Four portions of the acetylating reagent, each containing 2 mCi of ^3H-labeled acetic anhydride (400 mCi/mmol) in 20 μL of anhydrous dioxane, were added over a period of 1 h. The completed reaction mixture was passed through a column of Sephadex G-50 in 0.01 M NH_4HCO_3 to remove radioactive impurities and the protein solution lyophilized.

Succinylation of β_2-Microglobulin. The solution of ^3H-labeled β_2-microglobulin, 10 mg/mL, in 0.5 M $NaHCO_3$ was cooled in an ice bath. Four portions of solid succinic anhydride, each equivalent to a 20-fold molar excess of the protein lysine, were added successively. The reaction mixture was maintained at pH 8.5 with 1 N NaOH using a pH stat. The extent of succinylation was determined by the ninhydrin reaction. This assay showed that more than 95% of the amino groups had reacted. Gel filtration with Sephadex G-100 was used to isolate the modified protein.

[3] We are indebted to Dr. Martin Goldberg of the Hospital of the University of Pennsylvania for the suggestion that such urine would serve as a source of β_2-microglobulin and to Dr. Clyde Barker of the Hospital of the University of Pennsylvania for his cooperation in securing the urine collection.

Tryptophan Modification. The elimination of the fluorescence of β_2-microglobulin was effected by chemical alteration of the tryptophan residues with 2-hydroxy-5-nitrobenzyl bromide (Barman & Koshland, 1967). A solution (2 mL) containing 10 mg of succinylated, ^{3}H-labeled β_2-microglobulin and 7 M urea was adjusted to pH 3.0. Two portions (50 μL) of the reagent in anhydrous dioxane, at a final concentration (0.017 M) corresponding to a 20-fold excess over the tryptophan, were added to the stirred protein solution at room temperature. The reaction was complete within a few minutes, and the protein was isolated by gel filtration with Sephadex G-100 in 0.01 M NH_4HCO_3. The fraction of the tryptophans modified was determined by measurement of the optical density at 410 nm after adjustment to pH 10 (ϵ = 1.30 $\times$ 10^4 M^{-1} cm^{-1}). This assay showed that 1.6 of the 2 tryptophan groups had been converted to the expected product, although virtually all of the tryptophan fluorescence had been abolished.

Synthesis of N-(N^{α}-Iodoacetyl-N^{ϵ}-Dnp-L-lysyl)-p-aminophenyl β-Lactoside (Iodo-EK). The starting material was N-(N^{ϵ}-Dnp-L-lysyl)-*p*-aminophenyl β-lactoside in the form of the trifluoroacetate salt, prepared as previously described (Gopalakrishnan & Karush, 1975). To a 1-mL solution containing 93 mg (0.1 mmol) of redistilled dimethylformamide (DMF) was added 75 μL (0.5 mmol) of redistilled triethylamine. The solution was cooled in an ice bath and 46 mg (0.15 mmol) of *p*-nitrophenyl iodoacetate, prepared as described by Hudson & Weber (1973), were added. After incubation in the dark for 15 min, the product was precipitated with 10 mL of diethyl ether and the mixture kept in the dark overnight. The residue was redissolved in 1 mL of DMF and reprecipitated with 10 mL of ether three times. The residue was then triturated with 10 mL of ether and the granular solid washed two more times with 10 mL of ether. The product was air-dried and stored in the freezer. Analysis of the product by thin-layer chromatography (TLC) showed that the acetylation reaction was complete. Analysis by TLC of the reaction product formed with *N*-acetylcysteine indicated that at least 50% of the isolated synthetic product was the desired iodo-containing derivative.

Synthesis of N-(N^{α}-Acetyl-N^{ϵ}-Dnp-L-lysyl)-p-aminophenyl β-Lactoside (EK). The preparation of this monovalent ligand has been described previously (Gopalakrishnan & Karush, 1975).

Reduction and Alkylation. The conjugation of the EK ligand to the modified β_2-microglobulin was done in 0.2 M Tris-HCl, pH 8.0, and 4 M guanidine hydrochloride. The protein (10 mg/mL) was reduced with 2 mM dithiothreitol for 1 h under N_2 at room temperature and alkylated for 1 h with 8 mM iodo-EK. The alkylated protein was purified by repeated passage through a column of Sephadex G-100 in 0.01 M NH_4HCO_3.

Analytical Centrifugation. The determination of sedimentation coefficients was made with a Beckman Spinco Model E analytical ultracentrifuge equipped with schlieren optics. Protein solutions (3–5 mg/mL) in PBS–EDTA were centrifuged at 59 780 rpm at 20 °C.

Fluorescence Titration. Fluorescence measurements were made with a Perkin-Elmer double beam fluorescence spectrophotometer, Model 512. The excitation and emission wavelengths were 295 and 330 nm, respectively. The sample compartment was maintained at 22 °C with a circulating water bath. The antibody at a concentration of 1 $\times$ 10^{-6} M in PBS (3 mL) was placed in the fluorescence cell. A total volume of 0.2 mL of the ligand solution was added with a motor-driven micrometric Hamilton syringe to the stirred antibody solution and the fluorescence continuously recorded. Normal rabbit IgG was used as a control protein to correct for the nonspecific effects of dilution and attenuation. The value of Q_{max} was obtained by linear extrapolation of a plot of $1/Q$ vs. the reciprocal of the total ligand concentration.

Preparation of bivalent ligand

β_2-microglobulin

↓ Acetylation with ^{3}H-acetic anhydride. (0.2 acetyl group/protein molecule)

^{3}H-β_2-microglobulin

↓ Succinylation with succinic anhydride. (95% of lysines succinylated)

Succinylated-^{3}H-β_2-microglobulin

↓ Treatment with 2-hydroxy-5-nitrobenzylbromide. (80% of tryptophans modified)

Tryptophan-modified, succinylated-^{3}H-β_2-microglobulin

↓ Reduction in 4 M Gu-HCl, pH 8 and alkylation with N-(N^{α}-iodoacetyl, N ~Dnp-L-lysyl)-p-aminophenyl-β-lactoside. (2 EK groups/protein molecule)

Bis-EK,tryptophan-modified, succinylated-^{3}H-β_2-microglobulin

FIGURE 1: Flow sheet for the preparation of bis(EK), tryptophan-modified, succinylated [^{3}H]-β_2-microglobulin.

Complement Fixation Assay. The micro-complement fixation procedure of Wasserman & Levine (1961) was used. Sheep red blood cells (SRBC) and fresh frozen guinea pig serum were obtained from Rockland (Albertsville, PA) and hemolysin from Cordis Labs (Miami, FL). One volume of a 1:1000 dilution of the hemolysin was used to sensitize 1 volume of SRBC containing 1 $\times$ 10^9 cells/mL. A CH_{50} titration was performed for each batch of sensitized cells under the same conditions used in the complement fixation assay. For measurement of the complement fixation activity, a 3-mL reaction mixture containing 5.5 $\times$ 10^{-9} M IgM, 1.0 $\times$ 10^{-6} M EK, or 0.50 $\times$ 10^{-6} M bis(EK) and sufficient complement to give 90% hemolysis in Veronal buffer, pH 7.4, with 0.1% BSA was incubated at 37 °C for 1 h. Then 2.5 $\times$ 10^7 sensitized cells in 0.5 mL were added and the mixture was further incubated at 37 °C for 1 h. The suspension was finally centrifuged and the optical density read at 413 nm. Prior to its use for complement fixation, the IgM solution was centrifuged for 30 min at 10 000 rpm (12000*g*). This was followed by filtration of the protein solution (2 mg/mL) through a Millipore filter (0.22 μm).

Results

Characterization of the Bivalent Ligand [Bis(EK)]. The utilization of β_2-microglobulin as a monomeric carrier of the bivalent ligand requires several chemical modifications of the native protein. The series of chemical steps involved is depicted in Figure 1 together with the extent of each of the chemical changes. The labeling reaction with ^{3}H-labeled acetic anhydride resulted in the average acquisition of 0.2 acetyl group per protein molecule. The specific activity of the labeled protein at this stage was calculated from the optical density at 280 nm by using a value for $E^{1\%}$ of 16.8 (Berggård & Bearn, 1968). The specific activity was subsequently employed to calculate the number of EK groups per protein molecule. Succinylation of the protein was required to maintain its solubility after reduction and alkylation. However, to ensure that dimerization did not occur, it was necessary to convert at least 95% of the amino groups to the succinylated form. The need for tryptophan modification of the protein followed from the fact that its fluorescence would otherwise contribute to

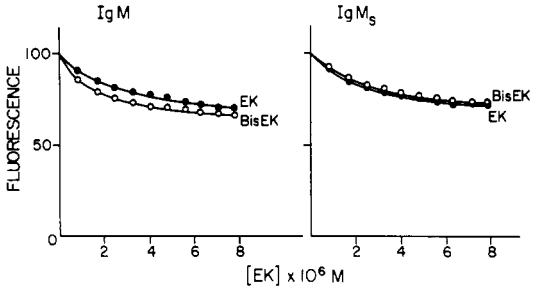

FIGURE 2: Fluorescence titration curves for IgM and IgMs with EK and bis(EK). The concentration of IgM (based on IgMs) was 0.57 × 10^{-6} M and that of IgMs was 0.988 × 10^{-6} M.

Table I: Summary of Binding Results from Fluorescence Quenching[a]

	bis(EK)			EK		
antibody prepn	K_0 × 10^{-6} (M^{-1})	Q_{max} (%)	a	K_0 × 10^{-6} (M^{-1})	Q_{max} (%)	a
IgM (1554 IV)[b]	2.51	38.2	0.83	0.51	36.8	0.82
IgM (945 V)				0.62	37.7	0.81
IgMs (1681 V)	1.44	34.5	0.77	0.62	39.2	0.75
Fabμ (945 V)	0.39	54.6	0.89	0.60	64.1	0.81

[a] K_0 is the association constant corrected for statistical factors. Q_{max} is the maximum quenching of the antibody fluorescence due to the binding of ligand. The value of a is the Sips heterogeneity index (Karush, 1962). [b] The numbers in the parentheses identify the animal and bleeding used for the preparation of the purified anti-lactose IgM antibody.

the fluorescence measured during the titration of the antibody with bis(EK). Rather than correct for this contribution it was considered that its elimination would result in data of improved accuracy. Finally, the extent of alkylation of the reduced protein with iodo-EK was obtained from the protein concentration measured by radioactivity and the concentration of EK groups based on the optical density at 365 nm by using a value of 1.52 × 10^4 for the molar extinction coefficient (Gopalakrishnan & Karush, 1975). A correction for the contribution of the modified tryptophan was made. This calculation yielded 1.98 EK groups per protein molecule.

Sedimentation Analysis. The possibility that bis(EK)–β_2-microglobulin was serving to cross-link IgM and IgMs antibody was evaluated by analytical ultracentrifugation. Solutions were used at a protein concentration of about 3.5 mg/mL. In the case of IgMs, a sedimentation value of 5.94 S was found without the ligand and 6.3 S with the ligand (3 × 10^{-5} M). This difference is probably due to the increased mass resulting from the binding of the ligand. For IgM, no significant difference in sedimentation behavior was brought about by the bivalent ligand (2.1 × 10^{-6} M).

Fluorescence Titration. The quenching of protein fluorescence due to binding of the ligands is shown in Figure 2 for the interaction of EK and bis(EK) with IgM and IgMs antibody. The fluorescence curves have been corrected for the nonspecific effects of dilution and attenuation due to the addition of the ligand solution by using normal rabbit IgG. The maximum quenching (Q_{max}) ranges between 35% and 40%. The exact values determined as described above are shown in Table I. The fluorescence data were used to calculate the fraction of sites occupied as a function of the free concentration of ligand. In the case of the bivalent ligand, the assumption was made that its binding to the antibody utilized two combining sites. The grounds for this assumption are given in the Discussion. The results of these calculations are presented in the form of Scatchard plots in Figure 3 for

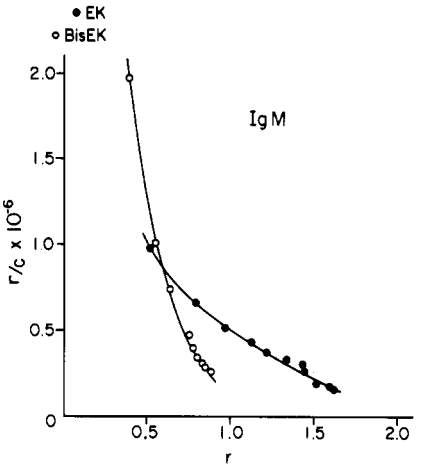

FIGURE 3: Scatchard plots for binding of EK (●) and bis(EK) (○) by IgM in which r is the average number of ligand molecules bound per monomer and c is the corresponding free concentration of the ligand.

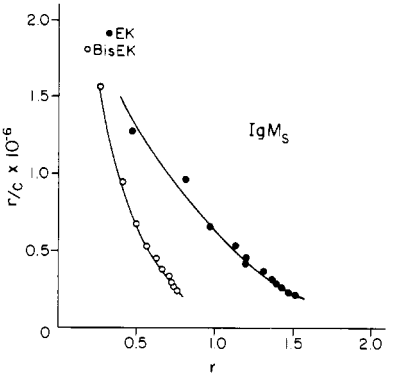

FIGURE 4: Scatchard plots for binding of EK (●) and bis(EK) (○) by IgMs. See Figure 3 for definition of r and c.

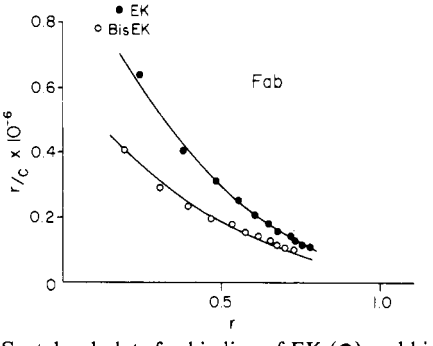

FIGURE 5: Scatchard plots for binding of EK (●) and bis(EK) (○) by Fabμ in which r is the average number of EK groups bound per molecule of Fabμ and c is the corresponding free concentration of EK groups.

IgM and Figure 4 for IgMs. It will be noted that for IgM the titration was carried to the extent of at least 80% occupancy of sites and IgMs to about 75% occupancy. In the calculation of the association constant for IgM, the equivalent concentration of IgMs was used to take into account a statistical factor of 5 arising from the pentameric form of IgM. The results of the fluorescence titration of Fabμ with the two ligands are shown in Figure 5. In this case Q_{max} exceeded 54%. The value of K_0 for the bivalent ligand was calculated by using the total concentration of the reactive groups, i.e., twice the concentration of the ligand. A summary of the intrinsic association constants, the values of Q_{max}, and the Sips heterogeneity indices (a) (Karush, 1962) are given in Table I.

Table II: Complement Fixation by the Complex of IgM Antibody and Bis(EK) Ligand[a]

IgM + EK	IgM + bis(EK)	ligand control: EK	ligand control: bis(EK)	antibody control	complement control	complete hemolysis
0.375[b]	0.070	0.403	0.281	0.385	0.425	0.665
0.385	0.066	0.425	0.320	0.394	0.427	0.670

[a] Reaction mixtures (3 mL) contained 1.64×10^{-5} μmol (5.5×10^{-9} M) of IgM, 3.0×10^{-3} μmol (1.0×10^{-6} M) of the EK ligand, and 1.5×10^{-3} μmol (0.50×10^{-6} M) of the bis(EK) ligand. [b] Optical density at 413 nm of the reaction supernatant.

Complement Fixation. The capability of the IgM complexed with the bivalent ligand to fix complement is demonstrated in the experiment summarized in Table II. The figures in the two rows are the optical density value for duplicate sets of tubes. There is substantial inactivation of complement by bis(EK) itself but only a minor loss due to the IgM preparation. After correction for these controls, the complex showed a 74% inactivation of the available complement. On the other hand, the complex with the monovalent ligand exhibited no activity.

Discussion

The optimal utilization of β_2-microglobulin as the carrier for the bivalent ligand required that it retain its monomeric form in nondisrupting solvents. The tendency of the reduced and alkylated protein to aggregate was evident from the fact that the product was insoluble at neutral pH. This observation is in accord with the conclusion reached by Isenman et al. (1975) from measurements of circular dichroism that, in the absence of the intrachain disulfide, β_2-microglobulin is unfolded in neutral, aqueous buffer. The monomeric form was stabilized, however, by increasing the negative charge of the protein through succinylation of the lysyl residues. Extensive modification of these groups was necessary as shown by the sedimentation coefficient of 3.0 S for bis(EK) in which only 25% of the lysyl groups were succinylated. This value contrasts with the figure 1.5 S found for the product in which more than 90% of the susceptible residues were converted to negatively charged residues. In the monomeric bis(EK), the net charge at neutral pH is highly negative, presumably with a value of −17 to −18 (Cunningham et al., 1973). We may surmise, therefore, that, in view of the observations of Isenman et al. (1975), our monomeric bis(EK) ligand is in the unfolded state but, on the average, significantly more extended than the equivalent polypeptide chain in the randomly coiled form.

Since the fluorescence quenching measurements do not directly demonstrate the formation of the cyclic 1:1 complex, further analysis of the system was necessary. Intermolecular cross-linking of IgM and IgMs by the bis(EK) was clearly ruled out by the sedimentation results as a significant factor in the fluorescence experiments, especially since the concentration of the antibody involved was reduced by more than tenfold compared with that in the sedimentation experiments. The issue that remains is whether the bound bivalent ligand is attached to the antibody through both of its reactive groups, i.e., whether the cyclic complex is formed. This matter could be resolved from the quantitative aspects of the fluorescence observations. Thus, if it was assumed that the cyclic complex was not formed, it was found that for the first point in the titration, and, in some instances, the second as well, the calculated value for the concentration of free liquid was a negative number. This anomaly did not appear in the titration with the monovalent ligand. Furthermore, with this assumption the intrinsic association constant was at least tenfold higher than the value obtained for the binding of bis(EK) by Fabμ. Consequently, we conclude that the monovalent interaction can contribute to the total binding only to a minor extent.

Two further comments are relevant to the evaluation of the fluorescence experiments. First, any differential effect of the large negative charge of the carrier on the binding constants was avoided by comparing the K_0's for binding of the bivalent ligand by IgM and Fabμ. Furthermore, the similarity of the values of K_0 for the binding of bis(EK) and the monovalent ligand EK (0.39×10^6 M^{-1} vs. 0.60×10^6 M^{-1}) demonstrates the minor effect of the properties of the carrier on the reaction. Second, the utility of a direct measurement of the extent of binding of bis(EK) to IgM, as is commonly done by equilibrium dialysis with ligands of low molecular weight, is quite obvious. However, because of the relatively large size of bis(EK), our extensive efforts to make such measurements by equilibrium dialysis and differential sedimentation were unsuccessful.

The most striking finding in this study is the absence of a large enhancement of the association constant for the binding of the bivalent ligand relative to that for the monovalent ligand. Although the cyclic complex is highly favored for the reaction of the antibody with bis(EK), the enhancement factor of 10^4 previously found for similar antibody in the neutralization of lactose-conjugated φX174 (Gopalakrishnan & Karush, 1974) was reduced to only several-fold in the present context. The major difference between the neutralization experiments and the present ones is the fact that in the former the reactive groups (the lactosyl moieties) were fixed with respect to their separation from each other. This condition was the result of their attachment to the proteins constituting the capsid of the viral particle. In contrast, because the bis(EK) molecule is in the unfolded state, many configurations are available to it which are associated with different distances between the reactive groups. Thus the formation of the cyclic complex must involve the loss of configurational entropy. Although we are unable to evaluate this loss quantitatively, the careful studies of Archer & Krakauer (1977) have demonstrated that a ring closure factor, representing the loss of configurational entropy in the conversion of the noncyclic complex to the cyclic form, may reach a value of 10^{-5}. This value was established for random coil, bivalent antigens in the molecular weight ranges of 25 000 to 75 000 and includes the loss of configurational entropy due to immobilization of the antibody in the cyclic complex. The loss of configurational entropy in cyclic formation for bis(EK) is probably substantially less than that represented by the closure factor of 10^{-5} because of the smaller size of the bivalent ligand and its deviation from a random coil. The loss is, nevertheless, of major significance as judged by the finding that the association constant for the binding of bis(EK) by IgM (1554 IV) antibody is only threefold larger than that for Fabμ, after correction for a statistical factor of 2 (Table I). Although the Fabμ was obtained from IgM (945 V), the similarity of their values of K_0 for the binding of EK justifies this comparison. The twofold lower value K_0 for the IgMs compared with that for IgM in the binding of bis(EK) may be associated with the fact that in the preparation of IgMs

most of the interheavy chain disulfides were cleaved. It may be noted that the values of K_0 for the binding of the monovalent ligand to IgM (945 V) and Fabμ derived from it show no significant difference. This result provides assurance that the binding site of the antibody remained functionally intact in the preparation of Fabμ from IgM antibody.

The complement depletion effected by the complex formed between bis(EK) and IgM antibody is a useful vehicle for relating specific antibody binding of antigen with effector function mediated by constant regions. The proximity of the four-chain subunits in the IgM pentamer provides favorable conditions for the appropriate juxtaposition of Fc regions if this rearrangement is, indeed, required for complement fixation. The rearrangement can evidently be brought about by the binding of bivalent ligand, although we have not yet established the minimum number of bivalent ligands bound per IgM molecule necessary for activation. Further analysis of the effect of variation of ligand concentration on the efficiency of complement depletion would be useful in establishing the need for such juxtaposition. This issue is under further investigation.

The requirement for the binding of a critical multiple of antigenic determinants to a single IgM molecule to initiate complement depletion has been inferred from a recent study by Humphries & McConnell (1977). In this investigation a spin-label lipid hapten was incorporated in the membrane of liposomes and complement depletion by bound anti-hapten IgM was measured. The efficiency of complement fixation increased with hapten density in the membrane, although the bulk concentration of hapten was maintained constant. It could thus be concluded that multiple binding enhances the activity of the membrane-bound IgM. In the experiment summarized in Table II, the average number of bivalent ligand molecules in the complex was calculated as 3–4. This range was based on the K_0 of 2.5×10^6 M^{-1}, measured at 22 °C, with a correction factor of 2.4 for the fact that the complement depletion assay was carried out at 37 °C calculated from an earlier study (Karush, 1957).

An interesting model for the activation of IgM by multivalent binding has been provided by Feinstein et al. (1971). From electron micrographs of IgM with and without particulate antigen, it appears that the free IgM exists in a planar form, i.e., with its $F(ab')_2$ regions in the same plane as the central $(Fc)_5$ disk. Binding to antigen causes displacement of the $F(ab')_2$ portions out of the plane, thus modifying the geometrical relationship between the central and peripheral regions. Such distortion, it is suggested, would allow for C1 binding and activation by IgM. A more detailed analysis based on the generalized structure of immunoglobulin domains has been provided recently by Beale & Feinstein (1976). In the case of the binding of bis(EK), we may similarly propose that the formation of the complex involves a partial conversion of the IgM molecule from the planar form to the table form. Such conversion would probably be facilitated if the bivalent ligand were to cross-link neighboring $F(ab')_2$ regions. Such cross-linking is compatible with dimensions of the table form of the IgM molecule (Feinstein & Munn, 1969). It finds support, furthermore, in the earlier conclusion by Rosse et al. (1967) that the antigen must combine with two adjacent subunits of IgM antibody for exposure of the C1 binding site.

Recent experiments by Brown & Koshland (1975) with anti-lactose IgM antibody have been interpreted as demonstrating that a monovalent ligand, associated with a bulky carrier (RNase), can activate the antibody to fix complement. It was then concluded that the activation of the antibody was due to an allosteric effect in the Fc region brought about by binding of the ligand. It has been pointed out by Metzger (1978) that there is an inconsistency between the concentrations of IgM and ligand at which complement fixation was observed and the association constant measured ($K_0 = 4.7 \times 10^4$). He has calculated that only 0.02% of the antibody sites would be occupied in the reaction mixture in which complement depletion occurred. An adequate explanation of this apparent paradox has not yet been provided. A more recent study by Chiang & Koshland (1979) has utilized another monovalent ligand in which great care was taken to exclude multivalent ligands. The earlier results were confirmed in that complement fixation was observed by the reaction mixtures containing IgM anti-lactose antibody and the monovalent ligand. Nevertheless, the issue of the limited occupancy of sites remains unresolved.

Finally, we note that Pecht et al. (1977) have reported that rabbit IgG antibody to the lysozyme loop will cause complement fixation when reacted with a bis-loop ligand. Their interpretation is based on the notion that a cyclic complex is formed leading to a structural alteration in the Fc region associated with effector function. Metzger (1978) has pointed out that in this study also there is an inconsistency in the experimental observations which challenges the validity of the interpretation. The problem here is that the optical changes (circular polarization of fluorescence) associated with the binding of bivalent antigen were unaltered with concentrations of antigen greater than equivalence, whereas complement fixation exhibited an optimum value for the antigen concentration and decreased beyond this value. This anomaly in the stoichometric relationships precludes an unambiguous interpretation of the experimental results. It appears, therefore, that the conventional view that antigen-mediated complement activation requires the juxtaposition of two Fc regions brought about by a multivalent antigen has yet to be successfully challenged.

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Isolation, Purification, and Reconstitution of a Proline Carrier Protein from *Mycobacterium phlei*†

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ABSTRACT: Membrane vesicles from *Mycobacterium phlei* contain carrier proteins for proline, glutamine, and glutamic acid. The transport of proline is Na^+ dependent and requires substrate oxidation. A proline carrier protein was solubilized from the membrane vesicles by treatment with cholate and Triton X-100. Electron microscopic observation of the detergent-treated membrane vesicles showed that they are closed structures. The detergent-extracted proteins were purified by means of sucrose density gradient centrifugation, followed by gel filtration and isoelectric focusing. A single protein with a molecular weight of 20 000 ± 1000 was found on polyacrylamide gel electrophoresis. Reconstitution of proline transport was demonstrated when the purified protein was incubated with the detergent-extracted membrane vesicles. This reconstituted transport system was specific for proline and required substrate oxidation and Na^+. The purified protein was also incorporated into liposomes, and proline uptake was demonstrated when energy was supplied as a membrane potential introduced by K^+ diffusion via valinomycin. The uptake of proline was Na^+ dependent and was inhibited by uncoupler or by sulfhydryl reagents.

Active transport of proline has been shown with the electron transport particles (ETP),[1] depleted ETP, cell membrane ghosts, and whole cells of *Mycobacterium phlei* (Hirata et al., 1971; Hirata & Brodie, 1972; Prasad et al., 1976). Proline transport in membrane vesicles exhibited a strict requirement for substrate oxidation as well as for Na^+ or Li^+ (Hirata et al., 1974b) and specific phospholipids (Prasad et al., 1975a). The uptake of proline proceeded against a concentration gradient with succinate, generated NADH, exogenous NADH, or ascorbate–*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TPD) as substrate. The transport of proline does not require a high-energy phosphate bond (Hinds & Brodie, 1974) since it proceeds in the absence of coupling factor or inorganic phosphate. It is inhibited by anaerobic conditions (Hirata et al., 1971). In addition to proline, the uptake of glutamine and glutamic acid has been demonstrated in the membrane vesicles from *M. phlei*, and it has been shown that the energy requirement and carrier protein(s) or binding site(s) involved in the uptake of glutamine and glutamic acid appeared to be different from those for the uptake of proline (Prasad et al., 1975b).

The active transport of metabolites across biological membranes has been studied in bacterial and mammalian systems. The energy source and the relationship between active transport and energy coupling have been investigated (Brodie et al., 1972; Kaback, 1974; Hirata et al., 1974a; Berger & Heppel, 1974) and reviewed (Simoni & Postma, 1975; Boyer & Klein, 1972; Harold, 1972; Kaback, 1972; Oxender, 1972). The existence of solute-specific membrane-associated proteins has been documented in bacterial systems for solute transport (Fox & Kennedy, 1965), solute binding (Piperno & Oxender, 1968), and group translocation (Kundig & Roseman, 1971a,b). Recently, carrier proteins, which may be defined as proteins which translocate solutes across the membrane, have been solubilized from bacterial and mammalian membranes by using detergents (Gordon et al., 1972; Kashara & Hinkle, 1976; Hirata et al., 1976; Shertzer & Racker, 1976; Crane et al., 1976; Amamuna et al., 1977; Lee et al., 1977a). The reconstitution of active transport with carrier proteins in-

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[1] Abbreviations used: ETP, electron transport particles; DETP, ETP depleted of the coupling factor latent ATPase; Tx-ETP, Triton X-100 treated ETP; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride.

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